INFLUENCE OF HEXOSE-PHOSPHATES AND CARBON CYCLING ON SUCROSE ACCUMULATION IN SUGARCANE *spp*.

by

Margaretha Johanna van der Merwe

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Supervisors: F.C Botha & J-H Groenewald

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Declaration

I, the undersigned, hereby declare that the work presented here are original and have not been submitted in its entirety or in part at any university for a degree.

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SUMMARY

Sucrose accumulation, marked by a continuous cycle of synthesis and degradation, is characterised by a shift of carbon away from the insoluble matter and respiratory intermediates into sucrose. Despite this shift, a significant proportion of carbon is returned to these pools by hexose-phosphate: triose-phosphate cycling and/or sucrose cycling. Little is known about the magnitude and behaviour of these cycles in sugarcane. Contradictory reports on the relationship between these two cycles have led to the evaluation of the link between the hexose-phosphate: triose-phosphate- and sucrose cycle. In addition, it still needs to be tested whether these cycles could significantly influence carbon partitioning within sugarcane internodal tissue.

In this work, a comprehensive metabolic profile was constructed for sugarcane internodal tissue by gas chromatography-mass spectrometry (GC-MS) in order to determine the steady state levels of a broad range of primary metabolites that are involved in these cycles. The power of GC-MS was illustrated by the detection of raffinose, maltose, ribose, xylitol, inositol, galactose, arabinose and quinic acid, which was quantified for the first time in sugarcane internodal tissue. Analyses were not solely based on the prevailing metabolite levels, but also on the interactions between these metabolites. Thus, in a complementary approach the metabolic flux between the two substrate cycles was assessed by ^{13}C nuclear magnetic resonance (NMR).

Analyses of transgenic sugarcane clones with 45-95% reduced cytosolic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) activity displayed no visual phenotypic change, but significant changes were evident in *in vivo* metabolite levels. Sucrose concentrations increased six and three-fold in young and maturing internodal tissue, respectively. Reduced PFP activity also resulted in an eight-fold increase in the hexose-phosphate: triose-phosphate ratio in the transgenic immature internodes. In addition, the hexose-phosphate: triose-phosphate cycling decreased in the immature internodes of the transgenic lines if compared to the immature control internode. However, there was no significant difference between the hexose-phosphate: triose-phosphate cycling in the mature internodal tissue of the transgenic and the control

lines. This illustrated that PFP mediates hexose-phosphate: triose-phosphate cycling in immature sugarcane internodal tissue.

Unpredictably, reduced PFP activity led to a ten-fold increase in sucrose cycling in the transgenic immature internodes. The combination of metabolite profiling and flux distribution measurements demonstrated that the fluxes through the sucrose and the hexose-phosphate pools were not co-regulated in sugarcane internodal tissue.

From these observations a model was constructed that implicates higher sucrose cycling as a consequence of increased sucrose concentrations.

OPSOMMING

Sukrose akkumulering, gekenmerk deur ‘n aanhoudende proses van sintese en afbraak, gaan gepaard met ‘n defnitiewe verskuiwing van koolstof vanaf onoplosbare materiaal en respiratoriese intermediate na die sukrose poel. Ten spyte van hierdie verskuiwing word ‘n beduidende bron van koolstof nogsteeds terug gesirkuleer na hierdie poele deur die heksose-fosfaat: triose-fosfaat- en/of die sukrose kringlope. Min is egter bekend oor die impak en geaardheid van hierdie siklusse in suikerriet. Teenstrydige resultate oor die verband tussen die twee siklusse het gelei tot hierdie studie wat daarop gemik is om die verhouding tussen die heksose-fosfaat: triose-fosfaat- en sukrose siklusse te ondersoek. Die bydrae van hierdie siklusse tot koolstof allokering na sukrose moet ook nog ondersoek word in suikerriet internodes.

In hierdie werkstuk is ‘n volledige metaboliese profiel opgestel met behulp van gas chromatografie massa spektrometrie (GC-MS) vir die bepaling van ‘n wye reeks primêre metaboliete betrokke by die siklusse in suikerriet internodale weefsel. Die krag van GC-MS is geïllustreer met die deteksie van raffinose, maltose, ribose, xylitol, inositol, galaktose, arabinose en kinien suur wat vir die eerste keer in suikerriet internodes gekwantifiseer is. Analises het nie net bloot die statiese metaboliet poele ondersoek nie, maar ook die interaksie tussen hierdie metaboliete ge-evalueer. Dus, in ‘n komplementêre aanslag is gebruik gemaak van ^{13}-koolstof kern magnetiese resonansie (KMR) om die fluks tussen die twee siklusse te bepaal.

Analises van suikerriet klone met 45-95% onderdrukte sitosoliese pirofosfaat: D-fruktose-6-fosfaat 1-fosfotransferase (PFF, EC 2.7.1.90) uitdrukking het geen fenotipiese verskille onthuldig nie, alhoewel *in vivo* metaboliet data betekenisvolle verskille aangetoon het. ‘n Ses- en drievoudige toename in sukrose konsentrasies is onderskeidelik aangetoon in die jong en ouer internodes. ‘n Verlaging in PFF aktiwiteit het gelei tot ‘n agt-voudige toename in die heksose-fosfaat: triose-fosfaat ratio in die transgeniese onvolwasse internodes. Die heksose-fosfaat: triose-fosfaat siklus het ook afgeneem in die onvolwasse internodes van die transgeniese lyne in vergelyking met dié van die onvolwasse internodes van die kontrole. Geen statistiese betekenisvolle verskil kon egter aangedui word in die heksose-fosfaat: triose-fosfaat siklus van die volwasse internodes

van die kontrole en transgeniese lyne. Gevolglik was afgelei dat PFF die heksose-fosfaat: triose-fosfaat siklus medieer in jong suikerriet internodale weefsel.

In 'n onverwagse wendsel het verlaagde PFF aktiwiteit gelei tot 'n tien-voudige verhoging in sukrose sirkulering in die onvolwasse internodes. Die kombinasie van metaboliet profiel generering en fluks distribusie bepalings het gedemonstreer dat die vloei deur die sukrose en heksose-fosfaat poele nie gekoördineerd ge-reguleer word in suikerriet internodes nie.

Vanaf hierdie observasies is 'n model opgestel wat verhoogde sukrose sirkulering impliseer as 'n gevolg van verhoogde sukrose konsentrasies.

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For friendships, old and new, and the steadfast support of my family, I am grateful to know you all. I am thankful for your unconditional love at all times. This thesis is dedicated to my mother, who has been a symbol of inspiration, dedication and style throughout her life to me. *I love you, mom*.

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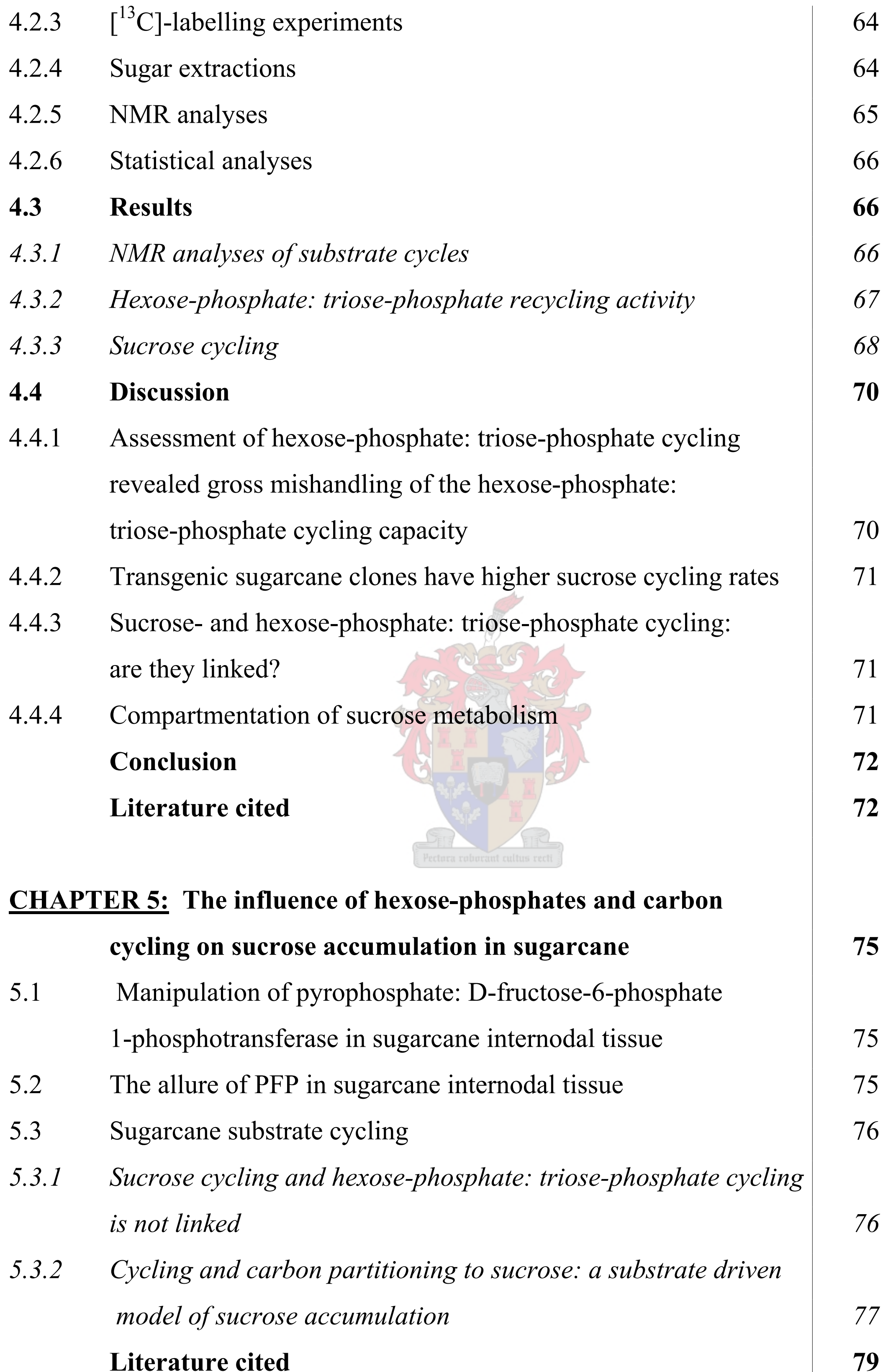

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LIST OF ABBREVIATIONS

^{o}C	degree centigrade
ADP	adenosine 5'-diphosphate
AEC	adenylate energy charge
AI	acid invertase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphte, toluidine salt
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
^{13}C	isotope-labelled carbon
CWI	cell wall invertase
ddH_2O	distilled deionised water
DFA	discriminate functional analysis
edn.	edition
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
EST	expressed sequence tags
fruc	fructose
FW	fresh weight
g	gram
x*g*	times gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
GC-MS	gas chromatography-mass spectrometry
Glc	glucose
G6P	glucose-6 phosphate
G6PDH	glucose-6-phosphate dehydrogenase (EC 1.1.1.149)
h	hour
HCA	hierarchical component analysis
HK	hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1)

HPLC	high performance liquid chromatography
i.e.	id est
IEX	ion exchange
KMR	kern magnetiese resonansie
M	molar
min	minute
MES	2-[N-morpholino] ethanesulfonic acid
MSTFA	N-methyl-N (trimethylsilyl)-trifluoroacetamide
NaCl	sodium chloride
NAD+	oxidised nicotinamide adenine dinucleotide
NADP	reduced nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
nd	not detected
NMR	Nuclear magnetic resonance
OPPP	oxidative pentose phosphate pathway
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependent phosphofructokinase (pyrophosphate:D-fructose-6-phosphate 1-phosphotrasnferase, EC 2.7.1.90)
PGi	phosphogluco-isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)
Pi	inorganic phosphate
PPi	pyrophosphate
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SE	standard error
SPE	solid phase extraction
SPS	sucrose phosphate synthase (UDP-glucose:D-fructose-6-P 2-α-D-glucotransferase. EC 2.4.1.14)

SuSy	sucrose synthase (UDP-glucose:D-fructose 2-α-D-glucosyl-transferase, EC 2.4.1.13)
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Unit (one unit of enzyme is the amount of enzyme that catalyzes the production of one μmol product per minute)
UDP	uridine 5'-diphosphate
UDP-glc	uridine 5'-diphosphate glucose
UGPase	uridine 5'-diphosphate glucose pyrophosphorylase
UTP	uridine 5'-triphosphate
UV	ultra violet
v	volume
V	Volt
w	weight

Chapter 1

GENERAL INTRODUCTION

In most plants sucrose is the main translocated form of organic carbon from the source. In addition, mature internodes of sugarcane (*Saccharum* sp. hybrids) accumulate approximately 12-16% sucrose on a fresh mass basis in the mature culm (Bull and Gasziou, 1963). However, projections based on culm morphology estimates that this concentration could potentially be doubled (Grof and Campbell, 2001; Moore et al., 1997). Increasing the sucrose yield has therefore been a key objective in several sugarcane improvement programmes.

In one such study transgenic sugarcane clones with reduced cytosolic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) activity accumulated 50% more sucrose in the immature internodal tissue than the wild type (Groenewald and Botha, 2001). In contrast, down-regulation of PFP activity in transgenic tobacco (Nielsen and Stitt, 2001; Paul et al., 1995) and potato (Hajirezaei et al., 1994) plants suggest that PFP does not play an essential role in carbon partitioning to either sucrose or starch pools. PFP catalyses the reversible reaction between fructose-6-phosphate (F6-P) and pyrophosphate (PPi) to fructose-1, 6-bisphosphate (F1, 6-P_2) and inorganic phosphate (Pi) (Carnal and Black, 1979). Earlier work also showed that a negative correlation exists between PFP activity and sucrose levels in the maturing sugarcane culm (Whittaker and Botha, 1999). Varieties that accumulate higher sucrose concentrations also have lower PFP activity (Whittaker and Botha, 1999). As mediator of the hexose-phosphate: triose-phosphate cycle in potato and tobacco (Dennis and Greyson, 1987; Fernie et al., 2000), it has been proposed that PFP contributes to the status of the hexose-phosphate pool in sink tissue by regulating the carbon flux between sucrose accumulation, respiration and/or the insoluble component (Whittaker and Botha, 1999; Hajirezaei et al., 1994).

Hexose-phosphate: triose-phosphate cycling is high in immature tissue of sugarcane, returning up to 50% of carbon from the triose-phosphate pool to the hexose-phosphate pool. With the onset of maturation there is a marked reduction in cycling, indicating that it might play a regulatory role in sucrose metabolism (Bindon, 2000). In

addition, cycling within the sucrose pool also decrease two-fold with maturation (Whittaker and Botha, 1997). Sucrose cycling has been proposed to play a pivotal role in metabolic flexibility and control of sucrose accumulation *in vivo* (Rose, 2001; Whittaker and Botha, 1997; Rohwer and Botha, 2001).

It has been proposed that these cycles operate in conjunction with each other in sugarcane internodal tissue, balancing the supply to sucrose storage on the one hand and sucrose utilization on the other (Moore and Maretzki, 1996). The link between these cycles and their role in carbon partitioning are, however, questioned in transgenic tobacco and potato plants (Nielsen and Stitt, 2001; Fernie et al., 2000; Paul et al., 1995; Hajirezaei et al., 1994). These results indicate that reduced PFP activity did not lead to enhanced sucrose or starch pools, although enhanced hexose-phosphates levels were evident. In addition, a decrease in the hexose-phosphate: triose-phosphate cycling led to no alteration in sucrose cycling.

In light of these discrepancies, it is evident that a more detailed examination of the metabolic consequences of a perturbation in PFP activity in sugarcane is essential. In this study previously generated sugarcane transgenic lines (Groenewald and Botha, 2001) displaying reduced endogenous PFP activity were analyzed on a broad biochemical basis. Firstly, the prevailing levels of a wide range of metabolite were determined by GC-MS. Quantification of phosphorylated intermediates was done by means of enzymatic and HPLC analyses (Chapter 3). Secondly, in a complementary approach, we determined the flux between metabolites by ^{13}C NMR (Chapter 4). These experiments highlighted the changes in substrate cycling that were the result of genetic modification. The new insights obtained from this investigation indicated that the two cycles in question was not co-regulated in sugarcane internodal tissue. In addition, it was suggested that the increased sucrose levels in these internodes were the result of increased substrate concentrations, and that sucrose cycling might be an important sink for elevated sucrose levels (Chapter 5).

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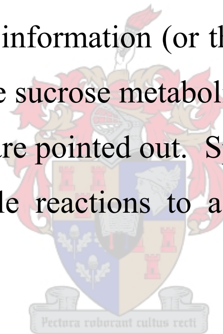
Chapter 2

A PERSPECTIVE ON SUCROSE METABOLISM IN SUGARCANE SINK TISSUE

Abstract

Sugarcane sucrose metabolism and its manipulation has been the focus of several research groups in the past. Despite these efforts, the principal mechanism(s) that control accumulation is still unclear. The major progress that has been made in elucidating sucrose metabolism over the last forty years is reviewed, and the distinguishing feature(s), which could discriminate between sugarcane genotypes that differ in their ability to accumulate sucrose, are highlighted. These traits might be important factors for breeders and molecular biotechnologists alike in the quest to improve the sucrose content of sugarcane. In this chapter vital information (or the lack thereof) regarding the known control mechanism(s) for sugarcane sucrose metabolism is highlighted and potential areas where further research is required are pointed out. Special emphasis is placed on the shift from the characterization of single reactions to a more holistic approach to unravel metabolic control mechanisms.



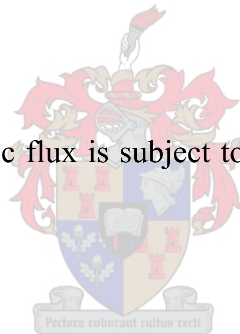
2.1 Introduction

Sugarcane is an important socio-economic crop that is capable of an impressive biomass yield and a large capacity to store sucrose (± 62% per dry weight biomass (Bull and Glasziou, 1963)). However, little is known about the key physiological and genetic determinants that control sucrose accumulation and hence the final levels of this metabolite in the culm. The power of the detailed characterization of the sucrose accumulation process as a whole has only been recently realised and the application of tools such as proteomics (Zhu et al., 2001; von Mering et al., 2002), transcripteomics (Vincentz et al., 2004; Shevchenko et al., 1996; Millar et al., 2001; Reinders et al., 2002; Chang et al., 2000), metabolomics (Fiehn et al., 2000; Roessner et al., 2000) and fluxomics (Sanford et al., 2002) greatly increases the choice of finding these control element(s).

In this review sucrose metabolism in sugarcane will focus solely on the culm tissue, i.e. the predominant sink organ of sugarcane. The reason for this is three-fold. Firstly, due to the nature of sucrose metabolism, genetic manipulation of the whole plant would probably result in a negative effect on growth and yield due to extra energy expenditures. Secondly, the demand of the system (i.e. sink strength) favours the accumulation of storage compounds (Hofmeyer et al., 1999). Thirdly, sugarcane (C4) source leaves produce abundant quantities of carbon to be exported to the sinks (McDonald, 2000). These plants are thus not source limited in terms of crop yield and productivity, and their differing abilities to store sucrose must lie in the sink tissue. For the purpose of the study, the term source relates to a plant organ that produce photosynthate in excess of its own needs, while the term sink relates to a plant organ that does not produce enough photosynthate and rely on import mechanisms to fulfil these carbohydrate needs.

2.2 Coarse vs. fine control

The magnitude of metabolic flux is subject to both long-term (coarse) and short-term (fine) controls.



2.2.1 Coarse control

Coarse control is energetically expensive (Plaxton, 1996) and involves the modulation of gene expression trough transcription, translation, mRNA processing, and –degradation, or protein turnover (Plaxton, 1996). The study of gene regulation through transcription and translation are well documented (for review, Plaxton, 1996) and techniques to quantify RNA are routinely used. However, considering proteomic and transcripteomic approaches it is apparent that a remarkable low correlation exists between actual RNA and protein levels (Gygi et al., 1999). It has been indicated that numerous posttranslational modifications, including protein phosphorylation and possibly *O*-glycolsylation and *S*-nitrosylation modify plant proteins significantly (for review, Huber and Hardin, 2004).

It has also been indicated that a large number of genes are expressed having no homology to known gene function across kingdoms (Casu et al., 2003; Carson et al.,

2002, see Fig 2.3). Identification of these gene functions is essential but is complicated by isozyme expression. Isozymes catalyze the same enzymatic reaction, but have distinct kinetic properties. They may reside in different compartments and may be differently expressed with developmental stage indicating that their expression could act as important control elements. Biotechnological approaches to assign gene function are therefore complicated by the available technologies that is not selective enough to down-regulate all isoforms in a single approach.

In comparison, very little is known about the role protein turnover plays in regulating enzyme activity. Protein half-life's are significantly shorter than those of a cell, ranging from 0.1 to > 200h (Dennis et al., 1997). This implicates that protein turnover can control the cell's protein abundance and hence it needs to be a finely coordinated process.

2.2.2 Fine control

Fine (metabolite) control is energetically inexpensive (Plaxton, 1996) and allows for great metabolic flexibility within the system. Usually these fine control mechanisms allow for an altered state, e.g. environmental condition or genetic modification, to retain its steady state levels. These mechanisms include 1) an alteration in substrate concentration, 2) variation in pH, 3) subunit association-dissociation modulation, 4) reversible covalent modification, 5) allosteric effectors and 6) reversible association of metabolically sequential enzymes (for review, Plaxton, 1996). They often interact with each other and modulation of metabolism usually involves the coordinated control of more than one or all of these factors (Plaxton, 1996). Elucidation of gene function and genetic improvement programs have been severely complicated by fine control exerted on enzyme expression. The elucidation of these mechanisms is thus essential. Since allosteric effectors constitute such a significant proportion of fine control, the application of metabolomics might aid us in the understanding of the fine-tuning mechanism in place for metabolism.

2.3 Sucrose metabolism in the sugarcane sink

Although the classical biochemical route of the sucrose accumulation process is known, research has not indicated which route(s) control the accumulation processes and/or which routes could be manipulated for a more "successful sugarcane". Work generated during the past four decades has pointed to two prominent features, namely: the occurrence of a continuous cycle of synthesis and degradation of sucrose (i.e. "futile" or substrate cycling) and the dominance of compartmentation of sucrose in the sugarcane sink.

2.3.1. Two major substrate cycles could be controlling sucrose accumulation

Sucrose accumulation is characterized by a cycle of continuous synthesis and degradation, termed futile cycling due to the apparent energetically wastefulness in terms of nucleotide triphosphate usage required during the process. Sucrose is accumulated against a concentration gradient, necessating energy in the form of these nucleotide triphosphates. However, opinion has shifted from a wasteful process to a necessary plant function in terms of its adaptability and possible metabolic control function (Dancer et al., 1990). In sugarcane, two major substrate cycles have been identified. One involves the synthesis and degradation of sucrose, i.e. sucrose cycling (Whittaker and Botha, 1997); while the other equilibrates the hexose-phosphate and triose-phosphate pools (Bindon and Botha, 2000).

Sucrose synthesis occurs exclusively in the cytosol facilitated by either/or the action of sucrose phosphate synthase (SPS, E.C 2.4.1.14) and sucrose synthase (SuSy, E.C. 2.4.1.13) (synthesis direction) (Fig 2.2). It appears that in immature tissue both of these enzymes contribute equally to sucrose synthesis (Botha and Black, 2000). With the onset of maturation SPS activity increases and exceeds that of SuSy threefold (Botha and Black, 2000), indicating that SPS carries the bulk of responsibility for sucrose synthesis in mature tissues (Batta and Singh, 1986; Wendler et al., 1990; Botha and Black, 2000). In addition, depending on the maturation stage, different kinetic forms of SPS exist in the internodal tissue (Botha and Black, 2000).

Sucrose degradation, on the other hand, is possible in the apoplastic space, cytosol or vacuole (Fig 2.2). It is facilitated by the action of the invertases (β-D-

fructofuranosidase, E.C 3.2.1.26), or SuSy (degradation direction). Depending on the pH optima, invertases are distinguished into two groups: 1) the insoluble acid invertases (CWI, cell wall invertases) operating in the apoplastic space or the soluble acid invertases (SAI) concentrated in the vacuolar space, and 2) the neutral invertases (NI) residing in the cytosolic sub cellular compartment. Invertase enzymes have been suggested as pivotal regulators of sucrose accumulation in sugarcane storage parenchyma (Gayler and Glasziou, 1972; Hatch and Glasziou, 1963; Sacher et al., 1963). In high-storing sucrose cultivars NI activity increases before the onset of sucrose accumulation in storage tissue (Hatch and Glasziou, 1963) and a significant correlation exists between the hexose pool and NI activity in mature internodes (Gayler and Glasziou, 1972). AI activity is also high in merismatic regions, but decreases by up to two orders of magnitude with maturation (Hatch and Glasziou, 1963).

SuSy favours neutral pH optimas and operate in the cytosol. Its activity decreases with maturity and three isoforms are currently known to exist in sugarcane (Schafer, et al., 2004).

Sucrose substrate cycling occurs via both the action of SPS and/or SuSy (synthesis direction) activity (in young tissue) and a concomitant action of either invertase and/or SuSy (degradation direction). Radiolabel distributions indicate that approximately 30% of the sucrose that is synthesised is returned to glucose and fructose in immature internodal tissue (Whittaker and Botha, 1997). Other results also indicate different sucrose cycling rates between high and low storing sucrose genotypes (Rose, 2001).

The sucrose substrate cycle operates in conjunction with another cycle further down the glycolytic pathway (Moore and Maretzki, 1996), although this link has been questioned (Fernie et al., 2001). This cycle, which equilibrates the hexose-phosphate: triose-phosphate ratio, has been hypothesised to be mainly controlled by the action of PFP in non-heterotrophic sugarcane tissue (Whittaker and Botha, 1997). In young internodal tissue, it appears that up to 50% of the carbon in the hexose-phosphate pool is returned from the triose-phosphate pool (Bindon, 2000), and this cycling decreases in older internodes. With the onset of maturation, sucrose futile cycling also decreases

(Rose, 2001). Thus sucrose, in conjunction with hexose-phosphate: triose–phosphate turnover might be key determinants in the ability of tissue to accumulate more sucrose.

Central and connecting both these cycles in sugarcane are the hexose-phosphate pool that operates close to equilibrium *in vivo* (Whittaker and Botha, 1997).

2.3.1.1 Assessing substrate cycles in vivo

Triose-phosphate: hexose-phosphate cycling can be followed by label distribution between C1 and C6, since randomization of carbon occurs at both triose-phosphate and hexose phosphate isomerasation. Following tissue incubation with labeled glucose substrates in carbon position 1, label detection in position 6 in fructose, sucrose or glucose will give an indication of triose-phosphate: hexose phosphate cycling (Fig 2.1).

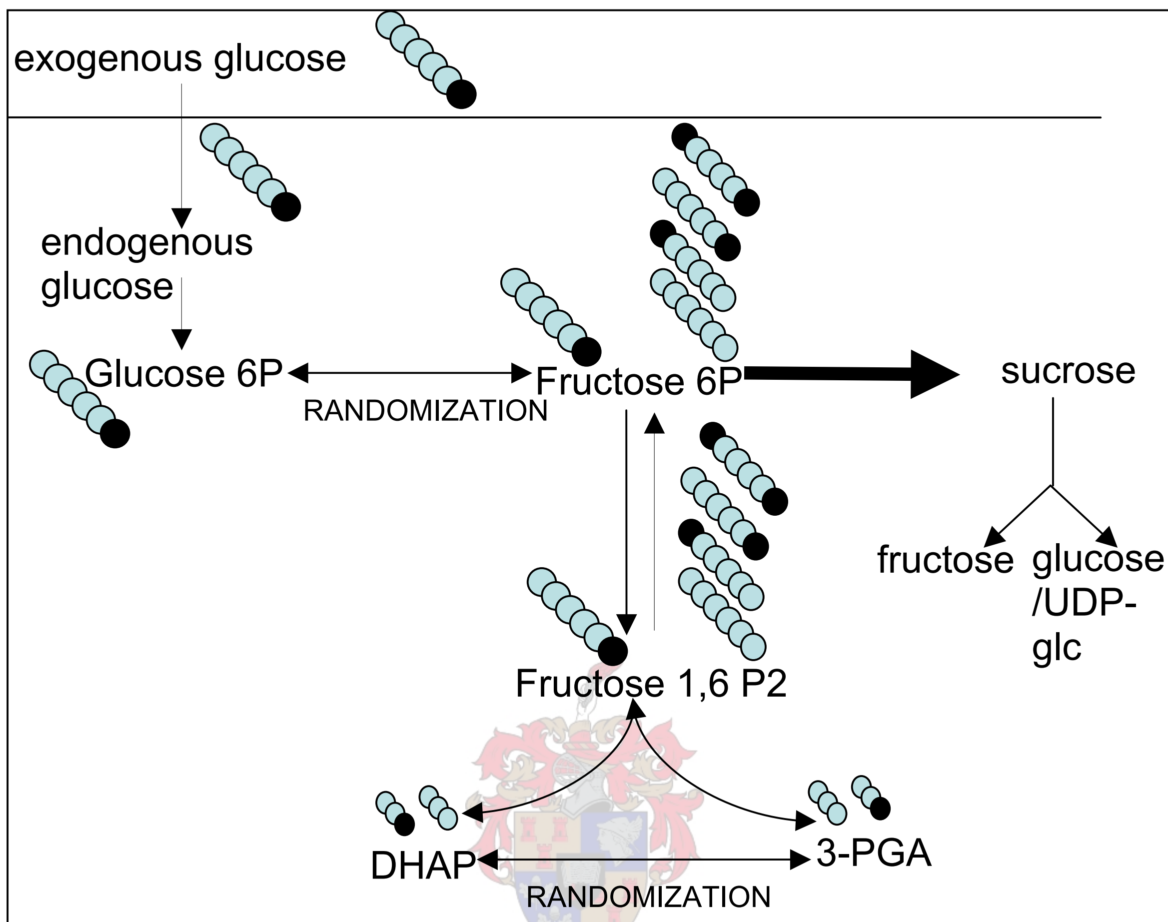


Fig 2.1 Illustration of the experimental setup of isotopomer discrimination following 1-^{13}C glucose labeling. Quantification of carbon annotations C1 relative to C6 in sucrose, glucose and fructose will indicate triose-phosphate: hexose-phosphate cycling and total label detection in fructose relative to total label in sucrose will assess sucrose cycling. DHAP=dihydroxyacetone phosphate, 3-PGA=3-phosphoglycerate, UDP-glc=uridinyl diphospho-glucose

Furthermore, sucrose cycling can be assessed with the label distribution within the fructose moiety when feeding with labeled glucose. Labeled fructose will only be liberated with sucrose degradation from invertase and/or SuSy (degradation direction) activity, and can be taken as an indication of sucrose degradation, and consequently sucrose cycling *in vivo* (Fig 2.1). Since sucrose cycling is a function of sucrose accumulation, sucrose degradation must be several order of magnitude lower than sucrose

synthesis (or maintaining a certain ratio; Zhu et al. 1997) in order for sucrose accumulation to occur.

2.3.2 Compartmentation of sucrose

Sucrose accumulation is characterised by concentration gradients, as well as intracellular compartments. These features ensure a more prevalent sucrose concentration in the more mature regions of the sugarcane culm tissue (Rosenfeld, 1956). The three compartments involved in the process of sucrose accumulation are identified as being the outer space (the apoplast and cell walls), the metabolic compartment (cytosol) and storage compartment (i.e. the vacuole) (Sacher et al., 1963) (Figure 2.2). In contrast to sugar beet where these gradients are, well-defined, limited information is available for sugarcane. In fact, some work indicate that the highest concentration exist in the cytosol (Welbaum and Meinzer, 1990).

Reduced carbon in the form of sucrose is translocated from the source and unloaded to the sink tissue. This unloading appears to be mainly facilitated via the apoplastic mechanism in the young tissue, while a more symplastic approach is followed in the more mature regions of the culm (McDonald, 2000, and references therein). This point of entry is potentially important since it is the first site where sucrose accumulation may be regulated with arrival at the sink tissue (see also section 2.5.1). Subsequently, sucrose is either unloaded into the cytosol or re-synthesised from the hexose moieties cleaved in the apoplast by CWI (Fig 2.2).

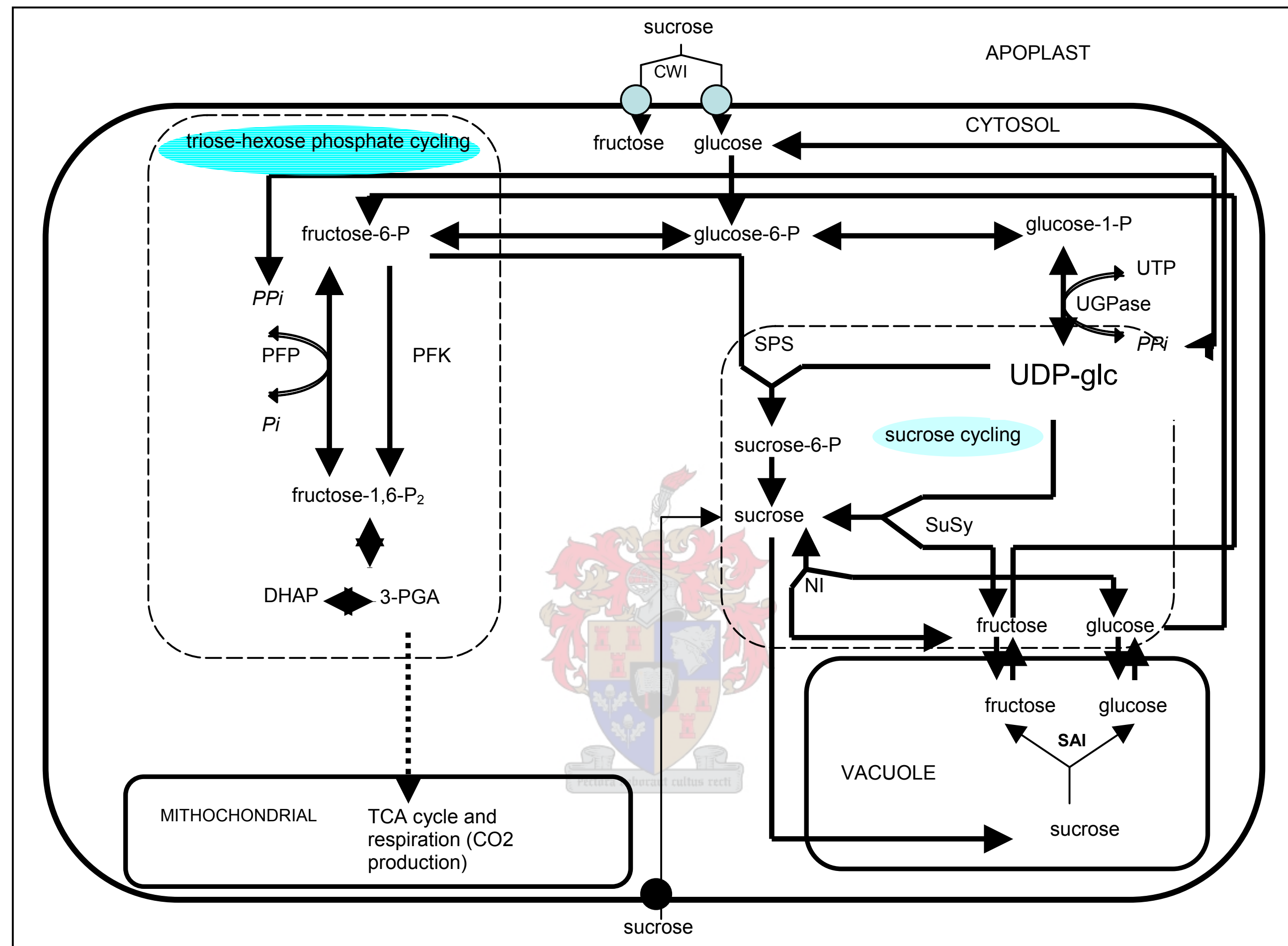


Figure 2.2 Sucrose metabolism in the sugarcane sink. *(Adapted from Krook et al., 2000)* CWI=cell wall invertase, SuSy=sucrose synthase, NI=neutral invertase, SAI=soluble acid invertase, SPS=sucrose phosphate synthase, PFK=phosphofructokinase, DHAP=dihydroxyacetone-phosphate,3-PGA=3-phospho-glycerate, PPi=pyrophosphate, Pi=inorganic phosphate, CWI=cell wall invertase, PFP= pyrophosphate: D-fructose-6 phosphate 1-phosphotransferase, TCA=tricarboxylic acid cycle, UTP= uridine 5'-triphosphate, UDP-glc= uridine 5'-tdiphosphate glucose, UGPase= uridine 5'-diphosphate glucose pyrophosphorylase

Depending on the distribution of sucrose between the cytosol and vacuole, it is clear that very different flux estimates of sucrose synthesis and degradation are possible (Bindon, 2000). Therefore, one of the pressing needs is the understanding of the spatial and sub-cellular compartmentation of sucrose, glucose and fructose, and the rest of the metabolites. Unfortunately, due to the nature of the culm the feasibility of micro-imaging techniques such as micro-autoradiography, tissue printing, single photon bioluminescence, and the introduction of chemical probes or even NMR imaging are limited.

2.3.3 A virtual sugarcane parenchyma cell

Most research efforts in genetically enhancing sucrose content has relied on the random modification of characterized reactions in the sucrose pathway and the subsequent analysis of the effect such a alteration has had on the plant metabolism. This approach is due to the limited information available on the regulation of sucrose accumulation, along with the complexity of the process. One way of following a more directed approach is by the development and application of tools that could accurately predict reactions that are important to sucrose accumulation in sugarcane. For this purpose, a kinetic metabolic model was constructed (Rohwer and Botha, 2001) simulating a part of the sucrose accumulation process (http://jjj.biochem.sun.ac.za). Thus far, features identified via this approach that may have significant effects on the sucrose accumulation process includes sucrose futile cycling which in turn, according to the model, are modulated by the actions of fructose uptake and phosphorylation, glucose uptake and/or the activity of neutral invertase. NI may break down up to 22% of the sucrose that is being synthesized. Refinement of the model is currently underway, and might prove to be a valuable aid in terms of its prediction capacity.

2.4 Genotypical differences

Modern sugarcane cultivars are polyploid hybrids of multispecific crossing of *Saccharum officinarum, S. barberi, S. sinense* and the wild species *S. spontaneum* and *S. robustum* (http://watson.fapesp.br/sucest.htm). Sucrose storing capabilities in these ancestral cultivars range from high (21% per fresh mass basis for *S. officinarum*

(Balakrishnan et al., 2000) to lower concentrations (<6% per fresh mass basis (*S. spontaneum)* (Bull and Glasziou, 1963) and <10% per fresh mass basis (*S. robustum*) (Ramana Rao et al., 1985). However, chromosomal evidence and *in situ* hybridization studies suggests that a large percentage of modern sugarcane hybrids retain *S. officinarum* qualities (Ming et al, 2001). Interspecific hybrids that vary in their ability to accumulate sucrose have subsequently been attributed to both morphological (Bull and Galsziou, 1963) and/or enzymatic (Hatch and Glasziou, 1963) factors. This contributes to numerous differences when screening genotypes for sucrose accumulation characteristics. The selectivity in genetic alteration of a single factor makes transgenic material a powerful biotechnological tool to assign gene function.

S. officinarum generally has low fiber content, thick stalks and is adapted to tropical conditions. *S.spontaneum*, on the other hand, is fibrous, thin-stalked and is geographically more adapted to a wider range of climates (Ming et al., 2001). Modern interspecific cultivars with high sucrose storing capabilities generally have thick stalks with a low fibre content and high moisture content (Bull and Glasziou, 1963), contributing to a general increase in the fresh weight mass of these stalks. Low sucrose accumulating interspecific hybrids, on the other hand, are thin, fibrous and has a low stalk mass. Therefore, a physical barrier may have been set for sucrose accumulation in the latter. There is no difference in leaf area and photosynthetic activity between interspecific hybrids that can accumulate different amounts of sucrose (Moore et al., 1997). Subsequently, differences in sucrose accumulation must lie in the translocation system and/or the accumulation and storage facility of the sink tissue.

Several enzymatic factors have been implicated in determining the sucrose capacity of a cell (see also section *2.3.1*). Cytosolic NI expression decreases during maturity in high storing varieties of sugarcane, while in low sucrose storing varieties NI activity is constitutively being expressed (Venkataramana et al., 1991). Further studies concentrating on varieties differing in their sucrose accumulation rates reveal that soluble acid invertase (SAI) activity decrease during maturation (Hatch and Glasziou, 1963; Gayler and Glasziou, 1972; Zhu et al., 1997), but contradicting results regarding this was also reported (Vorster and Botha, 1999). Final sucrose accumulation rate, it seems, is rather controlled by the difference between the expression of SPS activity and that of

soluble acid invertase (SAI) (Zhu et al., 1997). SPS activity is sometimes induced (Botha and Black, 2000), other times it remains constant (Zhu et al., 1997). Consequently, sucrose synthesis must exceed sucrose degradation in order for sucrose accumulation to occur. Therefore, under certain conditions, SuSy activity may either increase (Lingle and Smith, 1991) or decrease (Botha and Black, 2000) as long as sucrose synthesis exceeds degradation.

2.4.1 Coarse control reveals that the mature internode is still an active metabolic entity

Sugarcane research focused on the steps involved in sucrose synthesis, i.e. those catalysed by SPS and sucrose synthase (SuSy (synthesis direction)) activity. Comparing high and low storing sucrose genotypes, no significant difference was detected in sucrose synthesis rates. Differences in sucrose accumulation must therefore be attributed to 1) the rate of sucrose translocation from the source and/or 2) the degradation rates of sucrose in the sink tissue.

However, despite the onset of maturation the expression of enzymes involved in sucrose synthesis and cleavage and a majority of enzymes involved in glycolysis and the pentose phosphate pathway is evident in mature internodes (Casu et al., 2003; Carson et al., 2002). In addition, selected glycolytic metabolites also indicate that, with maturation, a large proportion of carbon is still directed to biosynthetic activities other than sucrose accumulation (Whittaker and Botha, 1999). This implicates that, despite the dedication to sucrose accumulation, the mature internodes are still metabolically active.

2.4.2 Fine control in sugarcane

Currently, little information is available on the fine metabolic control on sucrose metabolism in sugarcane. It has been indicated that protein phosphorylation (increase sensitivity to inhibition by Pi) controls SPS expression in sugarcane cell suspension cultures (Wendler et al., 1990; Goldner et al., 1991). This increase in sensitivity was alsoi evident in maturing internodal tissue (Botha and Black, 2000). In addition, sugarcane PFP is activated 60-fold by the addition of 1 μM exogenous $F2,6P_2$ (Lingle and Smith, 1991).

Current strategies to study metabolic fine control can greatly increase our knowledge concerning this topic (see section 2.5.2), but are still in early stages of development.

2.5 The dynamic power of the technologies that unravel coarse and fine control in sugarcane

2.5.1 Coarse control: the case for ESTs

Advancement in the field of genetic assessment of sucrose accumulation lies in the discipline of expressed sequence tag (EST) expression. ESTs are generated from bacterial or phage duplication of cDNAs. This approach reveal that during sucrose accumulation little change in the percentage of genes expressed concerned directly with carbohydrate metabolism are observed (Carson et al., 2002). In agreement, Casu et al. (2003) observe that these carbohydrate-modulating genes (CMGs) only constitutes about 2.1% (immature) and 2.5% (mature) of the entire EST dataset analysed. A larger correlation between the percentage of regulatory genes expressed and the level of maturity is displayed (Carson et al., 2002, see Figure 2.3). An increase of 12% is observed for cDNAs which are implicated in actions mainly concerned with coarse control (protease-, kinase activity, transporter proteins, ran proteins expression and ubiquitin (or –conjugated) activators of protein turnover) of metabolism. In addition, EST matches associated with protein synthesis increases greatly with the onset of maturation.

Furthermore, CMG data reveals a significant shift in mature ESTs towards sugar transporter matches (Casu et al., 2003). The study reveals that 27 EST matches are identified as hexose-phosphate transporters and five as sucrose transporters in the mature internodes. One of these putative transporters, PST type 2a, is localized in the phloem transport system, suggesting an efficient translocation system. It is also proposed that some transporter systems may act as additional sugar sensors (Barker et al., 2000). Sucrose transporters are suggested as potential control points of sucrose accumulation (Rohwer and Botha, 2001) as it is the first sites of entry of reduced carbon at the sink tissue. The identification and characterization of membrane-bound hexose transporters or

vacuolar sucrose importers might be promising avenues to explore in genetic sugarcane manipulation.

Apart from the evident increase in regulatory and transporter ESTs with maturity, there seems to be an 11% reduction in the expression of ESTs that are involved in central hexose, pentose and triose-phosphate metabolism (Casu et al., 2003) during maturation. Within this EST set, the most significant matches correlate with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNAs. The significance of this match in mature internodal tissue still needs to be assessed.

EST data generated from Carson et al. (2002) were limited due to an insufficient sugarcane EST database. Since the report, the SUCEST database for sugarcane has become available (http://exiba.dcc.unicamp.br), which consists of nearly 300 000 ESTs from 37 libraries constructed from various organs and tissues at different stages of development (Grivet and Aruda, 2001, http://exiba.dcc.unicamp.br).

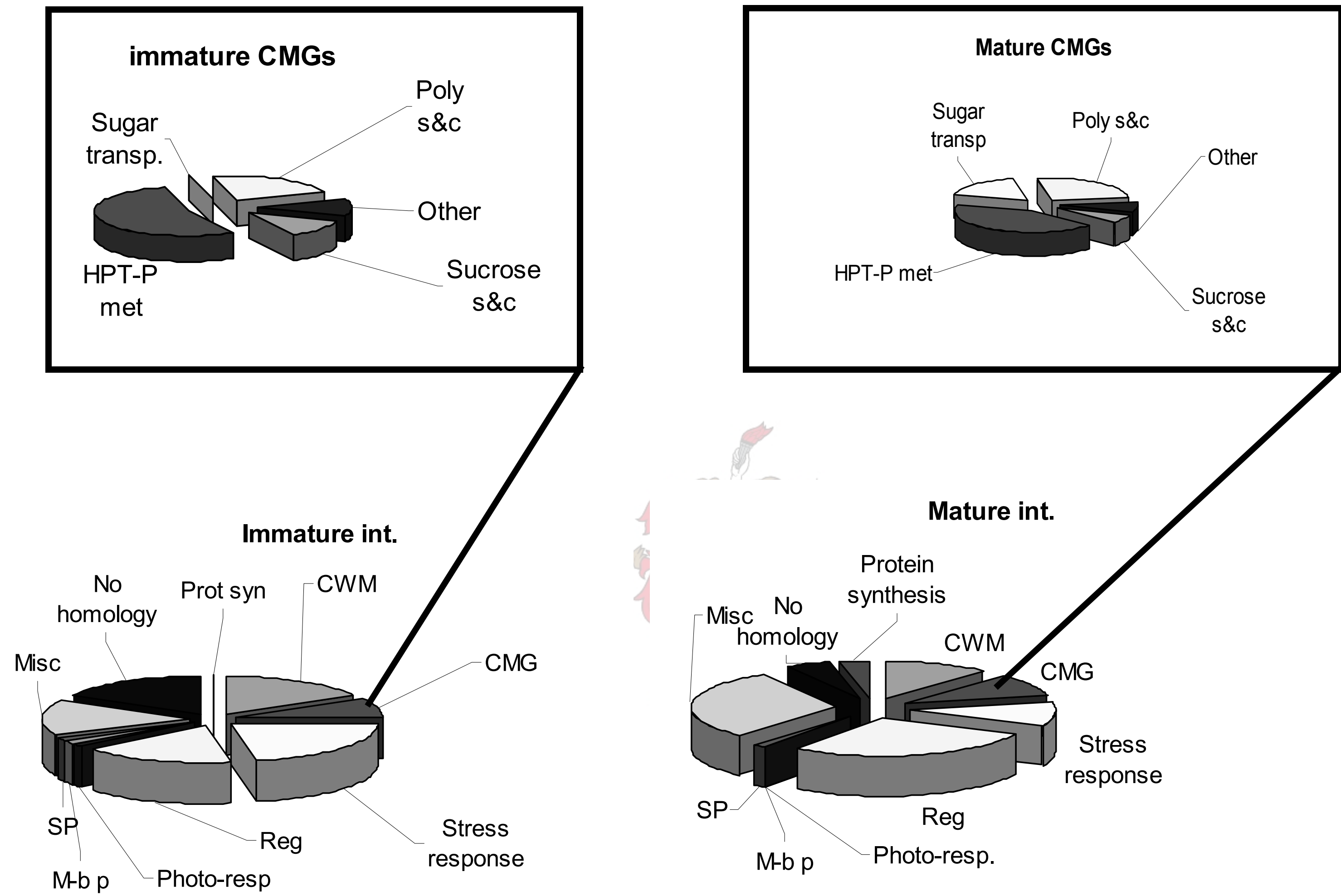


Figure 2.3: The expression of ESTs in sugarcane in immature and mature internodal tissue (Redrawn from data from Carson et al., 1997, and Casu et al., 2003). transp= transport; Poly=polysaccharide; s&c=synthesis and catalysis; HPT-P=hexose-, pentose- and triose-phosphate; CWM=cell wall metabolism; CMG=carbohydrate modulating genes; M-b p=metal-binding protein; SP=structural proteins; Misc=miscellaneous; Reg=regulatory elements; resp=respiration

2.5.2 Fine control: metabolomics

Metabolite profiling, the elucidation of the small molecule components of a cell or tissue or organ has emerged as a useful technique in plant biochemistry to study fine metabolic control. While it is not a novel technique *per se*, the advance of metabolomics into the holistic approach towards elucidation of plant function and control provides a great complementation toward approaches such as the genomic-, transcripteomic- and proteomic revolutions. If we regard the genome as representative of the potential in a cell, the proteome of what is being expressed in the cell, then the metabolome provides an understanding of the prevailing status of the cell.

The plant metabolome consist of approximately 5,000-200,000 compounds (http://www.hos.ufl.edu/meteng/HOS6231-2002) and no analytical technique exist that can quantify all these compounds. In addition to classical spectrometry and high pressure liquid chromatography (HPLC) techniques, the most commonly used analytical method for the simultaneous analysis of sugars, sugar phosphates and -alcohols, amino acids and organic acids is the profiling capacity of the bench-top gas chromatography coupled to mass spectrometry (GC-MS). These techniques have increased metabolic resolution from a few selected individual metabolites to an impressive analysis of approximately 150 metabolites in plant tissues (Roessner et al., 2000). Double derivatized samples are first fractioned by a gas chromatography and the resultant peak is then further scanned within the spectrometer to yield mass fragments with specific mass to charge ratios (m/z values). These are used to identify and quantify peak sizes (for further information, http://www.shsu.edu/~chemistry/primers.gcms.html). The choice of technology is motivated by the combination of chromatographic separation power, selectivity, sensitivity, the dynamic range of mass detection and the extremely high reproducibility (Roessner et al., 2000). Unfortunately, due to the changing nature of the metabolite pools in response to several conditions, biological variability within test samples contribute greatly to standard errors observed (Roessner et al., 2000). In addition, the half-life times of metabolites are short (ranging from hrs, minutes to seconds) and artefacts may arise in short intervals between harvest and extraction times.

Key in this metabolic profiling data is unlocking the information contained within. Simple statistical methods such as hierarchical cluster analysis (HCA, Fiehn

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et al., 2000; Roessner et al., 2000), principal component analyses (PCA, Fiehn et al., 2000; Roessner et al., 2000), discriminate function analysis (DFA, Raamsdonk et al., 2001) and simple correlation matrices (Roessner et al. 2000; Kose et al., 2002) are currently being used. HCA cluster subsets together based on their maximal similarity, while PCA separate data based on maximal difference.

In addition to steady state metabolite analysis, nuclear magnetic resonance (NMR) detection of stable isotope incorporation emerged as an alternative to study metabolic flux. NMR relies on the measurement and quantification of energy produced by nuclei spin with excitation from magnetism. Metabolic flux represents the interaction of prevailing metabolite levels in a system. One of the reasons for a stable isotope approach is the non-invasive manner of labeling, which reduces the risk of artifact formation arising from radio-label cell disruption. In addition, specific carbon atom labeling can be followed closely in order to gain a better understanding in the cycling capacities of the cells/tissues in question. The combination of these approaches could help to unravel fine control *in vivo* and were utilized in the current project.

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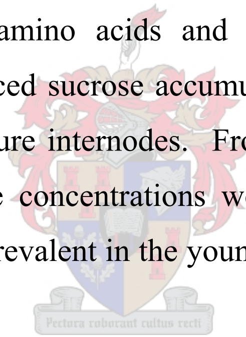
CHAPTER 3

Metabolic profiling of transgenic sugarcane clones with reduced PFP activity

Abstract

In an attempt to construct a metabolic snapshot of sucrose accumulation, transgenic sugarcane clones with reduced levels of cytosolic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity has been analysed at three stages of maturity. Four independent sugarcane transgenic clones representing extreme reduction in pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (5-55% remaining of control pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase) were analysed by gas chromatography-mass spectrometry. Although the clones displayed no phenotypical change, metabolic profiling indicated significant changes in the prevailing metabolite levels. Sucrose concentration increased six- and three-fold in immature and mature internodes, respectively. Analysis indicated that hexose-phosphates, triose-phosphates, amino acids and PPi levels underwent the most significant changes when enhanced sucrose accumulation was evident in the control maturing- and transgenic immature internodes. From these results it was suggested that increased hexose-phosphate concentrations were most likely to drive sucrose synthesis. This was especially prevalent in the young internodes, but diminished with maturation.



3.1 Introduction

Maturation of the sugarcane culm is associated with the pronounced accumulation of sucrose (Glazsiou and Gaylor, 1972; for review, Moore, 1995). The accumulation is associated with the metabolic (re)cycling of sucrose, hexose-monophosphates and triose-phosphates within the stem parenchyma of both young and mature tissues (Whittaker and Botha, 1997; Vorster and Botha, 1999; Bindon and Botha, 2000). This cycling allows for the continued functioning of glycolysis and other secondary processes involved in growth and development during later stages of maturity, and have been implicated in regulating sucrose metabolism (Bindon, 2000).

Cytosolic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) has emerged as a potential target for molecular manipulation of sucrose content in sugarcane. PFP catalyses the reversible reaction between fructose-6-phosphate (F6-P) and pyrophosphate (PPi) to fructose-1,6-bisphosphate (F1,6-P_2) and

inorganic phosphate (Pi) (Carnal and Black, 1979). The exact physiological function of PFP in plants still needs to be determined but it is implicated in equilibrating the hexose- and triose-phosphate pools (Dennis and Greyson, 1987; Fernie *et al.*, 2000). In addition, it is proposed that this cycle works in conjunction with the sucrose-hexose cycle, balancing the supply of sucrose on the one hand and the demand for carbon in respiration and biosynthesis on the other (Moore and Maretzki, 1996). In addition, PFP activity is inversely correlated with sucrose levels in sugarcane culms (Whittaker and Botha, 1997). This implicates that the enzyme could act as an important regulator of carbon flux between sucrose accumulation, respiration and/or the insoluble component (Whittaker and Botha, 1999; Hajirezaei *et al*., 1994). Down-regulation of PFP activity in transgenic tobacco (Nielsen and Stitt, 2001; Paul *et al.*, 1995) and potato (Hajirezaei *et al.*, 1994) plants suggests however that PFP does not play an essential role in carbon metabolism. In contrast, results from immature transgenic sugarcane clones suggest a 50% increase in sucrose content (Groenewald and Botha, 2001).

In light of this discrepancy, the need for further investigation is evident. Since metabolic changes occur in the sugarcane stem tissue during sucrose accumulation it is expected that genetic manipulation of the sugarcane genome will be accompanied by unforeseen changes in metabolite levels. Therefore, a full-scale metabolic profile was compiled in order to assess metabolic divergence within transgenic sugarcane clones altered in their PFP expression. Here we report that the reduction in PFP activity in sugarcane culms led to significant increases of sucrose and hexose-phosphates in the young internodes. In addition, a decrease in triose-phosphate- and amino acid content was evident. These alterations seemed to faze out with maturation.

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3.2 Materials and Methods

3.2.1 Plant material

Clones of *Saccharum* spp. hybrid variety NCo 310 with varying degrees of reduced PFP β expression were grown under prevailing environmental conditions in Stellenbosch, South Africa. Culms with approximately 14 aboveground internodes were randomly selected and harvested. Internodal tissue selected for analysis was excised from the stalk and the rind carefully removed. The first internode from

which the leaf with the first exposed dewlap originates was defined as internode one (Van Dillewijn, 1952).

The underlying tissue, spanning the core to the periphery, was rapidly sliced, homogenized and frozen in liquid nitrogen within 1h after harvesting. Four individual stalks were used as replicates.

3.2.2 Biochemicals

All auxiliary enzymes, cofactors and substrates used for enzyme assays and metabolite determinations were purchased from either Sigma Aldrich Fluka (SAF) Chemical Company (St. Louis, MO, USA) or Roche Diagnostics (Basel, Switzerland), unless stated otherwise.

3.2.3 Enzyme extraction and measurement

3.2.3.1 Protein extraction

The extraction procedure for the measurement of PFP activity was carried out at $4^{o}C$ according to the method of Lingle and Smith (1991). Crude extracts were prepared by homogenization of internodal tissue in liquid nitrogen. The ground tissue was suspended in ice-cold extraction buffer in a buffer volume to tissue mass of 2:1 and continuously stirred for 15 min. The standard extraction buffer contained 100mM Tris-Cl (pH 7.2), 2 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 2 % (m/v) PVPP and 10% (v/v) glycerol. Extracts were centrifuged for 5 min ($4^{o}C$) at 10 000 xg and the supernatants retained.

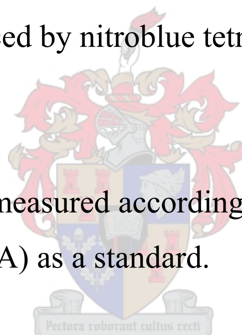
3.2.3.2 SDS PAGE and protein blotting

Polypeptides in crude protein extracts were separated by SDS-PAGE (Theodorou and Plaxton, 1996). Samples were resolved on a discontinuous 10% (m/v) polyacrylamide (acrylamide/bis-acrylamide, 37.5:1) gel followed by a 3% (m/v) stacking gel (Laemmli, 1970). A low molecular weight calibration kit for electrophoresis (Amersham Biosciences, Buckinghamshire, UK) was used as molecular weight standards. Samples were concentrated with a 10% (v/v) trichloroacetic acid (TCA) precipitation (Harris and Angal, 1989) and 20 μg crude protein was loaded per lane.

The resolved polypeptides were transferred for 4 min at 12V to a nitrocellulose membrane, Hybond™-C Extra (Amersham Life Science Ltd., Buckinghamshire, UK) using the Trans-Blot® SD system (Bio-Rad). The transfer buffer contained 48 mM Tris, 39 mM glycine, 0.0375% (m/v) SDS and 20% (v/v) methanol. The nitrocellulose membrane was subsequently blocked overnight in TBST (Tris-buffered saline buffer containing Tween, 137 mM NaCl, 20 mM Tris (pH 7.6), 0.1% (v/v) Tween-20) containing 4% (m/v) BSA at 4°C. Blots were probed for 5hrs, RT with potato (*Solanum tuberosum*) PFP-β antiserum (1:500 dilution in TBST buffer). Probed blots were washed three times with TBST for fifteen minutes each before 1h incubation in the secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG, 1:2000 dilution in TBST buffer containing 3% (m/v) fat free milk powder). The blots were rinsed once and washed twice for 10 min in TBST containing 0.05% (m/v) SDS followed by two 10 min washes with TBST-buffer. Cross-reacting polypeptides were stained with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, toluidine salt) and enhanced by nitroblue tetrazolium (NBT).

3.2.3.3 Protein determination

The protein content was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.



3.2.3.3 PFP activity

PFP activity was measured in the glycolytic direction according to Kruger *et al*. (1983). The standard reaction mixture contained 100 mM Tris-Cl (pH 7.5), 1 mM MgCl2, 10 mM Fru-6-P, 0.1 mM NADH, 10 μM Fru-2,6-P2, 1U aldolase, 10 U triose-phosphate isomerase and 1 U glycerol-3-P dehydrogenase. Activity was initiated by the addition of 1 mM PPi. NADH oxidation at 340 nm was followed on a Beckman DU®7500 spectrometer to quantify PFP activity.

3.2.4 Metabolite determination

3.2.4.1 GC-MS analyses

To analyse the levels of primary metabolites a GC-MS protocol for plant material (Roessner et al., 2000) were optimised for sugarcane tissue. Extraction procedures were optimised for the simultaneous determination of selected hydrophilic

metabolites including sugars, sugar phosphates, organic acids and amino acids. Recovery experiments indicated that among boiling ethanol (Vorster and Botha, 1998), trichloroacetic acid (Jelitto et al., 1992; http://www.jic.bbsrc.ac.uk/SERVICES/metabolomics/tcaether), and methanol/chloroform/water (Whittaker and Botha, 1999) extractions procedures, the latter confirmed the best overall recoveries. ± 100 mg homogenized internodal tissue stored at –80°C were extracted with 430µl 100% pre-chilled methanol, 30 µl 20 mM Na-Hepes (pH 8.5) containing 2 mM EDTA and 150µl 100% chloroform with 10 µl 3.94 µg. $µl^{-1}$ ribitol (in Hepes buffer) as internal standard. The mixture was incubated for 10 min at 28°C, centrifuged for 15 min at 4000 x*g* and the supernatant transferred to clean tubes.

100 µl of supernatant were dried down in a rotary evaporator, RT. The dried residue was subsequently redissolved and derivatized for 90 min at 30°C in 80 µl 30 $mg.ml^{-1}$ methoxyamine hydrochloride (in pyridine). Samples were trimethylated by a 30 min treatment at 37°C containing 140 µl MSTFA. 20 µl of a retention time standard mixture was also added prior to trimethylisation. The retention time standards consisted of 3.7% (w) heptanoic acid, 3.7% (w) nonanoic acid, 3.7% (w) undecanoic acid, 3.7% (w) tridecanoic acid, 3.7% (w) pentadecanoic acid, 7.4% (w) nonadecanoic acid, 7.4% (w) tricosanoic acid, 22.2% (w) heptacosanoic acid, 55.5% (w) hentriacontanoic acid dissolved in tetrahydrofuran to contain 23.8 ng of each alkane per injection. Peak integration was done by means of the ICIS algorithm and metabolite levels were quantified by the establishment of linear calibration curves as described by Roessner et al. (2000).

3.2.4.2 Enzymatic coupled assays

Metabolites determinations via spectrometry and HPLC analyses were extracted according to Whittaker and Botha (1997). Samples dried down under vacuum were resuspended in deionised distilled H2O. The following metabolites were measured enzymatically at 340 nm, using a DU® 7500 spectrophotometer (Beckman): PEP and pyruvate (Czok and Lamprecht, 1976); DHAP, G3P and F-1,6-P_2 (Michal and Beutler, 1974), sucrose, glucose and fructose (Bergmeyer and Bernt, 1974); Glc-6-P, Fru-6-P (Lang and Michal, 1974) and G1P, F-2,6-P_2 (Scott and Kruger, 1994) and PPi and Pi (Bergmeyer and Bernt, 1974; Drueckes, Schinzel and Palm 1995). Glc-6-P, F-6-P and G-1-P were measured by the consecutive addition of

0.1 U/assay 6-phosphogluconase, 0.1 U/assay hexose phosphate isomerase and 0.1 U/assay phosphoglucomutase to the standard reaction mixture (Lang and Michal, 1974). The amount of F-2,6-P2 were measured through the stimulation of potato tuber PFP. The assay buffer contained 25 mM Tris-Acetate (pH 7.8), 2 mM Mg-Acetate, 1 mM F-6-P (acid treated to remove traces of F-2,6-P_2), 0.24 mM NADH, 0.1 U/assay aldolase, 1.6 U/assay TPi, 4.5 U/assay GDH and 2.7 mU/assay potato PFP. The PFP reaction was initiated by the addition of 0.4 mM PPi in an assay volume of 230 µl. After 3 min in the plate reader, a volume of 20 µl containing the metabolite extracts (2 µl) or internal standards was added. The internal standards refer to metabolite extracts from which the endogenous F-2,6-P2 content was hydrolyzed by the addition of 0.1 volumes of 2M HCl, followed by a 15 min incubation period, RT. The extracts were then neutralized by the addition of an equal volume of 2 M NaOH. The solution were kept on ice for 2 min, followed by the addition of 10 mM Tris-Acetate (pH 7.8) to insure that the extracts contained the same amount of pre-mixed ions. The extracts were spiked with authentic F-2,6-P_2 (0.05 and 1 pmol) standards for linear calibration quantification of F-2,6-P_2 for each sample.

PPi and Pi concentration was determined colorimetrically with the addition of 2.4 U/assay inorganic pyrophosphatase and 0.02M $MgCl_2$ solution to the standard reaction mixture. The standard reaction mixture contained 40 µl ammonium molybdate reagent (15 mM zinc acetate and 10mM ammonium molybdate (pH 5.0) and 160 µl reducing agent (10% ascorbic acid (pH 5.0)). Pi and PPi concentration in sugarcane internodal tissue were calculated by comparing authentic standards from a linear curve to the absorbance (A) measured at 660nm at 34°C.

Coupling factors were desalted prior to use in the enzymatic assays by centrifugation at 10 000 xg, 4°C. The pellet was resuspended in ddH_2O.

3.2.4.3 Nucleotide and nucleotide sugar determinations

Extracted nucleotides and nucleotide sugars were filtered through Millex-GV4 (0.22 µm) units (Millipore) prior to injection onto the high-performance liquid chromatography (HPLC) system. Adenylates (ATP, ADP, and AMP) and uridinylates (UTP, UDP, UDPglucose) were separated in extracts (10 µl injection volume) by HPLC on a 150 x 4.6 mm Phenomenex Aqua (5µm) C18 125A column

(Torrance, CA, USA) according to a modified method of Viola *et al*. (1994). Nucleotides were detected by their absorbance at 254 nm and identified by co-chromatography with authentic nucleotide standards. The quantity of each nucleotide and nucleotide sugar was determined by comparing the sample peak areas to those of the corresponding standard. Mobile phases constituted of (A) 0.1 M KH_2PO_4/K_2HPO_4 and 5 mM tetrabutylammonium hydrogen sulphate (pH 6.0) and (B) 70% A and 30% methanol. The linear flow gradient employed was 0.9 ml.min^{-1} for A and 0 ml.min^{-1} for B for the first 10 min, after which the gradient was changed to 0.8 ml.min^{-1} (A) and 0.5 ml.min^{-1} (B). After 20 min the program was set to gradually refer back to the original flow rates. The column temperature was maintained at 30°C throughout.

3.2.4.4 Statistical analyses

HCA and PCA statistical analyses were conducted on the Statistica software package (StatSoft, Inc. (2003). STATISTICA (data analysis software system), version 6. www.statsoft.com.). Student t-tests (two-tailed) were performed on the Microsoft® Excel 2002 software package (Microsoft Corporation Inc., Seattle, USA). The term significant is used to indicate differences for which $P < 0.05$.

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3.3 Results

Protein content expressed on a cell basis does not differ between internodes (Bindon and Botha, 2000). This agrees with previous findings that the protein content per internode and total cell number remains stable in sugarcane (Botha et al., 1996). Results will therefore be compared on a soluble protein basis to compensate for changes in the volume of the cytosolic (metabolic) compartment. Results have also been confirmed by two separate experimental setups.

3.3.1 Evaluation of PFP activity in internodal tissue of the transgenic sugarcane clones

The expression profile of PFP in a series of previously generated transgenic clones (Groenewald and Botha, 2001) (OPu50_) was determined in order to select internodal tissue representing extremes in PFP expression. The PFP protein product was extracted from eight putative transgenic clones and subjected to enzymatic

coupled assays. Results indicated that all the transgenic sugarcane clones expressed less PFP protein on a soluble protein basis compared to that of the control (data not shown). Of these clones, the four representing the most severe reduction in PFP expression was selected for further biochemical analyses (Fig. 3.1). In addition, control protein blotting indicated a positive band that decreased in intensity with the onset of maturation, while polypeptide cross-reactivity stains in the transgenic lines ranged from slight detection to below detection level in all the lanes (data not shown). The transgenic sugarcane clones displayed no phenotypical change in regards to culm morphology.

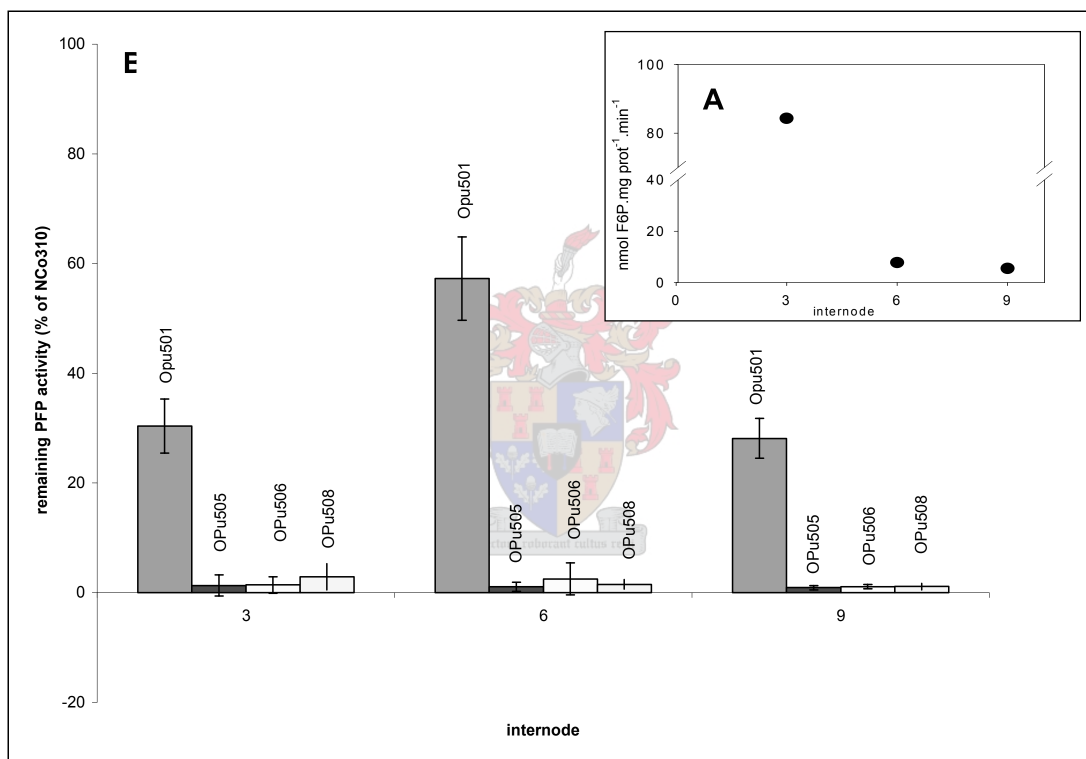

Fig 3.1 The expression of PFP activity in NCo 310 (**A**). PFP activity in four (OPu 501, 505, 506, 508) selected transgenic clones, expressed as a percentage of the activity present in NCo 310 (**B**). Values presented are the mean ± SE (n=4).

3.3.2 Metabolite analyses

3.3.2.1 Development of a method for metabolite analyses of sugarcane tissue

Genetic confirmation and quantification of the transgene resulted in biochemical analyses of metabolite levels by GC-MS. Heat labile metabolites were determined by enzymatic coupled assays and HPLC (Table 3.1).

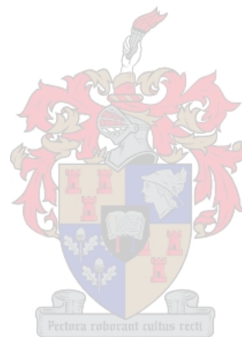

Table 3.1 *Metabolites recoveries in a methanol/chloroform plant extract from sugarcane NCo 310 internode 3. Metabolites were determined by GC-MS, HPLC and enzymatic analyses.* nd = metabolite was not detected in the sugarcane extract

Method of analyses	Metabolite	% Recovery		SE (n=4)
Enzymatic	Pi	91	±	0.7
	PPi	91	±	0.3
	F2,6-P2	86	±	2.6
	F1,6-P2	98	±	1.9
	pyruvate	72	±	1.9
	PEP	64	±	3.6
	DHAP	70	±	2.8
	3-PGA	85	±	1.0
	G6P	78	±	1.1
	F6P	86	±	1.7
	G1P	89	±	6.0
	sucrose	110	±	37.0
	glucose	101	±	6.0
	fructose	94	±	3.0
HPLC	ATP	91	±	3.4
	ADP	100	±	4.4
	AMP	82	±	6.2
	UDP	113	±	1.0
	UTP	81	±	4.7
	UDP-glc	97	±	4.5
GC-MS	succinate	94	±	10.7
	malate	105	±	10.8
	2-oxoglutarate	nd	±	
	tartrate	nd	±	
	shikimic acid	102	±	1.6
	citrate	118	±	9.5
	xylose	106	±	21.5
	arabinose	155	±	53.3
	ribose	116	±	6.8
	fructose	107	±	39.5
	glucose	156	±	35.5
	galactose	76	±	5.4
	sorbitol	98	±	15.9
	inositol	108	±	21.1
	sucrose	106	±	11.2
	maltose	97	±	4.3
	trehalose	117	±	6.6
	alanine	95	±	14.3
	valine	88	±	15.4
	serine	91	±	21.2
	leucine	nd	±	
	isoleucine	88	±	9.0
	glycine	150	±	4.4
	threonine	71	±	3.3
	methionine	nd	±	
	aspartic acid	83	±	13.3
	trans-4-hyroxy-proline	115	±	20.4
	glutamic acid	nd	±	
	phenylalanine	nd	±	
	asparagine	nd	±	
	glutamine	94	±	4.4
	lysine	nd	±	
	histidine	nd	±	
	tyrosine	nd	±	
	tryptophan	nd	±	
	cystine	nd	±	

For most metabolites recoveries above 80% were recorded, with the exception of pyruvate, PEP, DHAP, G6P and threonine (Table 3.1). To verify the methodologies selected metabolites were quantified with different methodologies and compared. The levels of sucrose, glucose and fructose were not statistically different when detected enzymatically or by GC-MS. Also, the levels of UDP-glucose were not significantly different between HPLC and enzymatic analyses (data not shown).

3.3.2.2 Comparison of metabolite data in developing internodal tissue

The metabolite levels at the three different stages of maturation (internode 3, 6 and 9) were compared in the NCo 310 control sugarcane culm (Table 3.2).

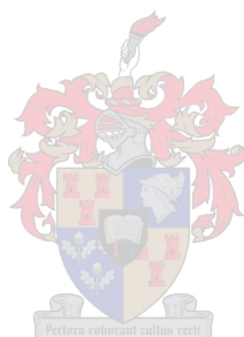

Table 3.2 *Levels of metabolites in internodes 3, 6 and 9 in the culm of NCo 310.* Values presented are the mean ± SE (n=4). nd indicates that the compound was not detected.

Metabolite	Metabolite concentration (nmol.mg^{-1} prot)					
	Internode					
	3		6		9	
2-oxoglutarate	42.0 ±	23	25.8 ±	26	nd ±	
aconitic acid	935.5 ±	18	222.2 ±	32	599.7 ±	495
ADP	14.2 ±	6	27.4 ±	3	13.0 ±	3
alanine	272.9 ±	185	343.0 ±	85	122.1 ±	58
AMP	18.8 ±	9	35.9 ±	3	35.2 ±	5
arabinose	15681.6 ±	5398	22686.3 ±	1302	15586.5 ±	2007
asparagine	nd ±		nd ±		nd ±	
aspartic acid	276.4 ±	171	500.5 ±	248	650.6 ±	642
ATP	41.4 ±	12	59.0 ±	12	69.5 ±	14
citric acid	60.8 ±	18	139.6 ±	26	60.6 ±	16
cysteine	41.5 ±	30	267.3 ±	267	1101.9 ±	1102
DHAP	151.3 ±	29	49.7 ±	8	32.2 ±	3
F1,6-P_2	29.4 ±	22	44.2 ±	10	45.3 ±	4
F2,6-P_2	0.2 ±	0	0.5 ±	0	0.2 ±	0
Frc6P	32.8 ±	5	50.6 ±	4	34.4 ±	3
fructose	195383.6	66459	29548.9	3271	101662.3	18428
Glc1P	14.8 ±	1	37.4 ±	7	32.7 ±	7
G3P	128.9 ±	17	50.5 ±	7	24.9 ±	2
Glc6P	59.9 ±	11	84.1 ±	14	41.9 ±	11
galactose	9216.5 ±	5717	42742.9 ±	7971	35674.7 ±	7439
glucose	675431.8 ±	241834	1160042.9 ±	338765	1434690.9 ±	502276
glutamic acid	294.5 ±	295	nd ±		43.2 ±	43
glutamine	2.8 ±	3	nd ±		12.8 ±	8
glycine	267.6 ±	170	385.7 ±	133	64.5 ±	34
histidine	nd ±		nd ±		nd ±	
inositol	1291.3 ±	769	690.7 ±	95	174.2 ±	45
isoleucine	429.9 ±	164	1388.9 ±	1297	547.8 ±	480
leucine	nd ±		nd ±		nd ±	
lysine	nd ±		nd ±		nd ±	
malic acid	582.9 ±	94	465.4 ±	170	175.6 ±	48
maltose	152.2 ±	95	48.2 ±	8	43.9 ±	24
methionine	74.1 ±	38	21.8 ±	13	21.2 ±	19
PEP	26.9 ±	4	51.8 ±	4	23.4 ±	4
phenylalanine	nd ±		nd ±		nd ±	
Pi	2318754.7 ±	395729	7294258.0 ±	1932082	4059555.0 ±	1283497
PPi	99.6 ±	23	403.0 ±	84	169.9 ±	31
pyruvate	34.5 ±	1	51.8 ±	4	26.3 ±	2
quinic acid	174.0 ±	69	162.7 ±	57	4.1 ±	2
raffinose	86.2 ±	86	6871.4 ±	2383	18952.8 ±	8616
rhamnose	nd ±		nd ±		nd ±	
ribose	8.1 ±	6	8.3 ±	2	1.2 ±	1
serine	108.2 ±	85	2038.8 ±	1935	18.3 ±	5
shikimic acid	77.9 ±	30	133.0 ±	11	7.3 ±	3
sorbitol	6.5 ±	5	1490.1 ±	370	1540.0 ±	198
succinic acid	60.3 ±	15	101.7 ±	20	2.8 ±	2
sucrose	74130.9 ±	15071	94551.7 ±	24850	172007.7 ±	49066
tartric acid	nd ±		nd ±		nd ±	
threonine	1123.4 ±	120	5538.4 ±	311	2296.5 ±	212
trans-4-hydroxy-proline	3415.9 ±	187	6161.8 ±	278	1019.1 ±	153
trehalose	347.3 ±	24	549.2 ±	24	2133.4 ±	167
tryptophan	nd ±		nd ±		nd ±	
tyrosine	nd ±		nd ±		107.4 ±	107
UDP	5.2 ±	2	4.8 ±	2	5.0 ±	1
UDP-glc	28.5 ±	2	27.1 ±	10	65.5 ±	18
UTP	7.2 ±	5	11.3 ±	2	10.5 ±	7
valine	464.3 ±	108	1749.7 ±	783	729.9 ±	630
xylitol	50476.7 ±	39362	20691.2 ±	3392	11115.3 ±	478
xylose	15.7 ±	9	nd ±		5.6 ±	5

3.3.2.2.1 Sugars, sugar alcohols and sugar phosphates

Sucrose concentration underwent the most significant change with maturation. Typical for sugarcane tissue the lowest concentration was found in internode 3. A six-fold increase in sucrose content was evident between the youngest and oldest internode. Glucose levels increased with maturity, while fructose levels remained constant (Table 3.2).

The concentrations of xylitol, galactose, arabinose and inositol were higher than those of raffinose and trehalose. Furthermore sugar and sugar alcohol concentrations varied greatly with maturation. Trehalose, raffinose and sorbitol levels increased with maturation, while maltose, galactose and inositol levels decreased with maturation (Table 3.2).

The sugar-phosphates displayed two definite trends. The triose-phosphates (DHAP, G3P) decreased with the onset of maturation. On the other hand, F2,6-P_2 levels were similar in internodes 3 and 9, but were two-fold higher in internode 6 than in the young and older internodes. Similarly, the highest concentrations of PEP, pyruvate and F1,6-P_2 was found in internode 6. The hexose-phosphate pool also exhibited the highest metabolite levels at internode 6 (Table 3.2).

3.3.2.2.2 Organic- and amino acids

Total organic acid content decreased with maturation as illustrated by the concentrations of 2-oxoglutarate, aconitic-, malic-, quinic-, shikimic- and succinic acid. Citric acid content were two-fold higher at internode 6 than in either internode 3 or 9 (Table 3.2).

On the other hand, amino acid concentration increased with maturation, with the highest concentrations at internode 6. The exceptions to this statement included the levels of alanine, glutamine and methionine (Table 3.2).

3.3.2.2.3 Nucleotides, nucleotide sugar, inorganic phosphate and pyrophosphate

ADP, UDP, PPi and Pi had the highest concentrations at internode 6. In contrast, AMP and UTP concentrations were higher in the young than the maturing internodes. ATP and UDP-glucose levels increased with maturation (Table 3.2).

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3.3.2.2.4 Mass action ratios

Metabolite ratios for selected glycolytic and sucrolytic reactions indicated no significant change in substrate ratios in the NCo 310 sugarcane culm. Ten-fold changes for SuSy, fructokinase and FBPase activity was, however, recorded between internode 3 to 6 (Table 3.3).

Table 3.3 Mass action ratios (Γ) for selected glycolytic and sucrolytic reactions in internodes 3, 6 and 9 of NCo 310 and transgenic lines (OPu 501, OPu 505, OPu 506 and OPu 508)*.

	PFK [F1,6-P2][ADP]/[F6P][ATP]	**PFP** [F1,6-P2][Pi]/[F6P][PPi]	**FBPase**[B] [F6P][Pi]/[F1,6-P2]	**HPi** [F6P]/[G6P]	**PK** [pyr][ATP]/[PEP][ADP]	**SuSy (deg)** [Frc][UDPglc]/[Suc][UDP]	**UDPgPPase** [UTP][G1P]/[UDPglc][PPi]	**PGM** [G6P]/[G1P]	**TPi** [DHAP]/[G3P]	**Aldolase** [G3P][DHAP]/[F1,6-P2]	**PEPase** [pyr][Pi]/[PEP]	**HK** [G6P][ADP]/[Glc][ATP]	**FK** [F6P][ADP]/[Fruc][ATP]	**Adenylate kinase** $[ADP]^2/[ATP][AMP]$
Keq=[A]	300-1800	3.3	174	0.36-0.51	$3.19X10^5$-$1.15X10^8$	0.15-0.56	2.9-3.6	19						
310 3	4.292	291.740	0.440	0.547	3.745	0.544	0.000019	4.0620	1.1740	0.2253	7064.651	0.000015197	0.000028756	0.25820
310 6	0.813	31.676	7.269	0.601	2.154	0.047	0.000019	2.2482	0.9854	0.0990	12729.537	0.000016832	0.000397376	0.35474
310 9	0.491	62.957	2.507	0.821	6.043	0.161	0.000015	1.2816	1.2915	0.0288	7444.030	0.000002721	0.000031544	0.06882
501 3	0.749	56.828	12.655	1.378	3.141	0.706	0.000031	1.0733	1.3963	0.0514	29758.741	0.000175319	0.000455346	0.25955
501 6	2.361	266.061	5.017	0.739	1.135	0.702	0.000008	1.2822	1.2637	0.0868	13445.433	0.000037042	0.000173853	0.48803
501 9	0.572	177.745	3.883	1.922	3.941	0.126	0.000018	0.8197	1.1657	0.0546	8758.657	0.000042872	0.000198777	0.21475
505 3	0.108	3.243	34.751	0.639	7.175	3.484	0.000001	5.3939	0.9612	0.1958	26954.660	0.000002168	0.000006314	0.19505
505 6	0.583	18.865	8.530	1.273	4.226	1.621	0.000004	1.1932	0.7542	0.1415	21002.513	0.000001685	0.000005662	0.82729
505 9	0.128	20.306	3.549	1.287	14.566	0.793	0.000026	0.9485	0.8483	0.0493	6625.869	0.000001015	0.000002633	0.04577
506 3	0.059	6.672	30.349	0.910	14.102	7.165	0.000005	2.0681	0.8453	0.1471	25422.381	0.000000287	0.000000449	0.02547
506 6	0.097	70.022	5.146	0.606	18.089	4.907	0.000039	1.3539	0.7094	0.0468	9037.079	0.000000766	0.000000511	0.00518
506 9	0.312	221.742	3.257	1.579	12.354	1.533	0.000036	0.6594	0.9294	0.0305	12540.385	0.000000702	0.000001186	0.00701
508 3	0.008	13.679	126.815	1.278	34.980	14.755	0.000206	0.6754	0.8418	0.1060	34754.581	0.000000006	0.000000067	0.01482
508 6	0.037	145.765	24.387	1.308	46.946	19.062	0.000031	2.9787	0.8368	0.0241	42683.629	0.000000402	0.000000564	0.00504
508 9	0.060	37.573	7.684	0.949	31.112	3.682	0.000003	1.1055	0.8352	0.0311	14325.283	0.000000387	0.000000468	0.00525

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* In order to compensate for 10% cytosolic volume and to mM metabolite concentrations the following data were used:
internode 3 – protein content: 2.28mg protein.g^{-1} fresh mass; FW:DW: 13.69
internode 6 - protein content: 1.50mg protein.g^{-1} fresh mass; FW:DW: 7.14
internode 9 - protein content: 1.22mg protein.g^{-1} fresh mass; FW:DW: 5.00

[A]Theoretical constants are from Whittaker, 1997; Farré et al., 2001, and references therein; and in mM

[B] FBPase theoretical constant and Γ are in M concentrations

3.3.2.3 Comparison of metabolite data in transgenic and wild-type transgenic sugarcane clones

In order to allow easy comparison the metabolic data were normalized in respect to that of the control (Table 3.4, 3.5 and 3.6) and the control internode assigned an arbitrary value of 1.

Table 3.4 *Relative metabolite levels in the culm of internode 3 of the control and antisense PFP transgenic sugarcane clones.* Metabolites were determined in methanol/ chloroform/water extracts. Values presented are the mean ± SE (n=4). nd indicates that the compound were not detected.

	310 3	501 3	505 3	506 3	508 3
2-oxoglutarate	1.00 ± 0.55	1.19 ± 0.00	0.59 ± 0.08	0.27 ± 0.13	0.08 ± 0.07
aconitic acid	1.00 ± 0.02	1.50 ± 0.97	6.44 ± 1.55	7.99 ± 0.29	5.90 ± 1.58
ADP	1.00 ± 0.43	1.47 ± 0.34	0.63 ± 0.24	0.19 ± 0.07	0.14 ± 0.03
alanine	1.00 ± 0.68	0.04 ± 0.01	0.74 ± 0.23	0.96 ± 0.59	0.05 ± 0.02
AMP	1.00 ± 0.45	1.60 ± 0.39	0.46 ± 0.11	0.39 ± 0.19	0.23 ± 0.02
arabinose	1.00 ± 0.34	1.05 ± 0.45	1.45 ± nd	0.97 ± 0.26	0.82 ± 0.09
aspartic acid	1.00 ± 0.62	1.53 ± 0.06	5.38 ± 3.28	5.18 ± 3.77	0.34 ± 0.15
ATP	1.00 ± 0.28	1.35 ± 0.27	1.16 ± 0.33	0.98 ± 0.47	1.40 ± 0.17
citric acid	1.00 ± 0.29	1.00 ± 0.07	1.39 ± 0.19	0.50 ± 0.19	0.31 ± 0.11
cysteine	1.00 ± 0.72	2.33 ± 0.77	18.60 ± 8.26	10.61 ± 6.68	0.13 ± 0.11
DHAP	1.00 ± 0.19	0.45 ± 0.05	0.34 ± 0.08	0.36 ± 0.11	0.19 ± 0.06
F1,6-P2	1.00 ± 0.11	0.76 ± 0.03	0.17 ± 0.02	0.28 ± 0.08	0.11 ± 0.03
F2,6-P2	1.00 ± 0.16	0.60 ± 0.09	0.70 ± 0.21	0.46 ± 0.11	0.61 ± 0.03
F6P	1.00 ± 0.29	4.74 ± 1.20	3.63 ± 1.00	3.98 ± 0.43	6.00 ± 1.20
fructose	1.00 ± 0.34	0.33 ± 0.17	9.07 ± 2.85	50.33 ± 26.69	252.75 ± 210.87
G1P	1.00 ± 0.19	7.12 ± 2.64	2.35 ± 0.92	4.70 ± 0.38	15.45 ± 1.71
3PGA	1.00 ± 0.13	0.38 ± 0.02	0.42 ± 0.18	0.50 ± 0.21	0.27 ± 0.05
G6P	1.00 ± 0.38	1.88 ± 0.15	3.11 ± 0.92	2.39 ± 0.10	2.57 ± 0.98
galactose	1.00 ± 0.62	0.93 ± 0.35	0.08 ± 0.02	0.99 ± 0.75	0.14 ± 0.04
glucose	1.00 ± 0.36	0.18 ± 0.04	11.96 ± 6.56	25.00 ± 16.72	618.02 ± 360.07
glutamic acid	1.00 ± 1.00	0.48 ± 0.34	nd ± nd	nd ± nd	nd ± nd
glutamine	1.00 ± 0.94	0.28 ± 0.19	0.08 ± 0.04	5.29 ± 4.06	0.05 ± 0.04
glycine	1.00 ± 0.64	0.38 ± 0.10	0.46 ± 0.01	2.68 ± 2.05	0.07 ± 0.03
inositol	1.00 ± 0.60	0.83 ± 0.10	0.05 ± 0.01	0.35 ± 0.19	0.09 ± 0.03
isoleucine	1.00 ± 0.38	1.21 ± 0.34	1.94 ± 0.64	4.31 ± 3.06	0.12 ± 0.06
malic acid	1.00 ± 0.16	2.11 ± 0.05	0.55 ± 0.14	0.95 ± 0.03	0.54 ± 0.05
maltose	1.00 ± 0.62	0.46 ± 0.18	0.14 ± 0.04	0.25 ± 0.16	0.01 ± 0.01
methionine	1.00 ± 0.52	0.28 ± 0.19	1.32 ± 0.90	0.82 ± 0.68	3.15 ± 2.69
PEP	1.00 ± 0.28	1.50 ± 0.47	1.09 ± 0.60	0.97 ± 0.68	0.49 ± 0.10
Pi	1.00 ± 0.26	4.61 ± 2.65	3.64 ± 2.47	4.84 ± 3.52	5.40 ± 2.95
PPi	1.00 ± 0.23	3.78 ± nd	15.04 ± 2.04	14.82 ± 2.59	2.15 ± 0.36
pyruvate	1.00 ± 0.05	1.37 ± 0.27	1.14 ± 0.43	0.72 ± 0.22	0.45 ± 0.18
quinic acid	1.00 ± 0.39	3.78 ± 0.29	0.16 ± 0.04	0.48 ± 0.38	nd ± nd
raffinose	1.00 ± 1.00	1.89 ± 1.34	0.42 ± 0.37	nd ± nd	nd ± nd
ribose	1.00 ± 0.74	0.44 ± 0.07	0.05 ± 0.04	0.84 ± 0.57	0.05 ± 0.04
serine	1.00 ± 0.78	0.21 ± 0.03	0.06 ± 0.03	16.88 ± 14.42	0.34 ± 0.19
shikimic acid	1.00 ± 0.38	3.62 ± 0.09	0.43 ± 0.05	0.55 ± 0.22	0.43 ± 0.24
sorbitol	1.00 ± 0.72	10.02 ± 7.09	0.01 ± 0.01	26.19 ± 20.20	3.47 ± 1.62
succinic acid	1.00 ± 0.26	0.42 ± 0.08	nd ± nd	0.02 ± 0.01	nd ± nd
sucrose	1.00 ± 0.60	0.44 ± 0.27	3.88 ± 2.48	6.88 ± 0.57	4.18 ± 1.24
threonine	1.00 ± 0.11	1.52 ± 0.21	2.72 ± 1.08	2.42 ± 1.56	0.06 ± 0.05
trans-4-hydroxy-proline	1.00 ± 0.55	0.99 ± 0.36	0.42 ± 0.13	2.78 ± 1.85	0.27 ± 0.12
trehalose	1.00 ± 0.69	0.56 ± 0.17	0.07 ± 0.06	0.01 ± 0.00	nd ± nd
tyrosine	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd
UDP	1.00 ± 0.35	0.95 ± 0.16	0.63 ± 0.02	0.22 ± 0.10	0.29 ± 0.03
UDP-glucose	1.00 ± 0.08	1.65 ± 0.96	1.73 ± 0.07	3.99 ± 0.85	5.19 ± 0.77
UTP	1.00 ± 0.63	1.43 ± 0.26	0.40 ± 0.00	3.52 ± 2.20	7.92 ± 1.55
valine	1.00 ± 0.23	0.81 ± 0.17	1.90 ± 0.84	2.53 ± 1.84	0.07 ± 0.04
xylitol	1.00 ± 0.78	0.33 ± 0.14	0.26 ± 0.07	0.09 ± 0.08	1.05 ± 0.80
xylose	1.00 ± 0.58	1.96 ± 0.60	nd ± nd	3.55 ± 2.79	0.15 ± 0.13

Table 3.5 *Relative metabolite levels in the culm of internode 6 of the control and antisense PFP transgenic sugarcane clones.* Metabolites were determined in methanol/ chloroform/water extracts. Values presented are the mean ± SE (n=4). nd indicates that the compound were not detected.

	310 6	501 6	505 6	506 6	508 6
2-oxoglutarate	1.00 ± 0.10	1.85 ± 0.40	0.98 ± 0.27	0.58 ± 0.31	0.55 ± 0.35
aconitic acid	1.00 ± 0.14	1.21 ± 0.10	61.76 ± 8.35	18.79 ± 4.56	7.97 ± 1.11
ADP	1.00 ± 0.10	1.70 ± 0.42	1.21 ± 0.29	0.11 ± 0.06	0.13 ± 0.04
alanine	1.00 ± 0.25	3.73 ± 2.03	2.78 ± 1.80	1.20 ± 0.74	1.33 ± 0.32
AMP	1.00 ± 0.09	1.70 ± 0.23	0.41 ± 0.13	0.62 ± 0.40	0.49 ± 0.16
arabinose	1.00 ± 0.06	1.79 ± 0.17	0.67 ± 0.14	1.05 ± 0.40	1.08 ± 0.21
aspartic acid	1.00 ± 0.50	1.93 ± 0.60	6.72 ± 3.51	4.17 ± 0.91	3.55 ± 0.57
ATP	1.00 ± 0.21	1.23 ± 0.09	1.52 ± 0.33	1.27 ± 0.09	2.23 ± 0.18
citric acid	1.00 ± 0.19	0.99 ± 0.28	1.19 ± 0.32	0.22 ± 0.09	0.40 ± 0.10
cysteine	1.00 ± 0.20	10.98 ± 4.92	4.37 ± 2.14	4.17 ± 0.53	7.58 ± 2.67
DHAP	1.00 ± 0.17	1.39 ± 0.15	0.92 ± 0.17	0.58 ± 0.32	0.60 ± 0.35
F1,6-P2	1.00 ± 0.22	1.73 ± 0.33	0.77 ± 0.27	1.00 ± 0.30	1.72 ± 0.42
F2,6-P2	1.00 ± 0.17	1.29 ± 0.20	0.26 ± 0.03	0.49 ± 0.16	0.42 ± 0.08
F6P	1.00 ± 0.15	0.82 ± 0.19	0.85 ± 0.39	0.71 ± 0.20	2.11 ± 0.75
fructose	1.00 ± 0.11	2.59 ± 1.09	47.43 ± 21.33	46.69 ± 13.43	83.51 ± 58.98
G1P	1.00 ± 0.37	1.17 ± 0.25	0.76 ± 0.08	1.17 ± 0.15	0.73 ± 0.24
3PGA	1.00 ± 0.13	1.09 ± 0.26	1.20 ± 0.56	0.81 ± 0.14	0.70 ± 0.12
G6P	1.00 ± 0.34	0.67 ± 0.15	0.40 ± 0.20	0.70 ± 0.05	0.97 ± 0.59
galactose	1.00 ± 0.19	0.66 ± 0.28	0.16 ± 0.07	0.34 ± 0.39	0.24 ± 0.03
glucose	1.00 ± 0.29	0.42 ± 0.18	3.19 ± 2.75	1.31 ± 0.18	2.28 ± 1.45
glutamic acid	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd
glutamine	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd
glycine	1.00 ± 0.34	1.59 ± 0.29	0.84 ± 0.54	2.57 ± 0.27	1.61 ± 0.18
inositol	1.00 ± 0.14	0.61 ± 0.10	0.50 ± 0.26	0.37 ± 0.12	0.18 ± 0.02
isoleucine	1.00 ± 0.93	1.92 ± 0.22	1.41 ± 0.75	1.34 ± 0.30	0.44 ± 0.05
malic acid	1.00 ± 0.36	1.77 ± 0.44	0.75 ± 0.10	0.87 ± 0.35	1.09 ± 0.23
maltose	1.00 ± 0.17	0.56 ± 0.20	0.11 ± 0.04	0.18 ± 0.02	0.09 ± 0.05
methionine	1.00 ± 0.60	6.48 ± 2.17	3.00 ± 2.17	7.31 ± 2.94	6.46 ± 4.10
PEP	1.00 ± 0.16	0.74 ± 0.09	0.55 ± 0.09	0.38 ± 0.13	0.67 ± 0.10
Pi	1.00 ± 0.40	1.45 ± 0.73	1.06 ± 0.34	1.00 ± 0.77	2.74 ± 1.99
PPi	1.00 ± 0.21	0.36 ± 0.03	1.60 ± 0.45	0.64 ± 0.19	0.49 ± 0.09
pyruvate	1.00 ± 0.17	0.54 ± 0.22	0.86 ± 0.09	0.27 ± 0.12	0.81 ± 0.16
quinic acid	1.00 ± 0.35	0.76 ± 0.18	0.73 ± 0.39	0.25 ± 0.03	0.26 ± 0.14
raffinose	1.00 ± 0.35	nd ± nd	0.07 ± 0.03	0.02 ± 0.04	0.01 ± 0.01
ribose	1.00 ± 0.27	1.05 ± 0.12	0.23 ± 0.15	0.21 ± 0.18	0.27 ± 0.04
serine	1.00 ± 0.95	0.07 ± 0.03	0.03 ± 0.01	0.66 ± 0.01	0.02 ± 0.01
shikimic acid	1.00 ± 0.08	1.15 ± 0.25	0.68 ± 0.30	0.23 ± 0.11	0.16 ± 0.07
sorbitol	1.00 ± 0.25	0.00 ± 0.00	0.09 ± 0.06	0.07 ± 0.06	0.10 ± 0.07
succinic acid	1.00 ± 0.20	0.30 ± 0.05	0.03 ± 0.02	0.03 ± 0.01	0.01 ± 0.01
sucrose	1.00 ± 0.20	0.49 ± 0.15	5.78 ± 4.07	6.96 ± 1.24	2.37 ± 0.75
threonine	1.00 ± 0.56	0.81 ± 0.17	2.05 ± 1.24	0.97 ± 0.11	0.28 ± 0.05
trans-4-hydroxy-proline	1.00 ± 0.45	1.33 ± 0.50	0.42 ± 0.12	1.53 ± 0.23	1.71 ± 0.26
trehalose	1.00 ± 0.43	0.01 ± 0.01	0.07 ± 0.07	0.02 ± 0.02	0.01 ± 0.01
tyrosine	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd
UDP	1.00 ± 0.37	2.40 ± 0.34	1.26 ± 0.27	0.49 ± 0.12	0.80 ± 0.14
UDP-glucose	1.00 ± 0.36	6.72 ± 1.00	5.31 ± 1.36	7.66 ± 1.78	9.20 ± 0.94
UTP	1.00 ± 0.19	0.84 ± 0.44	2.54 ± 0.68	8.48 ± 0.96	9.87 ± 1.20
valine	1.00 ± 0.45	1.09 ± 0.14	1.35 ± 0.83	0.92 ± 0.11	0.33 ± 0.03
xylitol	1.00 ± 0.16	1.29 ± 0.27	0.46 ± 0.08	1.13 ± 0.15	0.86 ± 0.18
xylose	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd

Table 3.6 *Relative metabolite levels in the culm of internode 9 of the control and antisense PFP transgenic sugarcane clones.* Metabolites were determined in methanol/ chloroform/water extracts. Values presented are the mean ± SE (n=4). nd indicates that the compound were not detected.

	310 9	501 9	505 9	506 9	508 9
2-oxoglutarate	nd ± nd	nd ± nd	nd ± nd	nd ± nd	nd ± nd
aconitic acid	1.00 ± 0.82	0.81 ± 0.39	7.20 ± 2.43	4.13 ± 1.69	0.78 ± 0.09
ADP	1.00 ± 0.25	2.16 ± 0.80	0.58 ± 0.08	0.44 ± 0.12	0.29 ± 0.12
alanine	1.00 ± 0.48	12.74 ± 11.84	20.32 ± 19.21	4.67 ± 2.08	2.06 ± 0.91
AMP	1.00 ± 0.13	0.76 ± 0.10	0.39 ± 0.11	1.25 ± 0.40	0.58 ± 0.12
arabinose	1.00 ± 0.13	1.84 ± 0.15	1.02 ± 0.20	2.51 ± 0.58	1.92 ± 0.51
aspartic acid	1.00 ± 0.99	5.87 ± 3.71	7.85 ± 2.98	1.45 ± 0.70	2.09 ± 0.90
ATP	1.00 ± 0.20	1.96 ± 0.31	1.32 ± 0.40	1.53 ± 0.08	1.89 ± 0.23
citric acid	1.00 ± 0.26	2.02 ± 0.43	1.63 ± 0.51	0.85 ± 0.21	1.02 ± 0.50
cysteine	1.00 ± 1.00	1.67 ± 0.25	0.68 ± 0.10	0.64 ± 0.13	1.38 ± 0.64
DHAP	1.00 ± 0.10	1.76 ± 0.41	1.05 ± 0.35	1.15 ± 0.49	0.81 ± 0.37
F1,6-P2	1.00 ± 0.09	1.81 ± 0.45	0.99 ± 0.13	1.73 ± 0.29	0.94 ± 0.25
F2,6-P2	1.00 ± 0.30	1.12 ± 0.29	0.77 ± 0.21	2.52 ± 0.34	1.46 ± 0.17
F6P	1.00 ± 0.19	1.72 ± 0.12	1.68 ± 0.51	0.79 ± 0.30	1.19 ± 0.25
fructose	1.00 ± 0.18	0.30 ± 0.11	8.92 ± 6.65	6.03 ± 3.90	12.27 ± 4.22
G1P	1.00 ± 0.44	1.15 ± 0.35	1.45 ± 0.80	0.80 ± 0.17	1.19 ± 0.75
3PGA	1.00 ± 0.10	1.95 ± 0.54	1.60 ± 0.31	1.60 ± 0.29	1.25 ± 0.25
G6P	1.00 ± 0.51	0.73 ± 0.24	1.07 ± 0.29	0.41 ± 0.10	1.03 ± 0.43
galactose	1.00 ± 0.21	0.52 ± 0.16	0.17 ± 0.05	1.15 ± 0.47	0.52 ± 0.10
glucose	1.00 ± 0.35	0.05 ± 0.02	1.28 ± 0.83	0.46 ± 0.15	1.11 ± 0.07
glutamic acid	1.00 ± 1.00	nd ± nd	nd ± nd	nd ± nd	nd ± nd
glutamine	1.00 ± 0.64	0.58 ± 0.39	0.39 ± 0.19	0.02 ± 0.02	0.39 ± 0.32
glycine	1.00 ± 0.52	9.34 ± 4.01	3.32 ± 1.76	6.79 ± 1.60	5.06 ± 1.26
inositol	1.00 ± 0.26	2.22 ± 0.26	1.46 ± 1.09	1.75 ± 0.47	0.59 ± 0.07
isoleucine	1.00 ± 0.88	3.75 ± 1.13	5.69 ± 3.21	1.82 ± 0.77	0.47 ± 0.17
malic acid	1.00 ± 0.27	7.63 ± 1.75	1.23 ± 0.28	1.99 ± 0.94	1.36 ± 0.46
maltose	1.00 ± 0.55	0.73 ± 0.40	0.67 ± 0.25	0.05 ± 0.02	0.11 ± 0.06
methionine	1.00 ± 0.87	3.15 ± 2.19	0.62 ± 0.62	5.24 ± 3.03	0.63 ± 0.38
PEP	1.00 ± 0.32	2.58 ± 0.31	0.88 ± 0.42	1.35 ± 0.30	1.69 ± 0.23
Pi	1.00 ± 0.47	1.63 ± 0.36	0.83 ± 0.22	2.86 ± 1.39	2.43 ± 1.82
PPi	1.00 ± 0.18	0.61 ± nd	1.52 ± 0.63	1.79 ± 0.45	3.24 ± 1.14
pyruvate	1.00 ± 0.17	1.86 ± 0.45	0.94 ± 0.29	0.80 ± 0.24	1.33 ± 0.09
quinic acid	1.00 ± 0.55	7.76 ± 4.91	5.26 ± 5.15	2.99 ± 1.23	0.91 ± 0.71
raffinose	1.00 ± 0.45	0.10 ± 0.07	0.11 ± 0.06	0.02 ± 0.01	0.01 ± 0.01
ribose	1.00 ± 0.47	1.55 ± 0.92	0.18 ± 0.18	1.36 ± 1.22	0.88 ± 0.35
serine	1.00 ± 0.27	5.07 ± 3.44	207.53 ± 206.46	2.57 ± 0.93	21.06 ± 20.40
shikimic acid	1.00 ± 0.44	9.41 ± 4.46	5.29 ± 4.84	6.70 ± 2.00	1.19 ± 0.82
sorbitol	1.00 ± 0.13	0.11 ± 0.11	0.10 ± 0.06	0.06 ± 0.06	0.19 ± 0.08
succinic acid	1.00 ± 0.87	2.98 ± 1.94	0.84 ± 0.53	0.42 ± 0.42	0.31 ± 0.31
sucrose	1.00 ± 0.16	0.24 ± 0.06	1.95 ± 0.19	2.60 ± 0.53	3.45 ± 1.16
threonine	1.00 ± 0.92	3.02 ± 1.91	6.57 ± 4.61	0.84 ± 0.27	0.67 ± 0.30
trans-4-hydroxy-proline	1.00 ± 0.75	7.02 ± 2.40	3.52 ± 1.03	3.16 ± 1.39	5.00 ± 2.45
trehalose	1.00 ± 0.78	0.27 ± 0.27	1.67 ± 1.40	0.01 ± 0.01	0.00 ± 0.00
tyrosine	1.00 ± 1.00	1.20 ± 1.20	89.07 ± 83.64	0.10 ± 0.10	0.26 ± 0.26
UDP	1.00 ± 0.20	2.39 ± 0.41	1.35 ± 0.24	0.60 ± 0.11	0.55 ± 0.07
UDP-glucose	1.00 ± 0.28	1.49 ± 0.26	1.45 ± 0.45	2.44 ± 0.74	3.50 ± 0.76
UTP	1.00 ± 0.65	0.93 ± 0.22	2.59 ± 1.03	12.80 ± 1.04	1.85 ± 0.50
valine	1.00 ± 0.86	2.01 ± 0.71	3.14 ± 1.72	1.14 ± 0.26	0.40 ± 0.07
xylitol	1.00 ± 0.04	1.98 ± 0.08	1.11 ± 0.32	1.94 ± 0.28	1.95 ± 0.50
xylose	1.00 ± 0.87	nd ± nd	nd ± nd	nd ± nd	nd ± nd

3.3.2.3.1 Sugars, sugar phosphates and sugar alcohols

The most striking change observed were the 6-fold and 3-fold increase in sucrose concentration in the young and mature internodes, respectively (Table 3.4, 3.5 and 3.6). In addition, there was also a significant increase in hexose-phosphate (G6P, F6P, G1P and UDP-glc) levels and a decrease in the triose-phosphate (DHAP, 3PGA) and F1,6-P_2 levels in the young internodes of all the transgenic clones (Table 3.4). In addition, F2,6-P_2 levels decreased significantly in the immature internodes (Table 3.4)

The sugars, trehalose, maltose and raffinose, and sugar alcohols, sorbitol, inositol and ribose, decreased within the transgenic line.

3.3.2.3.2 Organic-and amino acid

Total organic acid levels decreased within the immature transgenic lines. The levels of aconitic acid increased in all the transgenic lines (Table 3.4, 3.5 and 3.6).

In contrast, total amino acid concentrations increased in the transgenic lines (Table 3.4, 3.5 and 3.6). The exceptions to this included the concentrations of alanine, glutamine, glutamic acid and glycine in the immature internodes of the transgenic lines. With maturation the levels of alanine, glycine, isoleucine, serine and valine increased significantly in the transgenic lines (Table 3.6).

3.3.2.3.3 Nucleotide, nucleotide sugar, inorganic phosphate and pyrophosphate

The most consistent prevailing concentration was that of ATP, while UTP showed the most variability. Ratios between ATP: ADP and ATP: AMP indicated that no significant change was apparent in the transgenic lines (Fig 3.2). Furthermore, adenylate energy charge (AEC) calculations indicated no significant difference between the transgenic and control lines (Fig 3.3). Most lines exhibited a response between 0.7 and 0.9. The exception to this statement is the AEC of internodes 6 and 9 of the control, as well as internode 3 of the transgenic clone OPu 501.

There was, however, a 3 to 9 fold increase in UDP-glucose levels in the transgenic lines during all the stages of development compared to those of the control (Table 3.4, 3.5 and 3.6).

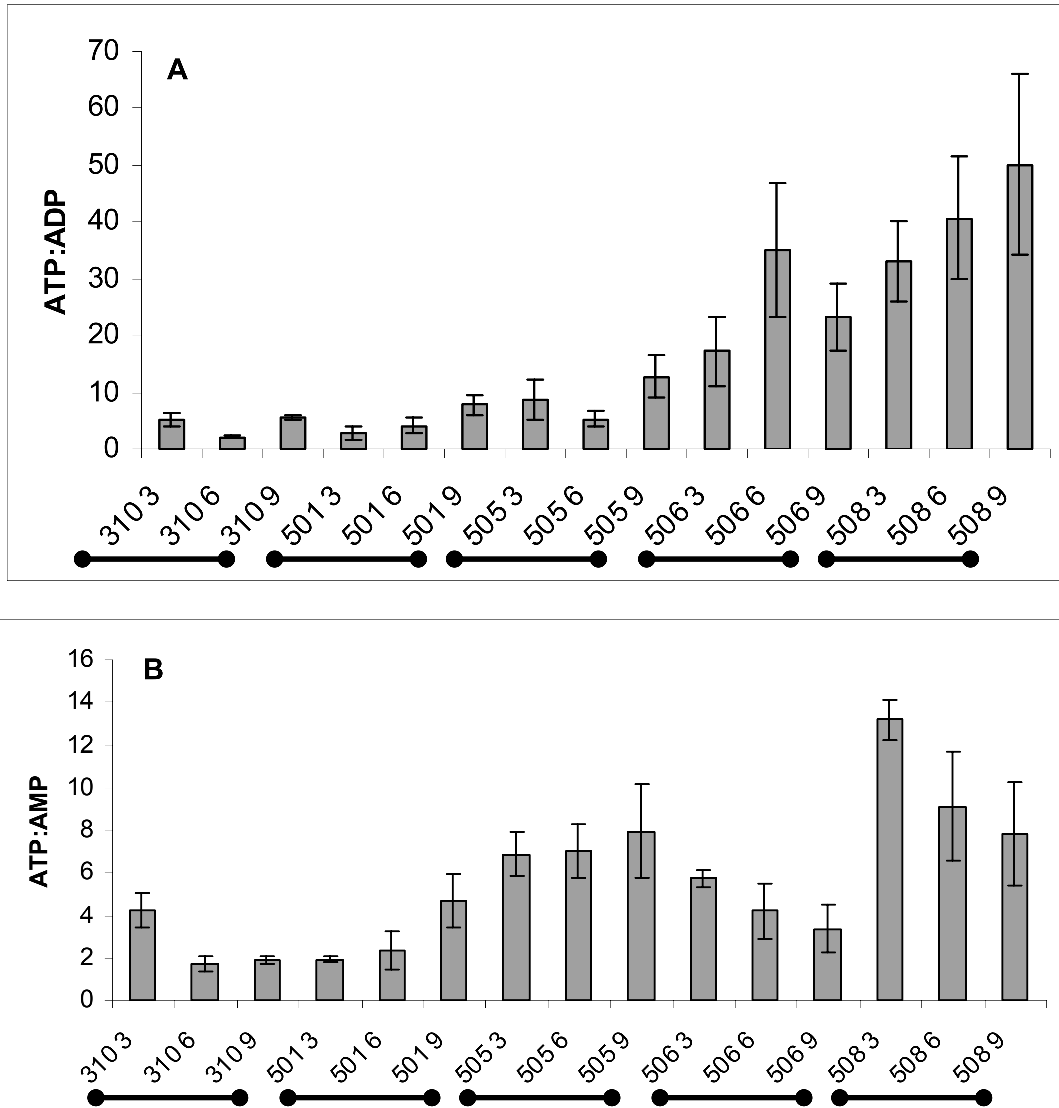


Figure 3.2: ATP: ADP (**A**) and ATP:AMP (**B**) ratio of transgenic clones (501, 505, 506 and 508) altered in their PFP expression compared to the control (310) in internodes 3, 6 and 9 (designated 3, 6 and 9).

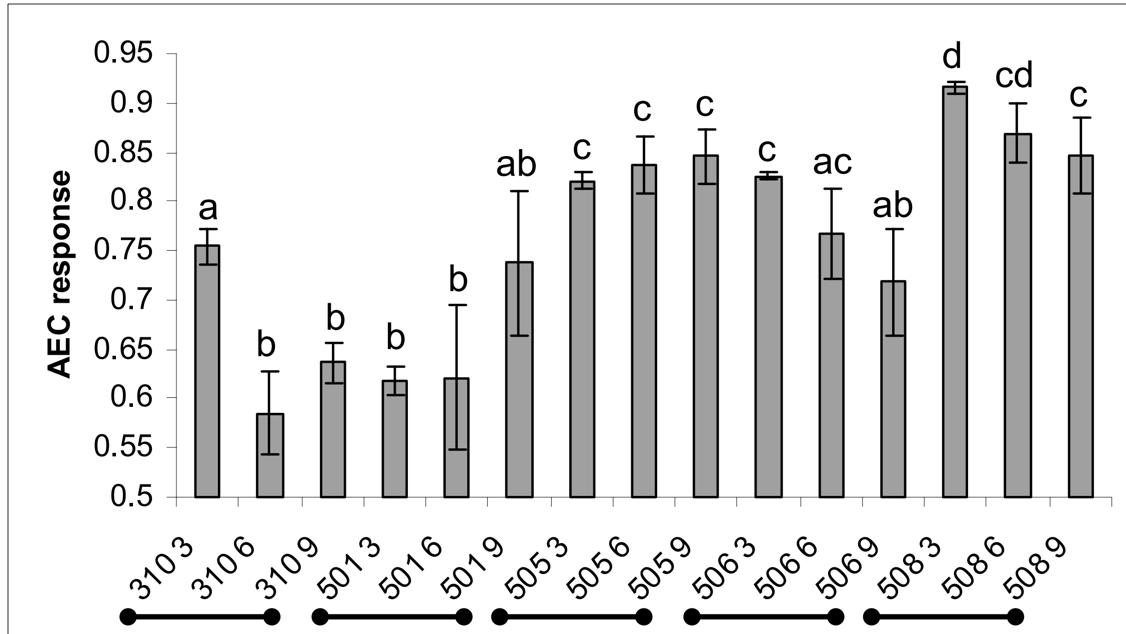


Figure 3.3 Adenylate Energy Charge (AEC) of control (NCo 310) and transgenic sugarcane clones (OPu 501, 505, 506 and 508) at the different internodal stages (designated 3, 6 and 9). AEC = ([ATP]+0.5[ADP])/([ATP]+[ADP]+ [AMP]) (Atkinson, 1977).

In contrast, the alternative energy donor of the cell, pyrophosphate (PPi) seemed to be altered significantly in the transgenic lines during the early stages of maturation. Reduced PFP activity lead to 15 times higher PPi concentrations in the young internodes (Table 3.4). These effects were not evident in the mature internodal regions.

3.3.2.3.5 Mass action ratios

Mass action ratios (Table 3.3) indicated only one significant more than a thousand fold change in enzyme regulation in the transgenic lines. Hexokinase (glucose dependent phosphorylation) mass action ratio in internode 3 of the transgenic line, OPu 508 increased 2 400-fold. In addition, this line also indicated a 430 times change in the fructokinase ratio, a 280 times change in the FBPase ratio and a 550 times change in the PFK ratio of internode 3.

3.3.2.3 Biological variability

A consideration in the interpretation of the metabolic data is the contribution of biological variability in regard to the metabolic variability within a sample. It has been previously reported that biological variability may account for as much as 1.5 to 10 fold the standard errors reported (Roessner et al., 2000). Principal component analyses (PCA) revealed that despite high variability, repetitions of the same internodal tissue still cluster together (Fig 3.4).

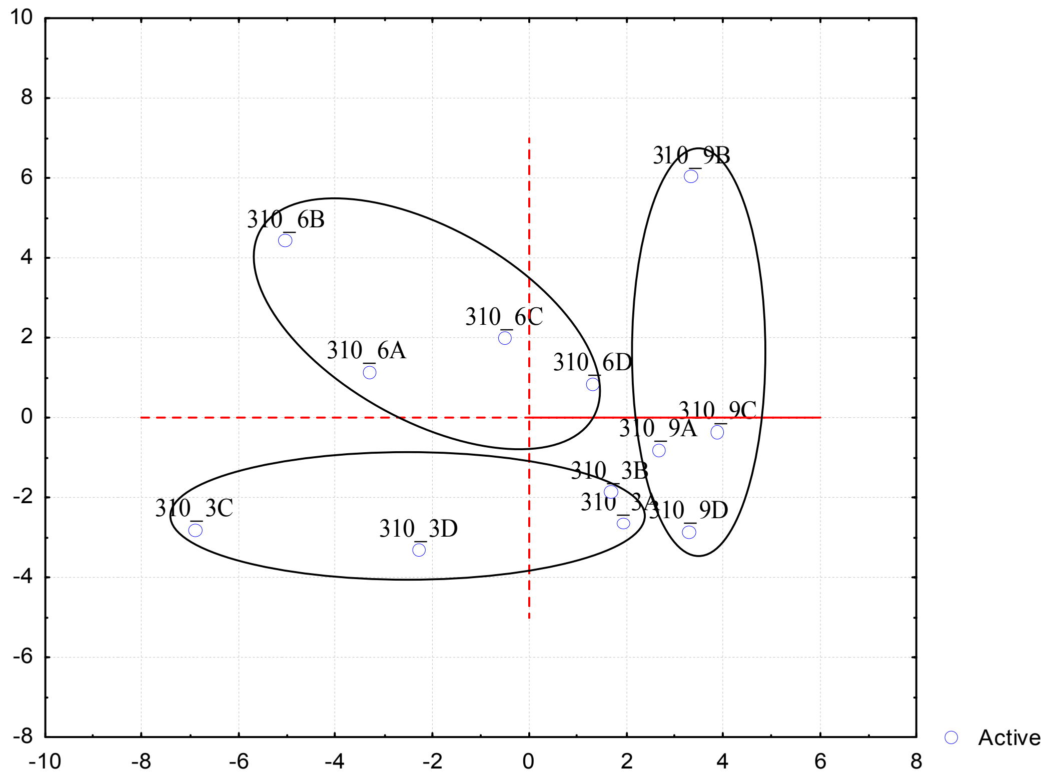


Figure 3.4 Principal component analyses of the control repetitions A, B, C and D at internode 3, 6 and 9. A two-factor plane was chosen as representative and the two vectors constituted 43.22% of the variance observed.

Considering the metabolome within the control, the repetitions indicated that sucrose and glucose were exempted from generalization (Fig 3.5).

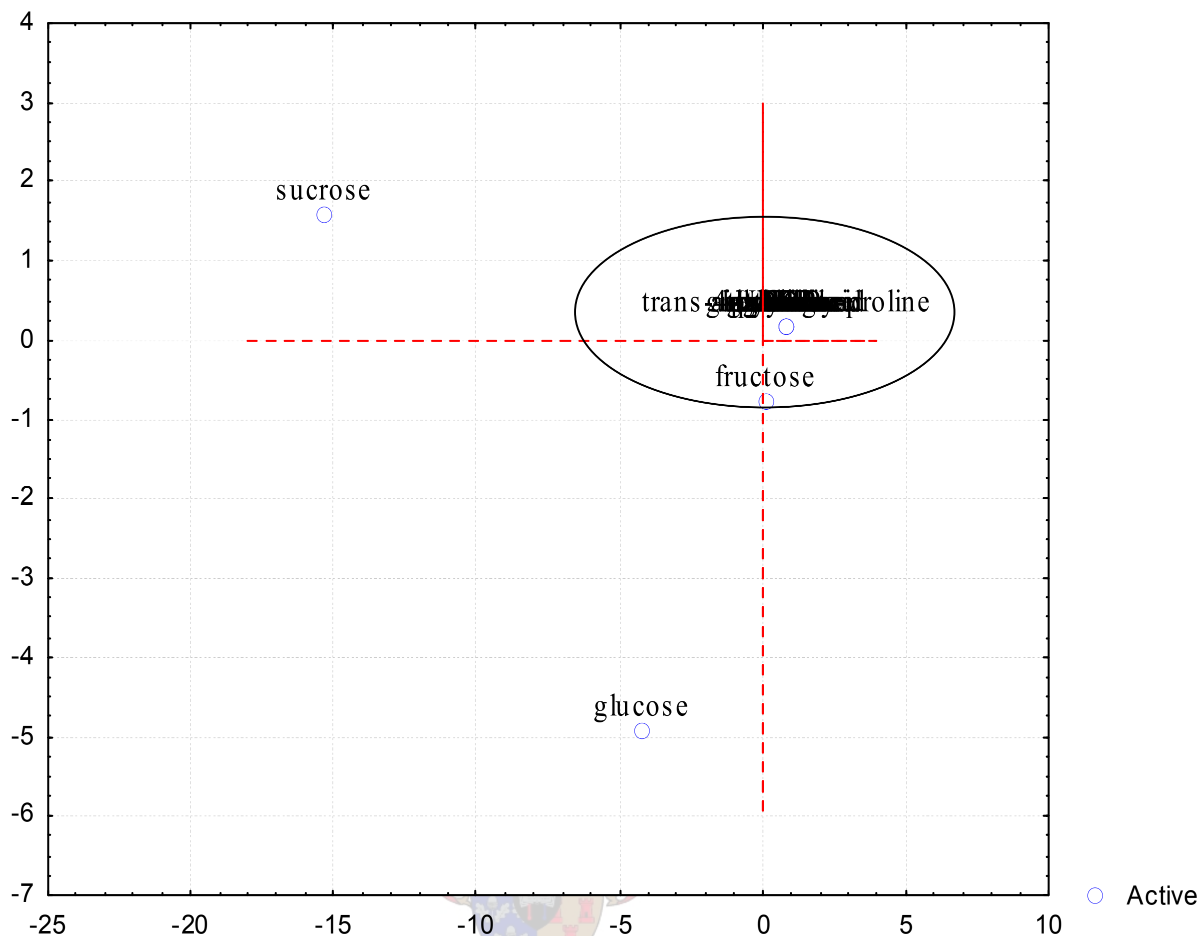
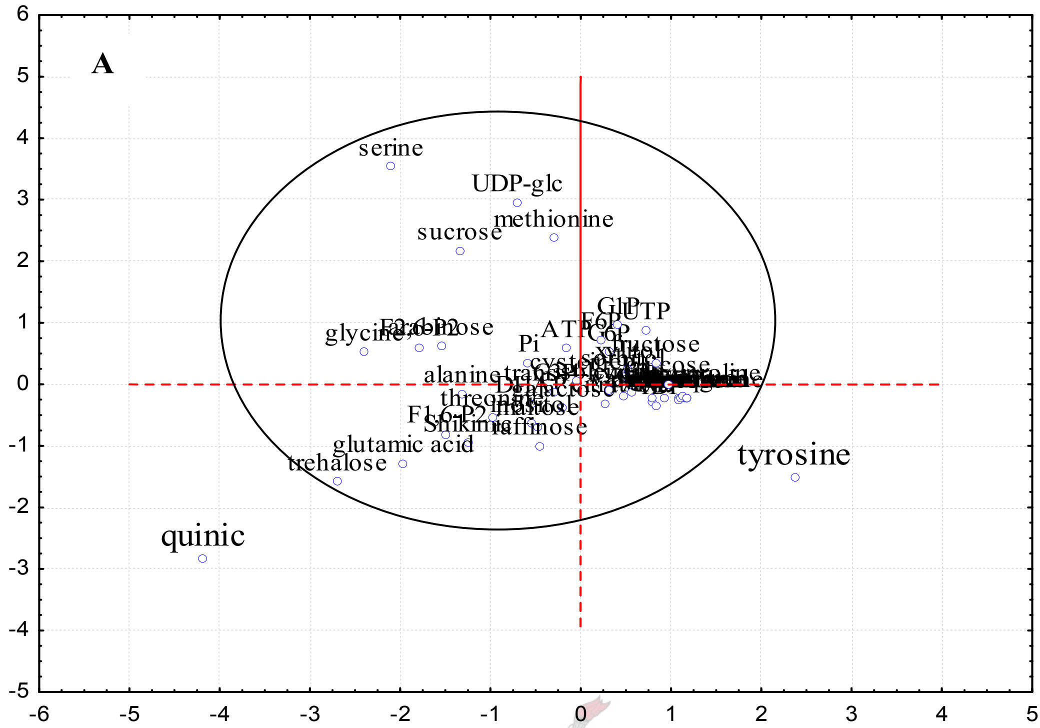
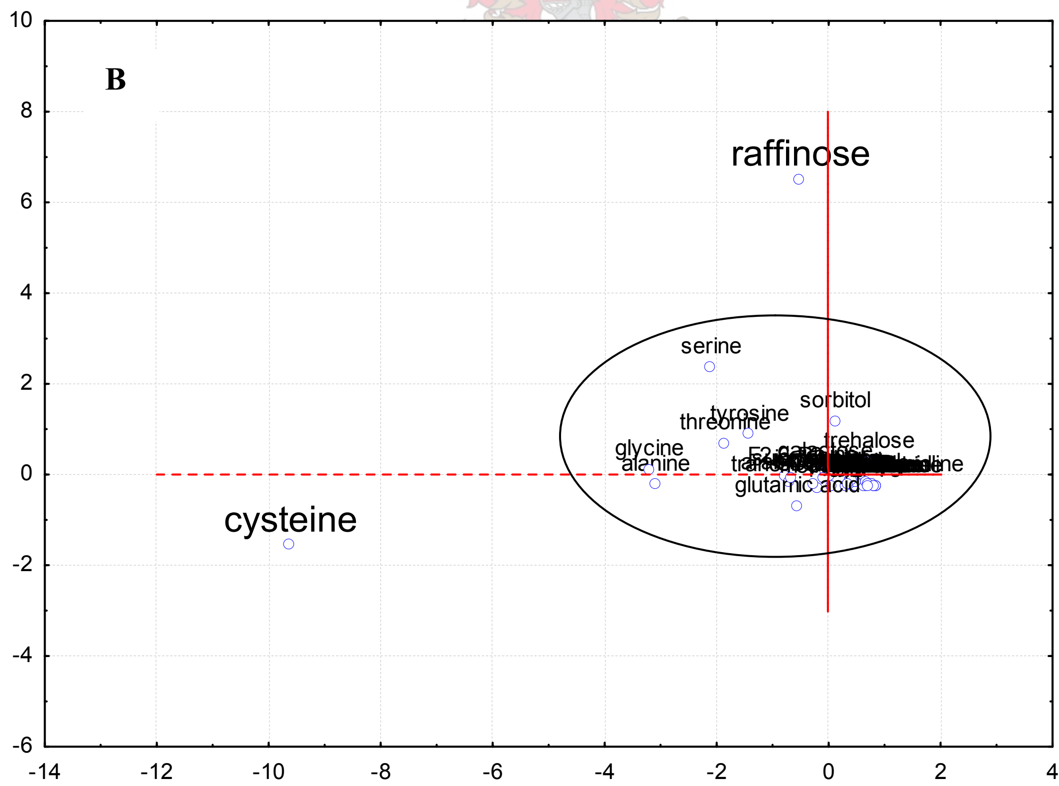


Figure 3.5 PCA of the metabolic subsets of the control plant. PCA vectors 1 and 2 were chosen for the best visualisation. These vectors constituted 99.83% of total representation.

3.3.2.5 Multivariate analyses of transgenic sugarcane clones

On the other hand, PCA analyses of the metabolome of the transgenic lines indicated far different deflectors (Fig 3.6). These included the levels of quinic acid and tyrosine in the immature internodes (Fig 3.6A), and cysteine and raffinose in the maturing internodes (Fig 3.6 B, C).


A
serine
UDP-glc
methionine
sucrose
UTP
fructose
Pi
alanine
glutamic acid
trehalose
raffinose
tyrosine
quinic


B
raffinose
serine
sorbitol
tyrosine
threonine
glycine
alanine
trehalose
glutamic acid
cysteine

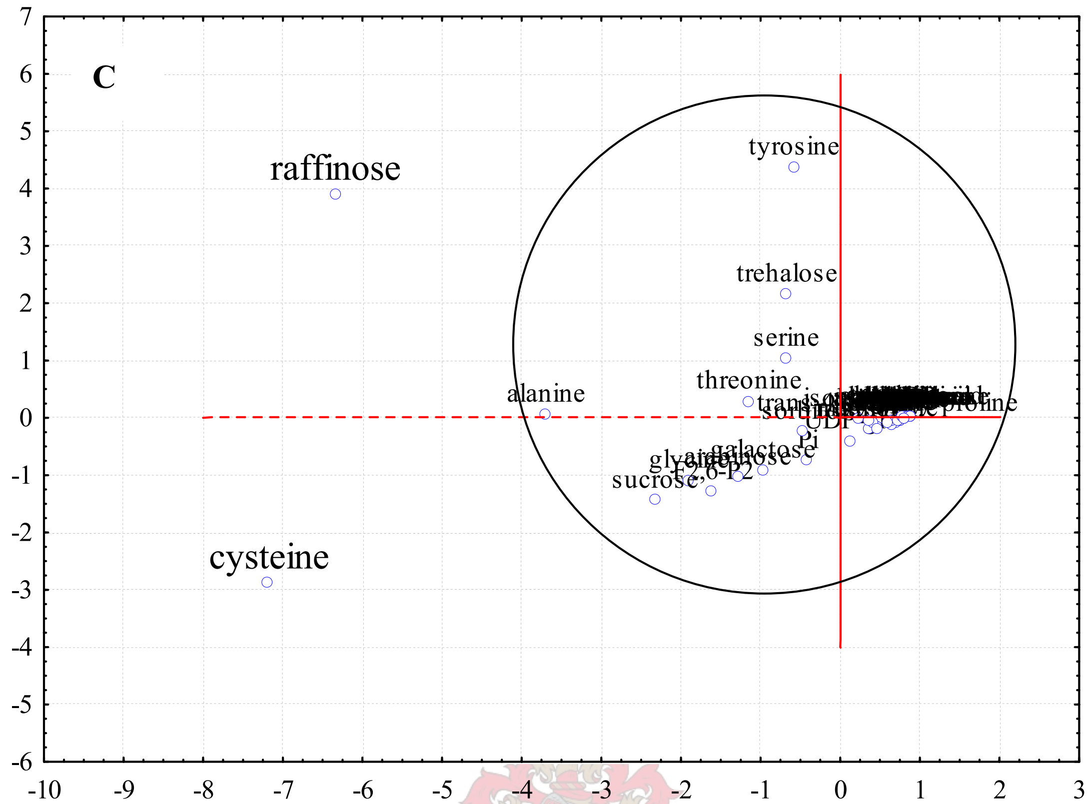


Figure 3.6 PCA of the transgenic metabolite data for internode 3 (**A**), 6 (**B**) and 9 (**C**). PCA vectors 1 and 2 were chosen at all stages of maturation for the best visualisation. These vectors constituted 52.73% in internode 3, 74.73% in internode 6 and 76.06% in internode 9.

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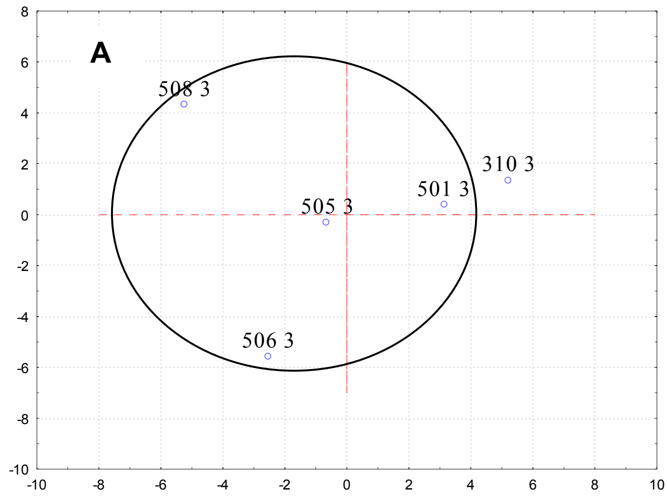
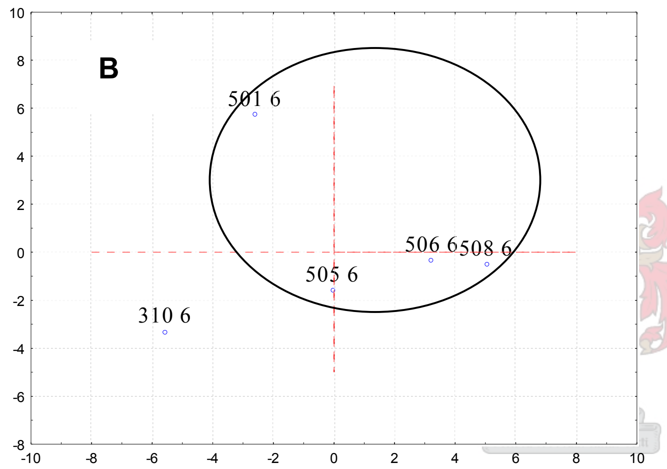
In terms of metabolic phenotypes, PCA analyses indicated that the transgenic clones formed more definite clusters with each other than with the control (Figure 3.7). This was especially prevalent in the more mature internodes (Fig 3.7 B, C). HCA analyses indicated similar results (data not shown).





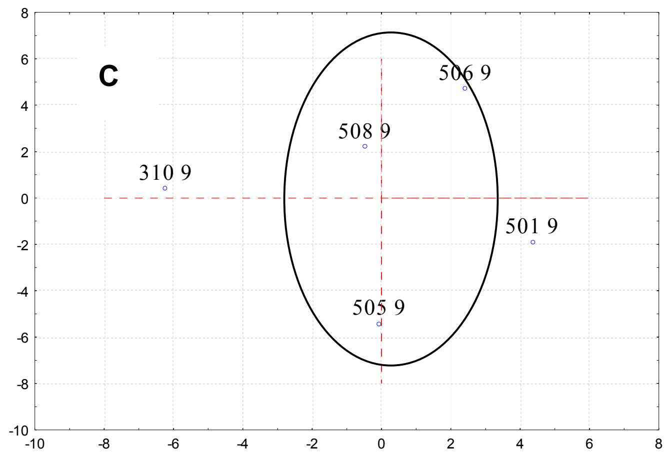


Figure 3.7 PCA analyses of the metabolic profiles of control *NCo 310* sugarcane and the transgenic clones, *OPu 501, 505, 506 & 508* in internode 3 (**A**), 6 (**B**) and 9 (**C**).

3.3.2.5 Metabolite correlations

Clique-matric vertices (Kose et al., 2002; www.mpimp-golm.mpg.de/fiehn/projekte/data-mining-e.html), where used to find positive linear correlations in the metabolic dataset and to visualise it in a clustering manner. From these cliques eight distinct metabolite clusters were identified in the transgenic sugarcane culm (Table 3.7). Four large-spanning cliques constituted of 1) amino acids (threonine, isoleucine, alanine, aspartic acid and valine), 2) DHAP, maltose, inositol, F1,6-P2 and G3P, 3) the hexose-monophosphate pool (G6P, G1P and G6P) and 4) ADP, shikimic- and quinic acid. The other four minor subsets indicate a pair-wise correlation between arabinose and F2,6-P2, UDP-glucose and fructose, sorbitol and raffinose, and glycine and proline.

Table 3.7 Simplified coloured matrix list of vertices forming cliques from transgenic sugarcane clones with reduced PFP expression. The R_{xy} threshold was defined at 0.8 for a metabolomics data set comprising of 780 metabolites, 20 individual plant samples at 3 different stages of maturity.

	threonine	isoleucine	alanine	aspartic acid	valine	F6P	G1P	G6P	DHAP	maltose	inositol	F1,6-P2	G3P	Shikimic	quinic	ADP	F2,6-P2	arabinose	UDP-glc	fructose	sorbitol	raffinose	glycine	trans-4-hydroxy-proline
Clique13									2	2	1	1												
Clique9									2	2			1											
Clique10	3	2	2																					
Clique12	3	2			1																			
Clique11	3		2	1																				
Clique1						2		1																
Clique2						2	1																	
Clique3																	1	1						
Clique4																			1	1				
Clique5														2		1								
Clique6														2	1									
Clique7																					1	1		
Clique8																							1	1

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3.4 Discussion

Limited reports are available on sugarcane metabolite data (Whittaker and Botha, 1997, Bosch et al., 2003). The metabolite data presented here is the first comprehensive attempt at quantifying the metabolites involved in glycolysis, amino acid, sugar and organic acid metabolism, as well as energy donor molecules (Table 3.2). This data was used to construct a metabolic picture of sucrose accumulation and to pinpoint possible key elements that might play a role in sucrose accumulation.

3.4.1 Transgenic maturing internodes are more metabolically active

The down-regulation of PFP activity resulted in no changes in the visual phenotype of the culm of the transgenic sugarcane clones. However, significant changes regarding the *in vivo* metabolites were evident and these changes were consistent with the percentage remaining endogenous PFP activity (Fig 3.1). In general, the metabolome of the immature transgenic clones with the highest remaining PFP activity (OPu 501),

exhibited the closest resemblance to the control metabolome (Fig 3.7). The transgenic lines with less than 5% remaining PFP activity (OPu 505, OPu 506 and OPu 508) seemed to cluster more closely to each other than to the control or OPu 501. The metabolite data thus corresponded to the reduction in enzyme activity *in vivo*.

A striking feature in the metabolite dataset was that most of the metabolite levels in the mature transgenic internodes were not significantly different from those of the control (Table 3.5, 3.6). PCA analyses indicated that metabolites that disparate from the norm were the same in internodes 6 and 9 (Fig 3.6 B, C), but distinctly different from internode 3 (Fig 3.6 A). The factors that contribute to this restoration are unclear. However, AEC responses (Fig 3.3) suggested that transgenic maturing internodes with <5% remaining PFP activity (Fig 3.1) were more metabolic active than the same internode of the control. In addition, sucrose accumulation in these internodes was accompanied by a decrease in the organic acid pool as well as the rest of the sugar pool (Table 3.2, 3.4, 3.5, 3.6). If we assume that these pools constitute large enough carbon reserves then a redirection of carbon might be responsible for increased carbon allocation to sucrose.

3.4.2 A possible link between sucrose accumulation and amino acid metabolism in sugarcane internodal tissue

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Sucrose accumulation has been linked to *de novo* synthesis of amino acids (Roessner-Tunali et al., 2003). In sugarcane, it appeared that amino acid levels were highest at internode 6 in the control line (Table 3.2). This was attributed to an increase in the concentrations of the amino acids cysteine, glycine and serine, which are synthesised from the glycolytic precursor 3-phosphoglycerate. In contrast, alanine levels decreased with significant sucrose increases (Table 3.2, 3.4, 3.5 and 3.6). Alanine has a strong linear relationship with amino acids that diverge from either the common precursor pyruvate or oxaloacetate (Table 3.7). In the transgenic lines a decrease in alanine was associated with a significant increase in aspartic acid and valine (Table 3.4). In addition, the strong linear relationship between glycine (derived from 3-phosphoglycerate) and proline (2-oxoglutarate) suggested that these amino acids either have a high degree of control or that they are themselves under strict control.

3.4.3 Reduced PFP activity led to a significant increase in sucrose levels in the young internodes.

In order to construct a metabolic picture of the potential relationship between sucrose accumulation and PFP activity we considered metabolites that 1) control PFP activity, 2) on which PFP activity may have a direct influence and 3) how they relate to our current knowledge of sucrose metabolism in sugarcane internodal tissue.

3.4.3.1 Reduction in PFP activity did not lead to increased activation by F2,6-P_2

Decreasing F2,6-P_2 levels were consistent with increased G6P, F6P and G1P levels, and decreased 3-PGA levels (Table 3.4). F6P activates and 3-PGA inhibits PFK-2 activity, which is responsible for F2,6-P_2 production (for review, Dennis et al., 1997). In the absence of FBPase activity in sugarcane internodal tissue (Bindon, 2000), we would expect elevated F2,6-P_2 levels to activate the residual PFP activity (for review, Stitt, 1990). However, this was not evident in the transgenic lines. This was also apparent from the mass action ratio of PFP, which indicated no significant change between the transgenic and control lines (Table 3.4). However, a 550 times change in the mass action ratio for PFK was evident in one of the immature transgenic (OPu 508) internodes (Table 3.5). But no change in maximum catalytic activity was reported for PFK in transgenic potato tubers with reduced PFP activity (Hajirezaei *et al.*, 1994). Thus the perturbation in the system was not restored by an increase in PFK activity and/or a reversal of PFP to a netto gluconeogenic flux.

3.4.3.2 Pyrophosphate utilisation

Reduced PFP activity led to a significant increase in PPi levels in the immature internodes of the transgenic lines (Table 3.4). It has been proposed that PFP regulates PPi concentration (Black et al., 1987; ap Rees et al., 1985; Stitt, 1990) and that PPi may be related to changes in sucrose accumulation in sink tissue (Taiz, 1986). PPi can be utilised in three reactions as an alternative energy donor. 1) Conversion of F6P to F1,6-P_2 via PFP (Carnal and Black, 1979), 2) sucrose degradation via SuSy and the subsequent

UGPase reaction and 3) vacuolar sucrose loading via the energisation of the tonoplast by H^+-PPase.

With the onset of maturation, PPi levels increased in the NCo 310 control suggesting that it might play a role in sucrose accumulation (Table 3.5). In the transgenic lines, six-fold higher sucrose concentrations were accompanied by 15-fold increases in PPi levels in the young internodes. However, a three-fold increase in sucrose concentrations was not consistent with a change in PPi levels in the mature internodes of the transgenic lines (Table 3.5). In addition, reduced PFP activity in transgenic potato tubers also shows no alteration in PPi levels; although a slight elevation in sucrose levels are evident (Hajirezaei et al, 1994). Potato tubers over expressing *E.coli* inorganic pyrophosphatase (PPase) indicate that a decrease in PPi levels lead to an increase in sucrose levels (Geigenberger et al., 1998).

The absence of a linear relationship or conflicting steady state metabolite levels do not necessary refute a relationship between two metabolic entities. The varying prevailing PPi levels in the above-mentioned studies could imply that sucrose accumulation is possible in all instances as long as PPi supply were met and utilised effectively. On the other hand, this study did not discriminate between vacuolar and cytosolic sucrose concentrations, while PPi is located exclusively in the cytosol (Farre et al., 2001).

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3.4.3.3 Reduced PFP activity led to enhanced substrate availability for sucrose synthesis

With the onset of maturation, significant metabolite changes occurred in the maturing culm (Table 3.2). Increased sucrose concentrations were accompanied by a decrease in the triose-phosphate pool and a two-fold increase in the hexose-phosphate pool in internode 6. Although PFP constitutes a reversible reaction an increased hexose-phosphate: triose-phosphate ratio in the transgenics suggested that the net flux was in the glycolytic direction in sugarcane immature internodal tissue.

Sucrose cycling and hexose-phosphate: triose-phosphate cycling decreases significantly at internode 7 (Whittaker and Botha, 1997; Bindon, 2000). This decrease is signified with an increase in hexose-phosphate concentrations. This suggests that the hexose-phosphate pool might be responsible for increased sucrose concentrations.

Furthermore, in the transgenic lines, significant increases in hexose-phosphates also led to significant increases in sucrose concentration in the young internodes (Table 3.4). Mass action ratios for hexokinase and fructokinase showed a change in the immature transgenic internodes (Table 3.3), suggesting that sucrose degradation was enhanced in these internodes. The increased hexose-phosphate pool was therefore probably the result of reduced PFP activity and/or increased sucrose degradation. With maturation, the hexose-phosphate pool was not significantly different from the control (Table 3.5, 3.6).

Therefore, if the increased sucrose pool was directly correlated to the enhanced hexose-phosphate levels in the young internodes, this beneficial effect will be diminished in mature cane as the metabolites restore to their steady state levels (Table 3.6). Further analyses of mature field grown cane should provide valuable insight into this.

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Chapter 4

ISOTOPIC ASSESSMENT OF HEXOSE-PHOSPHATE: TRIOSE -PHOSPHATE AND SUCROSE CYCLING IN TRANSGENIC SUGARCANE CLONES WITH REDUCED PFP EXPRESSION

Abstract

Contradicting results regarding the link between cytosolic substrate cycling has led to the evaluation of metabolic flux within the sucrose and hexose-phosphate: triose-phosphate pools using transgenic sugarcane clones with reduced pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity.

Label (re)distribution between the sucrose and hexose moieties, and hexose-phosphate and triose phosphate pools in sugarcane tissue discs were followed by ^{13}C nuclear magnetic resonance technology. Feeding the discs with 1-^{13}C glucose revealed a decrease in hexose-phosphate: triose-phosphate cycling in the transgenic immature internodes. The level of hexose-phosphate: triose-phosphate cycling in the control internodes and transgenic maturing internode were comparable. The values of label exchange indicated full randomization of label, indicating that the methodology used could lead to gross misinterpretation of hexose-phosphate: triose-phosphate cycling. Unpredictably, sucrose cycling increased ten-fold in the transgenic immature internode. A significant increase was also evident in the transgenic maturing internodes compared to the control. These results suggested that the two cycles are not linked by being co-coordinately controlled.

4.1 Introduction

In plant cells, including the sugarcane culm, substrate cycling is a well-known phenomenon. Substrate cycling, the continuous synthesis and degradation of a metabolic entity, has been implicated in several physiological roles, including control of respiration (Dancer et al., 1990), sugar signaling (Cortès et al., 2003) and control of sucrose accumulation (Rohwer and Botha, 2001).

Sugarcane sucrose metabolism is characterized by a sharp increase in sucrose content within the first 10 internodes (Moore, 1995; Moore and Maretzki, 1996; Botha et al., 1996; Whittaker and Botha, 1997), with the concurrent redirection of carbon away from proteins, structural carbohydrates, amino acids, phosphorylated intermediates and respiration into sucrose (Whittaker and Botha, 1997). In sugarcane, it has been postulated that sucrose levels are controlled by two cycles that govern this interconversions and redistribution of carbon, namely hexose-phosphate: triose-phosphate cycling and sucrose cycling (Sacher et al., 1963; Batta and Singh, 1986; Wendler et al., 1990; Whittaker and Botha, 1997; Bindon and Botha, 2000).

Hexose-phosphate: triose-phosphate cycling returns 50% of carbon entering the triose phosphate pool to the hexose-phosphate pool in sugarcane immature internodal tissue (Bindon, 2000). The sharp decrease in the extent of triose-phosphate cycling with the onset of maturation, indicate that it might play a role in carbon allocation to sucrose accumulation (Bindon, 2000).

Similarly, sucrose cycling in sugarcane immature internodal tissue is high and has been implicated in allowing great metabolic flexibility when metabolic demand is great in carrot cell suspension cultures (Krook et al., 2000a). Sucrose cycling decreases significantly between young and mature internodal tissue with the onset of maturation (Whittaker and Botha, 1997). Interspecific hybrids that accumulate more sucrose per fresh mass basis also have lower sucrose cycling abilities (Rose, 2001). This implicates that sucrose cycling can be one of the controlling factors of sucrose accumulation *in vivo* (Whittaker and Botha, 1997; Rose, 2001).

A reduction in both cycles with the onset of maturation suggests that these two cycles work in conjunction with each other (Moore and Maretzki, 1996). However, decreased hexose-phosphate: triose-phosphate cycling resulted in no change in the sucrose cycle in heterotrophic tobacco calli (Fernie et al., 2001), suggesting that no link exists between the two cycles. However, the term link did not distinguish between a physical co-ordination of the cycles (Moore and Maretzki, 1996) and co-regulation of the cycles (Fernie et al., 2001). The suggestion that the regulation of these cycles is co-coordinated in sugarcane internodal tissue therefore still needs to be confirmed.

Since the hexose-phosphate: triose-phosphate cycle is thought to be primarily mediated by PFP in sugarcane (Whittaker and Botha, 1999), the existence of modified sugarcane clones which produces less PFP product per protein basis (Groenewald and Botha, 2001) is a novel biotechnological tool to assess these cycles within the maturing culm tissue. It was indicated that higher sucrose concentrations were evident within the transgenic immature internodal regions (Chapter 3; Groenewald and Botha, 2001), and mature regions (Chapter 3). Changes in prevailing metabolite levels suggested that the cycles changed in response to the genetic alteration (Chapter 3) but failed to indicate how these cycles interacted with each other. Since sucrose accumulation is accompanied by a decrease in both substrate cycles within maturing internodes, we expected that the increase in sucrose concentrations in the transgenic lines would also be resultant of a decrease in both the cycles.

In order to determine flux between the hexose-phosphate: triose-phosphate pool and sucrose pool, carbon distribution patterns of isotopically labeled 1-^{13}C glucose were followed in sugarcane internodal tissue discs.

4.2 Material and Methods

4.2.1 Plant material

For the purpose of this study internode one of the culm was defined by the internode to which the leaf with the first dewlap was exposed (Van Dillewijn, 1952), with lower internodes subsequently numbered with increasing numericals. Young (internode 3 and 4) and mature(internode 6 and 7) internodes from both a transformed commercial variety (NCo 310) and a transgenic clone (OPu 506) with reduced PFP expression (Fig. 3.1) were harvested from prevailing environmental conditions in Stellenbosch, South Africa and processed immediately after harvesting. Longitudinal sections, 3 mm in diameter for immature tissue and 5 mm for mature tissue, were excised with a cork borer and sliced in sections comprising 1 mm in thickness, and washed in distilled deionised water. Repetitions of tissue discs were taken of each sample from three individual sugarcane culms.

4.2.2 Biochemicals

1-^{13}C glucose was purchased from Isotec Inc. (Miamisburg, Ohio, USA). The Millex-GV4 (0.45 µm) filter units were purchased from Millipore (Billerica, Massachusetts, USA) and 250 mm x 4.6 mm Supelco Supelclean™ LC-SAX and LC-SCX (5µm) SPE columns were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.2.3 [^{13}C]-labeling experiments

Tissue discs (*4.2.1*) were washed after excision four times in distilled deionised water. Excess liquid was removed by blotting and tissue discs (20 per sealed 100 mL Erlenmeyer flask containing 650µl incubation buffer (50 mM MES (pH 6.5), 50mM 99.9% enriched 1-13C glucose and 50 mM fructose)) were incubated for 5h with constant shaking (150 rpm) on a rotary shaker at 28°C. Sampling was done by removal of 10 tissue discs after 3h and 5h, respectively, for sugar and label incorporation determinations.

After labeling, tissue were rinsed and washed for five times in wash buffer (50 mM MES, pH6.5). Excess buffer was removed and sugars extracted with a hot ethanol/water extraction (Vorster and Botha, 1998).

4.2.4 Sugar extractions

Sugars were extracted as previously described (Bindon, 2000). The tissue discs were soaked at 70°C, overnight in 70% (v/v) ethanol and 30% (v/v) HM buffer (100 mM HEPES (pH 7.8), 20 mM $MgCl_2$). The discs were crushed and incubated for a further 2h. Extracts were centrifuged at room temperature for 10 min at 10 000 x*g* and the supernatant collected. The supernatants were filtered through PVDF 0.45µm Millipore filters and the filtrates loaded onto preconditioned (bed weight 100 mg, volume 1 ml) ion exchange (IEX) columns (LC-SCX and LC-SAX). The IEX columns were placed in tandem (LC-SCX tube on top) for solid phase extraction according to the manufacturer's recommendations. Samples were eluted with 4 ml ddH_2O. Complete elution of the

neutral fraction was achieved by washing the anion exchange columns four times with 1 ml ddH2O and combining all the eluate fractions.

The collected neutral fraction were dried down under vacuum, redissolved in deuterium oxide (D_2O) to a final amount of 20mg/g fresh mass and subjected to NMR analyses.

4.2.5 NMR analyses

NMR spectra were recorded on a 600MHz Varian ^{Unity}*Inova* spectrometer equipped with an Oxford magnet (14.09 T). A standard pulse sequence was used for collecting ^{13}C spectra at a frequency of 150 MHz. An acquisition time of 1.3 sec and a 1 sec pulse delay were used with a pulse angle of 43 deg. Number of scans varied from 5500 to 65500 depending on the sample. Chemical shifts were normalized relative to the field lock of D_2O. Reference spectra for sucrose, fructose and glucose were collected under the same conditions. Resonance assignment and integration were followed for glucose, fructose and sucrose spectras (Viola, et al., 1991; Krook et al., 2000a; Krook et al, 2000b). Peak areas at 104.29 ppm (sucrose-fructosyl C2), 98.68 ppm (β fructose C2-5) 96.53 ppm (β glucose C1), 92.78 ppm (sucrose-glucosyl C1), 81.97 ppm (sucrose-fructosyl C5), 81.30 ppm (β fructose C2-5), 77.03 ppm(sucrose-fructosyl C3), 76.57 ppm (β glucose C5), 76.40 ppm (β glucose C3), 76.04 ppm (β fructose C2-5), 75.08 ppm (β fructose C2-5), 74.76 ppm (β glucose C2), 74.61 ppm (sucrose-fructosyl C4), 73.18 ppm (sucrose-glucosyl C3), 73.01 ppm (sucrose-glucosyl C5), 71.67 ppm (sucrose-glucosyl C4), 70.77 ppm (β fructose C2-5), 70.22 ppm (β glucose C4), 69.81 ppm (β fructose C2-5), 68.20 ppm (β fructose C2-5), 64.52 ppm (β fructopyranose C1), 63.99 ppm (β fructopyranose C6), 63.33 (β fructofuranose C1), 62.96 ppm (sucrose-fructosyl C6), 61.97 ppm (sucrose-fructosyl C1), 61.38 ppm (β glucose C6) and 60.73 ppm (sucrose-glucosyl C6) were integrated. Initially both anomers were considered during spectra analysis. Due to a low detection signal from the α anomer for fructose and glucose, only the β anomer was considered for the further quantification of the sugars.

4.2.6 Statistical analyses

Student t-tests (two-tailed) were performed on the Microsoft® Excel 2002 software package (Microsoft Corporation Inc., Seattle, USA). The term significant is used to indicate differences for which $P < 0.05$.

4.3 Results

4.3.1 NMR analysis of substrate cycles

Labeling times of 3h and 5h were chosen as previous results indicated that isotopic steady state can be reached after 3h (Bindon, 2000). The change in label distribution between 3h and 5h was used for all calculations. Since the exchange rate between the hexose-phosphates are in the order of $k \sim 0.1\text{-}1\ s^{-1}$ (Roscher et al., 1998), and between hexoses range from $k \sim 0.1\text{-}1\ h^{-1}$ (Fernie et al., 2001), a 3h labeling times have resulted in isotopic equilibrium of the hexose-phosphate pool. Since hexose-phosphates concentrations are at least two orders of magnitude lower than that of hexoses (Chapter 3), the time course for flux of label from the hexose-phosphates to hexoses would be adequate to measure relative hexose-phosphate: triose-phosphate cycling.

The absence of measurable label in position C3 indicated that the cyclic flux through the non-oxidative pentose phosphate pathway was negligible in sugarcane internodal tissue.

Enrichment was calculated as defined by Viola et al. (1991) and Fernie et al., (2001). Spectra of the enriched samples were superimposed on natural abundant spectra of glucose, sucrose and fructose. Carbon position 3 was normalized in respect to the natural abundant isotopes. Once these relative enrichments were determined in the samples, the degree of enrichment was determined by calculating the increase in peak height over the natural abundance. The relative redistribution of isotope from the 99.9% enriched 1-^{13}C glucose to other carbon positions were calculated to give an indication of the percentage redistribution to carbon positions C1 and C6 of the hexosyl moieties in sucrose, as well as carbon redistribution to C1 and C6 of fructose and C6 of glucose.

4.3.2 Hexose-phosphate: triose-phosphate recycling activity

The exchange of label from position C1 to C6 expressed as the percentage labeled C6 carbons from total (C1 + C6) carbons were taken as indicative of the cycling between the triose-phosphate and hexose-phosphate pool. In this regard, label return from fructose was taken to represent cytosolic label exchange.

In the control immature internode, redistribution in fructose accounted for a return of almost 50% of label following feeding with 1-^{13}C glucose (Table 4.1). For every randomized triose-phosphate molecule returned to the hexose-phosphate pool a second unrandomised molecule is also recycled (Bindon, 2000). This would imply that a 100% recycling were evident in this particular internode. Similarly, label distribution in the control and transgenic maturing internodes also indicated a label return approximating 50%, indicating full randomization of label through the hexose-phosphate: triose-phosphate cycle (Table 4.1).

In contrast, transgenic immature internodal tissue indicated a 25% reduction in label distribution compared to the other internodes (Table 4.1). Interestingly, this reduction led to a two-fold decrease in the C1:C6 ratio (Table 4.1).

Table 4.1 Label redistribution from C1 to C6 within the immature (internode 3 and 4) and maturing (internode 6 and 7) internodes of NCo 310 control and OPu 506 transgenic lines. Values are the percentage labeled carbon position of fructose relative to 1-^{13}C glucose and are the mean ± SE (n=3)

	Control immature			Control maturing			Transgenic immature			Transgenic maturing		
Fructose C1 to C6 label distribution	49.60	±	2.53	45.67	±	2.21	36.56	±	9.15	51.28	±	7.66
Fructose C1:C6 ratio	1.03	±	0.11	1.20	±	0.11	0.55	±	0.20	1.05	±	0.33

Following the same approach as above it was seen that label distribution within the sucrose pool was reduced approximately two-fold in the isotopic enrichment of C1 compared to C6 within the control internodal stages (Table 4.2). The immature transgenic line exhibited a 25% decrease in the ratio between the hexose-phosphate: triose-phosphate cycling calculated from the fructose moiety compared to those of

calculated from sucrose. A similar increase in the ratio was observed in the transgenic maturing internode (Table 4.2).

Table 4.2 Sucrose C1 to C6 label redistribution in the control and transgenic culm at different stages of maturation, and the relative ratio between fructose C1 to C6 label distribution and sucrose C1 to C6 label distribution. Values are the percentage labeled moiety relative to 1-^{13}C glucose and are the mean ± SE (n=3)

	Control immature	Control maturing	Transgenic immature	Transgenic maturing
Sucrose C1 to C6 label distribution	21.02 ± 1.33	18.51 ± 2.62	25.12 ± 2.64	16.87 ± 4.16
Fructose C1 to C6 label distribution/ Sucrose C1 to C6 label distribution	2.36 ± 0.02	2.47 ± 0.01	1.46 ± 0.03	3.04 ± 0.02

4.3.3 Sucrose cycling

Sucrose cycling was calculated by the flux of label into sucrose and the return of total label (C1+C6) in fructose. The relative increases in sucrose concentration with maturation, as well as within the transgenic lines (Chapter 3) were compensated for by determining the specific activity of sucrose and glucose (Fig 4.1 and 4.2, respectively). Flux determinations thus considered both relative sucrose pool size, as well as the labeling time for the tissue discs after isotopic equilibrium was assumed. From these results it was evident that within the transgenic lines very different rates of flux exist at both stages of maturation analyzed. In the immature internodes a ten-fold increase in flux into fructose was evident, while, with the onset of maturation, a six-fold increase was evident in the transgenic lines compared to the corresponding control internode (Fig 4.1).

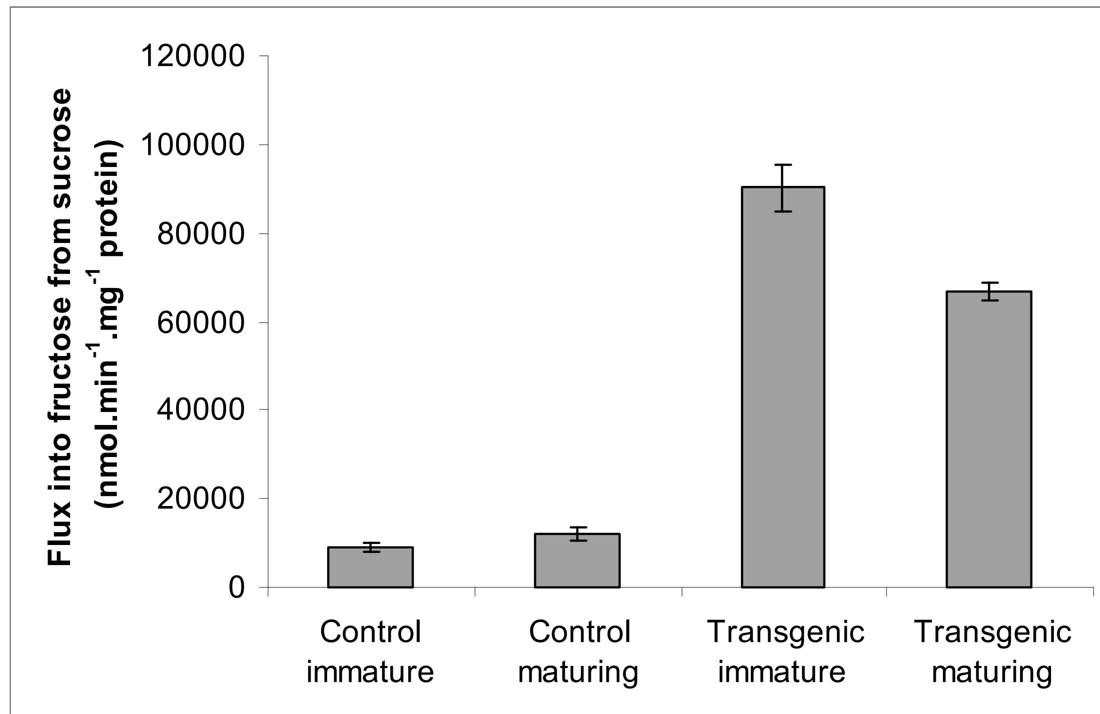

Fig. 4.1 Flux (nmol.min^{-1}.mg^{-1} protein) into fructose from the sucrose pool in immature and maturing sugarcane internodes of control (NCo 310) and transgenic (OPu 506) lines. Values are mean ± SE (n=3).

In comparison, flux distribution into sucrose from the glucose pool also indicated a significant fifteen-fold increase in the transgenic immature internode compared to the control immature internode (Fig 4.2). This significant increase was still evident in the maturing internode of the transgenic line, although the four-fold increase was less severe than the exacerbation experienced in the immature internodes.

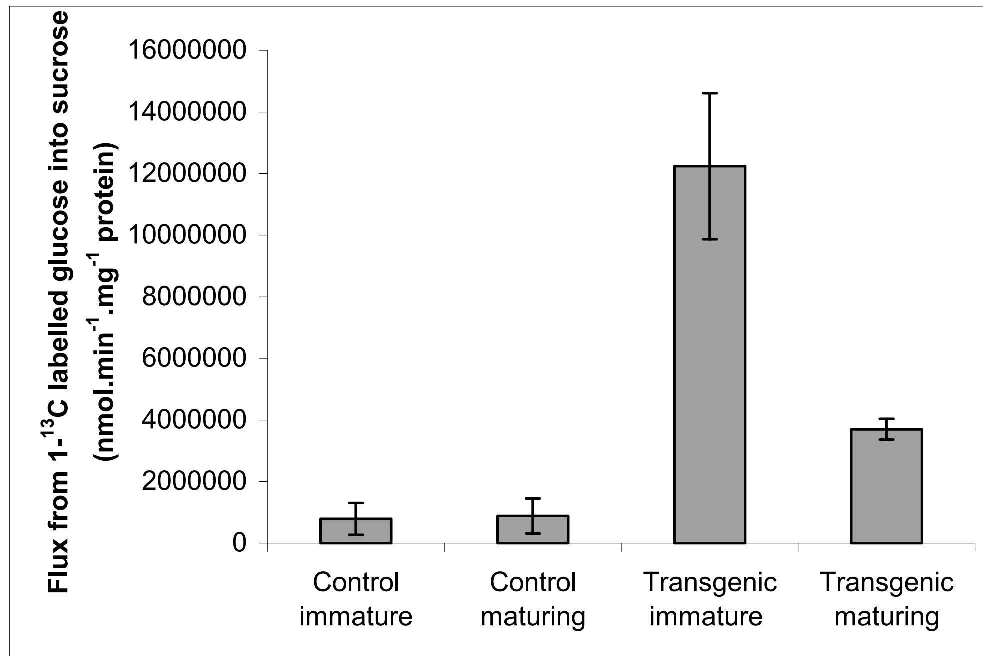

Fig. 4.2 Flux (nmol.min^{-1}.mg^{-1} protein) into sucrose from 1-^{13}C 99.9% enriched glucose in immature and maturing sugarcane internodes of control (NCo 310) and transgenic (OPu 506) lines. Values are mean ± SE (n=3).

4.4 Discussion

4.4.1 Assessment of hexose-phosphate: triose-phosphate cycling revealed gross mishandling of the hexose-phosphate: triose-phosphate cycling capacity

C1-C6 label exchange within the immature control culm indicated that a high degree of label distribution and hence hexose-phosphate: triose-phosphate cycling was evident in the sugarcane culm (Table 4.1; Bindon, 2000). A 100% cycling was observed in three out of the four conditions (control immature and maturing internodes, and transgenic maturing internodes) investigated (Table 4.1). This signifies the severe underestimation of the hexose-phosphate: triose-phosphate cycling in the sugarcane glycolytic pathway. In addition, C1:C6 ratios (Table 4.1) also indicated a close 1:1 relationship in these lines. This implicated that either full randomization between C1 and C6 occurred in the afore-mentioned conditions before sucrose hydrolysis commenced and/or that the method used to investigate hexose-phosphate: triose-phosphate cycling in sugarcane internodal tissue was insufficient to provide an accurate reflection on hexose-phosphate: triose-phosphate cycling *in vivo*.

Unexpectedly, the transgenic immature internodes revealed a decrease in the C1:C6 ratio of fructose (Table 4.1). Since negligible label return in C3 of fructose refuted the contribution of the oxidative pentose phosphate pathway (OPPP) to label enrichment in C6 two possible explanations were explored. Firstly, the experimental design and the conduction there-of were flawed by allowing too many metabolite intermediates and enzymatic conversion steps between the hexose phosphate and fructose pools. The second consideration involves the enhancement of PFK activity in the transgenic immature internodes. This would result in a loss of C1 in the respiratory pathway, resulting in less labeled C1 fructose compared to C6 fructose.

A significant decrease in the hexose-phosphate: triose-phosphate cycling with a reduction in PFP activity indicated however that PFK activity was insufficient to restore the steady state in the young internodes (Table 4.1). In light of the fact that FBPase activity is not present in sugarcane internodal tissue (Bindon, 2000), it appeared that PFP operated in a netto glycolytic direction in the transgenic immature internodes (Table 4.1). PFP is thus considered to be the primary mediator of hexose-phosphate: triose-phosphate cycling in sugarcane internodal tissue. It was evident from the data that hexose-phosphate: triose-phosphate activity was more readily required when the genetic perturbation did not allow for high metabolic activity (as seen from the immature internodes, Table 4.1) compared to the more mature regions of the culm. This was also observed in carrot cell suspension cultures (Krook et al. 2000a).

4.4.2 Transgenic sugarcane clones have higher sucrose cycling rates

With the onset of maturation, sucrose cycling decreased, suggesting that PFP regulates the balance between supply to sucrose and the demand of carbon to respiration in sugarcane (Bindon, 2000, data not shown). PFP activity has been negatively correlated with sucrose concentration (Whittaker and Botha, 1997) and a direct relationship exists between PFP activity and total respiratory flux (Whittaker and Botha, 1997). However, short term labeling of sugarcane internodal discs revealed similar sucrose fluxes in the control internodes (Fig 4.1 and 4.2). This implied that the cycling between these two entities were not significantly different.

Reduced PFP activity in the transgenic lines indicated a significant increase in sucrose cycling within the immature internodes. Contrary to prediction, reduced PFP activity did not lead to decreased rates of sucrose cycling. In hindsight, a decrease in hexose-phosphate: triose-phosphate flux would result in a significant pool of hexose-phosphates accumulating (Chapter 3). This could drive sucrose synthesis and/or degradation, resulting in an increase in sucrose cycling. In this study, this led to a ten-fold increase in sucrose cycling in the young internodes. This trend continued in the more maturing internodal regions, although less pronounced. This was probably due to 1) lower respiratory flux requirements and 2) the ability of the system to restore steady state values (Chapter 3).

4.4.3 Sucrose- and hexose-phosphate: triose-phosphate cycling: are they linked?

From the established patterns of sucrose cycling and hexose-phosphate: triose-phosphate cycling it appeared that no direct association could be established between the cycles. In the control lines no significant change in the hexose-phosphate: triose-phosphate cycling was accompanied by a slight increase in the sucrose cycling with maturation. In contrast, reduced PFP activity restricted the hexose-phosphate: triose phosphate cycling capacity slightly (Table 4.1). In contrast, a significant fifteen-fold increase in sucrose cycling was evident in the transgenic immature internodes (Fig 4.1 and 4.2). In the transgenic maturing internodes, elevated levels of hexose-phosphate: triose-phosphate cycling and reduced sucrose cycling (compared to the transgenic immature internodes) were not in the same order of magnitude. These results suggested that the regulation of the two cycles in question was not coordinated.

4.4.4 Compartmentation of sucrose metabolism

If we assume that cytosolic and vacuolar sucrose and fructose pools are in equilibrium with each other, and that the sucrose and fructose pools are in equilibrium with each other, then it is evident from Table 4.2 that compartmentation had a significant influence on the magnitude of these cycles. A two-fold reduction in hexose-phosphate: triose-phosphate cycling was evident. With the assumption as stated above, we could then assume that at least half of the sucrose pool was in the vacuolar metabolic

compartment in the control internodes. With maturation, it was seen that in the NCo 310 control this ratio was significantly elevated (Table 4.2). Due to its relative low sucrose yield capacity, we predict that this value might be significantly enhanced in greater sucrose storing genotypes.

In the transgenic immature internodes, there was a 25% reduction in the ratio, hence less sucrose are probably stored in the vacuole. Interesting, this was accompanied by increased sucrose concentrations (Chapter 3). In light of the fact that high adenylate energy charge (AEC) values were reported in these internodes (Chapter 3), more cytosolic sucrose was probably a result of high metabolic requirements in the cytosolic compartment. In addition, transgenic maturing internodes indicated significantly fructose C1 to C6: sucrose C1 to C6 ratio. These internodes accumulated more sucrose in their vacuoles, and also had elevated levels of sucrose. With the restoration of steady state levels (Chapter 3) we predict, however, that this value will not be significantly different between the control and transgenic mature internodes.

Conclusion

A reduction in endogenous PFP activity resulted in decreased hexose-phosphate: triose-phosphate cycling in immature internodal tissue. It was evident that PFP is the mediator of the hexose-phosphate: triose-phosphate cycling and that it operates in a netto glycolytic direction when high metabolic activity was required. It appeared, however, that the methodology used to follow C1: C6 label distribution to be an adequate measure of cycling could not be confirmed. Sucrose cycling, on the other hand, increased significantly in the transgenic internodes. Alternatively, when comparing the fructose C1:C6 label exchange and sucrose C1:C6 label exchange a relative idea of sucrose compartmentation was assumed. From the cycling capacities of both the hexose-phosphate: triose-phosphate and sucrose cycling it was concluded that the two cycles were not co-regulated.

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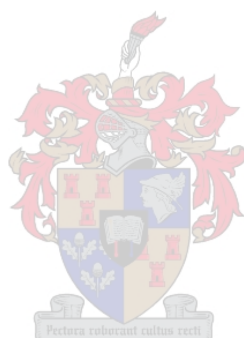

Chapter 5

The influence of hexose-phosphate and carbon cycling on sucrose accumulation in sugarcane

A GENERAL DISCUSSION

5.1 Manipulation of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase in sugarcane internodal tissue

Cytosolic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) activity assays and protein blotting confirmed the down regulation of PFP in transgenic sugarcane tissue (Fig 3.1). The effects of the manipulation were specific and direct as evident by three lines of evidence. Firstly, no phenotypic change was observed in the culm or leaves of the transgenic lines. Secondly, the maintenance of prevailing nucleotides levels (Table 3.4, 3.5, 3.6 and Fig 3.2) and healthy adenylate energy charge (Fig 3.3) indicated that the perturbation did not affect the primary energy metabolism. Thirdly, the similarity of the metabolite ratios of the selected sucrolytic and glycolytic reactions (Table 3.3) indicated no major pleiotropic effects in gene regulation brought about by the technology. However, although the extent of decrease proved to be successful for observation of metabolic changes (Table 3.4, 3.5 and 3.6), the method did not remove all PFP activity (Fig 3.1B). Nevertheless, a 95% reduction (Fig 3.1B) indicated that the technology was effective in down-regulating all the isoforms of PFP in sugarcane internodes.

5.2 The allure of PFP in internodal sugarcane tissue

The down-regulation of PFP activity resulted in an increased hexose-phosphate: triose-phosphate ratio (Table 3.4) and a decrease in hexose-phosphate: triose-phosphate cycling (Table 4.1) in the immature tissues of the transgenic lines. It was concluded that PFP activity mediates hexose-phosphate: triose-phosphate cycling in sugarcane immature internodal tissue (Chapter 4) and operate primarily in the glycolytic direction in these internodes (Chapter 4). These conclusions are similar to that drawn by Bindon (2000) and Whittaker and Botha (1999).

The hexose-phosphate: triose-phosphate cycle was not significantly different between the mature and immature internodes of the control and the mature internodes of the transgenic lines. This suggests that PFP activity is a limiting factor for hexose-phosphate: triose-phosphate cycling only in the immature internodes.

5.3 Substrate cycling

The interaction of sucrose- and hexose-phosphate: triose-phosphate cycling and their role in sucrose accumulation have not been assessed in sugarcane internodal tissue. In our approach we considered both static and dynamic interactions between metabolites. However, when comparing prevailing metabolite levels and flux data it should be kept in mind that compartmentation was not taken into consideration in these analyses. In addition, a 5h labelling experiment discriminates against vacuolar metabolism. These factors might significantly change how we view the cycle(s) and the factors that influence them. Assessment of sub-cellular metabolite levels are a pressing need in sugarcane storage parenchyma.

5.3.1 Sucrose- and hexose-phosphate: triose-phosphate cycling is not linked

No significant pattern in the cycling rates could be established between the hexose-phosphate: triose-phosphate- and sucrose fluxes of the immature control and transgenic internodes (Table 4.1, Figure 4.1). Since PFP exerted its effect more explicit at this culm section (section *5.2* and chapter 3) it was expected that, if a strong link existed between these cycles then, the most significant changes would also occur here.

Interestingly, neither hexose-phosphate: triose-phosphate- nor sucrose cycling changed significantly with the onset of maturation in the control lines (Table 4.1, Figure 4.1). This is in contrast to a general decrease in cycling previously reported by Whittaker and Botha (1997) and Bindon (2000). Also, in the maturing transgenic internode, similar hexose-phosphate: triose-phosphate substrate cycling rates were observed than those of the control maturing internode, but the sucrose cycling flux was significantly higher in the transgenic maturing internode compared to that of the control. This was the internodal section where the prevailing metabolites approached steady state (Table 3.5),

and thus no significant different cycling rates were expected. This further illustrates the fact that the two substrate cycles shared no obligatory link.

The reaction rate of any reaction is governed by two factors, namely i) the catalytic capacity of the reaction and ii) substrate availability. Analysis of the immature transgenic metabolome indicated that a reduction in the flux between F6P and F1,6-P_2 resulted in a 1.4-fold decrease in hexose-phosphate: triose-phosphate flux leading to a three- to six-fold increase in the hexose-phosphate pools (Table 3.4). Increasing the hexose-phosphate pool increased the reaction rate towards sucrose synthesis due to higher substrate concentrations. This yielded three- to six-fold higher sucrose concentrations (Table 3.4). Since the reaction rate is proportional to the concentrations of the molecules involved (*law of mass action*) higher sucrose levels (as a substrate) would result in higher sucrose degradation rates. The netto effect, as seen in the transgenic immature internodes, was a significant increase in sucrose cycling.

In comparison, enhancing the capacity of the hexose-phosphate: triose-phosphate cycling in transgenic tobacco calli with increased levels of F2,6-P_2 resulted in no alteration in the sucrose cycle (Fernie et al., 2001). This illustrated that an increase in the catalytic capacity of the hexose-phosphate: triose-phosphate cycle resulted in a change in the flux pattern between the two systems, but did not change the speed at which sucrose cycling was operating.

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5.3.2 Cycling and carbon partitioning to sucrose: a substrate driven model of sucrose accumulation

PFP activity in control tissues decreased 8-fold from internode 3 to 6 (Fig 3.1) leading to no reduction in hexose-phosphate: triose-phosphate cycling (Table 4.1). This resulted in a 1.5-fold increase in sucrose and hexose-phosphate concentrations (Table 3.2). In contrast, transgenic internode 3 had similar hexose-phosphate: triose-phosphate cycling rates and PFP activities than those observed in the control internode 6 (Table 4.1, Fig 3.1). This resulted in a significant 6-fold increase in sucrose and hexose-phosphate concentrations (Table 3.4). Thus it appeared that elevated sucrose concentrations in sugarcane sinks was a result of increased substrate availability caused by the perturbation

in the system. This increase in substrate could be attributed to two sources, namely sucrose synthesis and sucrose degradation.

An increase in the hexose-phosphate and hexose pools (Table 3.4) resulted in higher substrate availability for sucrose synthesis by either SPS and/or SuSy. The elevation of G6P (activator) levels (Table 3.4), suggested that SPS activity might be up-regulated. If we assume that SPS is responsible for the bulk of sucrose synthesis, then the accumulation of UDP-glucose and F6P levels might indicate that SPS activity could not cope with the excess substrate available.

Increased hexose and UDP-glucose levels (Table 3.4) in the transgenic lines indicated a stimulation of sucrose degradation via either SuSy or invertase. The increase in PPi levels suggested that SuSy breakdown is not favored in the young internodes, but the mass-action ratio (Table 3.3) of SuSy indicated no change in regulation occurred at this step and therefore might be (partially) responsible for the breakdown. Maximum catalytic activity of SuSy is, also, sufficient to sustain catalyses of the reaction in transgenic potato tubers with reduced PFP activity (Hajirezaei et al., 1994).

On the other hand, increased NI activity might be responsible for increased hexose pools observed. This would attribute to the significant change in regulation at the hexose phosphorylation step (Table 3.3), but its role and contribution is purely speculative. If NI activity is, however, responsible for sucrose degradation in the young internodes then this might contribute further to an increase in the hexose-phosphate pool. The enhanced hexose-phosphates (Table 3.4) can increase substrate availability for carbon allocation to other pools (Fig 5.1). It appeared that the sucrose pool was the strongest competitor for these extra carbons, since it was the metabolite that showed the most significant increase in the transgenic lines (Table 3.4). Enhanced sucrose levels probably resulted in higher sucrose cycling as was observed in the transgenic lines. This indicated that several competing sinks for sucrose might be activated at this point (Fig 5.1). These might include sucrose cycling and/or loading of vacuolar sucrose.

The precise mechanism of sucrose/hexose loading into the vacuole has not been evaluated and it has to be established if the event occurs actively or passively. Enhanced PPi levels (Table 3.4), however, questions the loading of sucrose in the vacuole via H^+-PPase activity when high sucrose concentrations were present, but needs further

verification. The carbon allocation to these sinks will probably be determined by their catalytic capacity or affinity to the substrate.

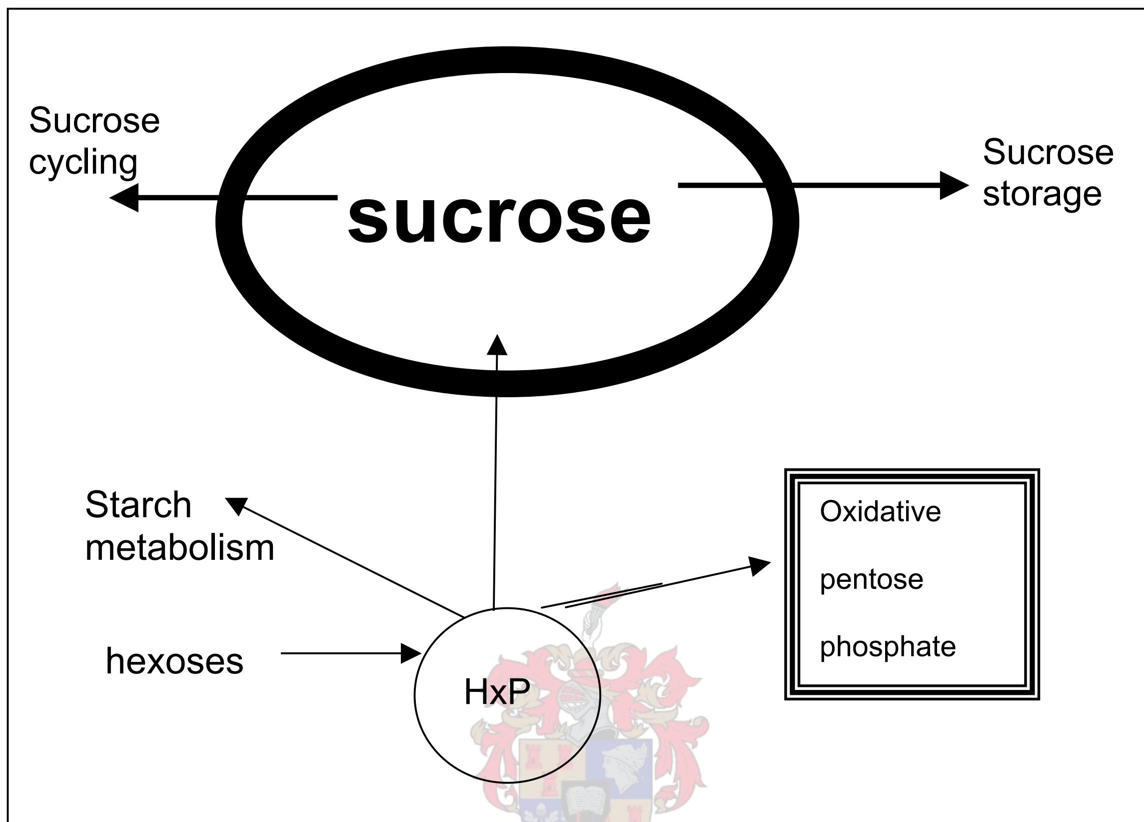

Fig 5.1 The substrate driven model for sucrose accumulation. HxP=hexose-phosphates

In conclusion, hexose-phosphate: triose-phosphate cycling could not be linked to sucrose cycling. It was implicated that sucrose cycling is a phenomenon of higher cytosolic sucrose concentrations. This was, however, not the only mechanism that was postulated for higher substrate utilisation. Vacuolar sucrose loading might also compete for the excess substrate available.

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CURRICULUM VITAE

Margaretha Johanna Van Der Merwe

Personal Details

Identity no.:	7912250145082
Nationality:	South African
Date of Birth:	1979/12/25
Sex:	Female
Marital status:	Single
Email Address:	plantb16@sun.ac.za

Personal Profile

Masters student with major areas of expertise in GC-MS, HPLC, spectrophotometric, and immunological-detection technology. Strong analytical abilities, problem solving and people-management skills. Fluent in English and Afrikaans, moderate in isiXhosa and Spanish.

Skills Summary

Specialist Knowledge

Compilation of sugarcane metabolite profiles via GC-MS, HPLC and spectrophotometric methods.

Skills and Abilities

Experienced in protein extractions, immunological-detection methods, radio-active and stable isotope labelling (NMR detection). DNA and RNA quantification/ detection methods. Undergraduate and postgraduate training in genetic transformation and tissue-culture techniques. Undergraduate knowledge in seed biotechnology, plant secondary metabolism, selected aspects of plant respiration and mitochondrial isolations, lipid metabolism, and nitrogen, sulphate and phosphate metabolism.

Computer Skills

Microsoft Word, PowerPoint and Excel, Statistica and Sigma plot literate.

Also, undergraduate training in Visual Basic, Microsoft Access and CorelDraw.

Other

Drivers licence

Research History

January 2003 – current

M.Sc. – Institute for Plant biotechnology, Stellenbosch

Project title: "The influence of hexose-phosphates and carbon cycling on sucrose accumulation in sugarcane"

- **Skills:** GC-MS, HPLC and spectrophotometric skills. Interpretation of data via statistical analyses.
- 13C isotope labelling and detection via NMR imaging.
- **Achievements/Improvements made:** Optimisation of the above-mentioned skills for sugarcane tissue. The application and improvement of current processing methods in elucidating metabolite identification. Compilation of metabolic profile in transgenic sugarcane clones.

  Assessing sucrose cycling and triose-phosphate cycling in transgenic clones.

  Also, attended HPLC introductory workshop, as well as a Varian solid phase extraction workshop.

Aug 2003-Jan 2004 & Jul 2004-Jan 2005

Contract lecturer – University of Stellenbosch, Stellenbosch

Lecturing final year undergraduates in "Carbohydrate metabolism in the sink tissue".

- **Responsibilities:** Compilation and presentation of selected course material during established contact sessions (6 week-course).
- Compilation and demonstration of practicals (6x3hrs) concerned with carbohydrate metabolism.
- Compilation and assessment of examination papers.

Aug 2003-October 2004 & September 2004-November 2004

Practical Leader – University of Stellenbosch, Stellenbosch

Lecturing and demonstrating practicals for first year students in Biology 144: The evolution of plants (part of the undergraduate course Biodiversity and Ecology)

January 2001 – December 2003

Practical demonstrator – University of Stellenbosch, Stellenbosch

Assisting in first year practicals (Biology 124 (Cell biology), 144 (Biodiversity and Ecology) and 154 (Functional biology (form and function of plants))

Education

Further Education

December 2002 – University of Stellenbosch

Hons BSc – Plant biotechnology – *cum laude*

Project title: "Metabolic profiling of transgenic sugarcane clones with reduced PFP expression"

Subjects: Integrated Plant Metabolism, Plant molecular biology

December 2001 – University of Stellenbosch

BSc – Plant biotechnology – *cum laude*

Subjects: Biochemistry, Plant Biotechnology, Genetics, Microbiology, Chemistry, Botany

Project title: "Determination of malate and sugar levels in developing grape berries" (Biotechnology 334: project consisting of four months)

School

January 1994 – December 1998

Goudini High School, Rawsonville

Matric (Grade 12) exemption

Subjects: Afrikaans, English, Science, Mathematics, Biology, Geography, Art

Awards

2003-2004: National Research Foundation Prestige Scholarship for Master's study

2002: National Research Foundation Grant holders Scholarship for Honor's study

2001: SASEX Book prize for best Third Year student in Plant biotechnology, University of Stellenbosch, South Africa

1998: Dux pupil (Goudini High School, Rawsonville, South Africa)

Leadership

1999: Second year committee (treasurer: Sonop ladies residence, Stellenbosch, South Africa)

2001: House Committee member of Sonop ladies residence, Stellenbosch, South Africa

1998: Head girl of Goudini High School, Rawsonville, South Africa
Student representative council (Goudini High School, Rawsonville, South Africa).