MANIPULATION OF NEUTRAL INVERTASE ACTIVITY IN SUGARCANE

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

The main goal of this project was to elucidate the apparent role of neutral invertase (NI) in sucrose accumulation in sugarcane. In the first part of the study putative transgenic cell lines (transformed with antisense NI constructs) were characterised to confirm the stable integration and expression of the transgene. Batch suspension cultures were used to initiate replicate cultures of several of these transgenic lines as well as a control, and the metabolism of the cultures during a 14 day growth cycle was examined.

The transgenic lines had substantially reduced levels of NI activity. While the activities of the other invertases remained unchanged, the activity of sucrose synthase (SuSy) was significantly higher in the transgenic suspension cultures relative to the control. Throughout the growth cycle, sucrose concentrations in the transgenic lines were consistently higher, and glucose and fructose concentrations lower, than the control. The transgenic cultures also exhibited a decreased growth rate in comparison to the control. Labelling studies confirmed a decrease in the *in vivo* rate of invertase-mediated sucrose hydrolysis in the transgenic lines, as well as indicating a decline in the partitioning of carbon to respiratory pathways in these cultures.

In the second part of the study, which focussed on greenhouse-grown transgenic plants, similar results were reported. NI activity was significantly decreased, and SuSy activity increased in all of the tissues sampled. The sucrose concentration and purity were also higher in the transgenic tissues, while the *in vivo* sucrose hydrolysis rate was lower. Allocation of carbon to respiration was lower in the transgenic plants, suggesting that a decrease in sucrose breakdown reduces the availability of hexoses for growth and respiration. Overall, the results suggest that NI plays a key role in the control of sucrose metabolism, and that changes in the activity of this enzyme have far-reaching effects on cellular metabolism.

The fact that the trends reported in the whole-plant studies parallel those of the suspension cultures confirms that suspension cultures can be used as a model system in metabolic engineering research in sugarcane. Thus the possibility now exists to analyse large numbers of transgenic lines in a quicker time frame and at a reduced cost in comparison to conventional methods.

OPSOMMING

Die hoofdoelwit van hierdie projek was om die potensiële rol, wat neutrale invertase (NI) in sukrose-akkumulering in suikerriet speel, te ontrafel. In die eerste deel van hierdie studie is transgeniese lyne (wat met antisens NI-konstrukte getransformeer is) gekarakteriseer om die stabiele integrasie en uitdrukking van die transgeen te bevestig. Duplikaat lot-suspensie kulture is van verskeie van hierdie transgeniese lyne en 'n kontrole lyn geïnisieër, en die metabolisme van die kulture is gedurende 'n 14-dag groeisiklus bestudeer.

Die transgeniese lyne het ’n duidelike verlaging in NI-aktiwiteit getoon. Terwyl die aktiwiteite van die ander invertases onveranderd gebly het, het die aktiwiteit van sukrosesintase (SuSy), relatief tot die kontrole, verhoog. Gedurende die verloop van die groeisiklus was sukrose konsentrasies konstant hoër, en glukose en fruktose konsentrasies laer as díe van die kontrole. Die transgeniese kulture het ook 'n laer groeitempo in vergelyking met die kontrole getoon. Merkerstudies het 'n vermindering in die *in vivo* tempo van invertase-bemiddelde sukrosehidrolise in die transgeniese lyne bevestig en ook op 'n afname in die verdeling van koolstof na die respiratoriese weë in hierdie kulture gewys.

In die tweede deel van hierdie studie, wat gefokus het op glashuis-gekweekte transgeniese plante, is soorgelyke resultate verkry. NI-aktiwiteit was noemenswaardig laer en SuSy-aktiwiteit hoër in al die weefsels was geanaliseer is. Die sukrosekonsentrasie asook die suiwerheid was hoër in die transgeniese weefsels, terwyl *in vivo* sukrosehidrolise teen 'n laer tempo plaas gevind het. Verdeling van koolstof na respirasie was laer in die transgeniese plante, wat aandui dat 'n afname in die afbraak van sukrose die beskikbaarheid van hekoses vir groei en respirasie verminder. Ten slotte, die resulate dui daarop dat NI 'n sleutelrol speel in die beheer van sukrosemetabolisme en dat veranderinge in die aktiwiteit van hierdie ensiem ’n vêrreikende invloed op selulêre metabolisme uitoefen.

Die feit dat die tendense in die heel-plant studies vergelykbaar is met díe van die suspensiekulture, bevestig dat suspensiekulture as 'n model sisteem vir metaboliese manipuleringseksperimente in suikerriet gebruik kan word. Dus bestaan die moontlikheid nou om groot hoeveelhede transgeniese lyne oor 'n korter periode en teen laer kostes te analiseer.

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PREFACE

This dissertation is presented as a compilation of 5 chapters. In Chapter 1 the general aims and motivation for this study are introduced. Chapter 2 is a review of the literature that is applicable to the field of study. Chapters 3 and 4 are the experimental chapters that delineate the exact aims and outcomes that together comprise the full body of experimental work endeavoured in this research. Chapter 5 concludes the work with a general discussion aimed at integrating the work presented in the preceding chapters into a coherent whole. Each chapter that will be submitted for publication is written according to the style of the particular journal as listed below.

Chapter 1	General Introduction. Will not be submitted for publication. Style: Plant Physiology
Chapter 2	Literature Review: Overview of enzyme activities involved in the cycle of sucrose synthesis and degradation, particularly in the sugarcane culm and sugarcane suspension culture cells. Will not be submitted for publication. Style: Plant Physiology
Chapter 3	Down-regulation of neutral invertase activity in sugarcane cell suspension cultures leads to increased sucrose accumulation. Submitted to Functional Plant Biology
Chapter 4	Down-regulation of neutral invertase activity in transgenic sugarcane plants reduces respiration and futile sucrose cycling in the internodes. Submitted to The Plant Journal
Chapter 5	General discussion. Will not be submitted for publication. Style: Plant Physiology

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LIST OF ABBREVIATIONS

AI	acid invertase
ATP	adenosine 5'-triphosphate
bp	base pairs
Bq	Bequerel
BSA	bovine serum albumin
^{o}C	degrees centigrade
^{14}C	radio-labeled carbon
$^{14}CO_2$	radio-labeled carbon dioxide
CWI	cell wall invertase
ddH_2O	double distilled water
DTT	1,4-dithiothreitol
DNA	deoxyribo nucleic acid
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
g	gram
x g	gravitational force
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
Hr	hour
kDa	kilo Dalton
L	litre
M	molar
MES	2-[N-morpholino] ethanesulfonic acid
min	minute
NAD^+	oxidised nicotinamide adenine dinucleotide
NI	neutral invertase (β-fructofuranosidase, EC 3.2.1.26)
nm	nanometer
O_2	oxygen
OD	optical density
PCR	polymerase chain reaction
PEP	phospho(enol)pyruvate

PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependant phosphofructokinase (pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90)
Pi	inorganic phosphate
PVPP	polyvinylpolypyrrolidine
RNA	ribonucleic acid
rpm	revolutions per minute
sec	seconds
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)
TCA	tricarboxylic acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate
v	volume
w	weight

CHAPTER 1

GENERAL INTRODUCTION

Central carbon metabolism in most sugar- or starch- storing plants is a vital area of study from an agricultural perspective. The value of these particular crops is directly related to the capacity of specialized sink tissues to synthesize and accumulate commercially valuable storage carbohydrates. Sugarcane is no exception: This C4 grass can accumulate high sucrose concentrations in the specialised parenchyma cells of the internodal culm cells (Moore & Maretzki, 1997). It is an important agricultural plant, especially in South Africa, where the sugar industry produces 2.5 million tons of sucrose per season, generating an annual average direct income of R 6 billion. The SA sugar industry contributes R 2 billion to the country's foreign exchange earnings on an annual basis, providing employment for some 350 000 individuals, either directly or indirectly in the manufacturing industry (www.sasa.org.za).

Worldwide, sugarcane research is a diverse field, focussing on a wide array of desired traits. One of the most important goals of many of these programs is the improvement of the cost-effective production of sucrose, i.e. sucrose yield per ton cane. Initially these challenges were met by traditional breeding approaches, which were later assisted by molecular marker technologies. However, the breeding and selection process remained fairly time-consuming and labour-intensive. It has also been suggested that the natural genetic potential of commercial sugarcane varieties has been exhausted, and thus further attempts at improving sucrose yield by breeding and selection will prove fairly futile (Grof & Campbell, 2001). In recent years, with the advent of the genomic era, the technology and resources that came into being made it possible to target genes in a more strategic and specific manner. Now the challenge of increasing sucrose yield in sugarcane can be met by careful down- or up-regulation of the enzymes that are involved in sucrose metabolism. It is hoped that genetic manipulation approaches would be more effective in producing new varieties with specific desired traits.

Commercial sugarcane varieties have the capacity to store up to 25% of their fresh weight as sucrose under optimum growth conditions (Moore & Maretzki, 1997). A popular theory is that the control of sucrose accumulation in the culm is exerted via a 'futile cycle' of sucrose

synthesis, breakdown, re-synthesis, etc. (Wendler *et al*., 1990). In this cyclical pathway, sucrose is synthesized in the cytosol by the enzyme sucrose phosphate synthase (SPS; EC 2.4.1.14) and subsequently hydrolysed by the invertases (β-fructofuranosidase; EC 3.2.1.26) or broken down to fructose and UDP-glucose by the enzyme sucrose synthase (SuSy; EC 2.4.1.13). Sucrose content during internode development can thus be roughly correlated with the difference between SPS and invertase activities (Zhu *et al*., 1997). As the sucrose concentration in the culm will depend on the balance between the sucrose synthesis and hydrolysis rates, it is reasonable to assume that the hydrolytic enzymes, particularly those of the cytosolic compartment where sucrose synthesis occurs, will play some part in determining the overall sucrose concentration in the storage tissues.

Of course sucrose accumulation is a highly complicated process, and there are many other factors to consider, i.e. the role of the storage compartment, environmental factors etc. In fact, the biochemistry of sucrose accumulation is still unresolved in its entirety (Moore, 2005). In this project, the focus was on the role of one of the sucrose-hydrolysing enzymes, namely neutral invertase (NI). This cytosolic enzyme is likely to be partially responsible for the cycle of degradation and synthesis of sucrose that has been found in sugarcane suspension cells (Wendler *et al*., 1990), in the immature sugarcane stem (Sacher *et al*., 1963) and in sugarcane tissue discs (Glasziou, 1961; Bindon & Botha, 2002). Using an antisense approach, we hoped to partially silence the gene encoding the NI protein. By decreasing the *in vivo* activity of this enzyme the potential exists to decrease the rate of sucrose hydrolysis and increase steady-state sucrose concentrations in the culm of transgenic plants.

The second component of our study was the establishment of sugarcane cell suspension cultures as a viable system for growing and analysing transgenic sugarcane lines. Cell suspensions of sugarcane are heterotrophic, representing typical sink cells. They also have the ability to store up to 25% of their dry weight as sucrose (Thom *et al*., 1982). Our goal was to investigate SPS, SuSy and invertase activities as well as metabolite levels in the transgenic suspension cultures throughout a 14 day culture period. During this time, the suspension cultures exhibit similar growth and sucrose-accumulating trends to maturing sugarcane internodes, namely an initial period of rapid growth followed by a period of sucrose accumulation (Thom *et al*., 1982). A suspension culture approach has never been followed before in sugarcane transgenic studies, and it offers several advantages over standard approaches. Probably the most important of these is regarding the time frame in which

preliminary analyses of the transgenic lines can be completed: While it may take 2 years to generate mature transgenic plants from callus, suspension cultures are ready for analysis in just a few weeks. In addition, large numbers of transgenic lines can be grown in a relatively small space under constant conditions, which overcomes the complications of greenhouse- and field- grown plants where the creation of uniform environmental treatments is a near impossibility. Overall, this means that homogenous replicates of the transgenic lines can be created for rapid, reproducible sampling – and at a reduced cost compared to conventional methods.

The goals of this project thus include the characterisation of transgenic sugarcane lines transformed with the antisense NI construct, both in suspension cultures and in greenhouse-grown plants. This characterisation will include PCR and Northern analyses (to confirm the integration and expression of the antisense sequence), determination of various enzyme activities (NI in particular), quantification of sucrose, glucose and fructose levels and finally the determination of *in vivo* sucrose hydrolysis rates and partitioning of carbon between the major metabolite pools. It is our expectation that a decrease in NI activity in the transgenic lines will lead to changes in metabolite concentrations, carbon partitioning and sucrose cycling in at least some of the tissues sampled. The results obtained from the greenhouse-grown plants will be compared with those of the suspension cultures to determine if the latter does in fact serve as a suitable model system for transgenic research.

To conclude, an overview of all the aims and outcomes of this study is presented in the context of the various chapters in which they were dealt with.

Chapter 2: Sucrose metabolism in sugarcane and the role of neutral invertase.

Aim: To present the background of this study as a review of applicable literature, including a critical discussion on the apparent role of NI in the complex process of sucrose accumulation in the internodal culm of sugarcane.

Outcomes: A broad overview of sucrose accumulation is presented, including the metabolism, transport and storage of this key metabolite in the context of sugarcane in particular. The roles of the various enzymes involved in the synthesis and degradation of sucrose are briefly discussed, with particular emphasis on the enzymes involved in the breaking down of sucrose.

As the specific focus of this project is on NI, a detailed report is presented regarding the molecular and kinetic properties of NI, as well as the potential role of NI in sucrose metabolism in sugarcane. An overview of suspension cultures as an experimental system is also given with specific reference to the advantages of using these cultures as a model system in the study of sucrose accumulation.

Chapter 3: Down-regulation of neutral invertase activity in sugarcane cell suspension cultures leads to increased sucrose accumulation.

Aim: To verify the potential role of NI in sugarcane sucrose metabolism by using suspension cultures as a model system for the preliminary analysis of transgenic lines in which NI activity has been down-regulated by antisense technology.

Outcomes: NI activity was successfully down-regulated in several transgenic sugarcane lines using antisense constructs. Reduced NI activity increased sucrose concentrations and decreased glucose and fructose concentrations in almost all the tissues that were sampled. These changes were more pronounced in the immature cultures, but no significant differences were apparent in the mature cultures. The increased accumulation of sucrose appeared to be at the expense of growth. This observation was supported by the results of labelling studies which indicated a decrease in carbon partitioning to the respiratory metabolite pools.

Chapter 4: Down-regulation of neutral invertase activity in transgenic sugarcane plants reduces respiration and futile sucrose cycling in the internodes.

Aim: To determine the effect of down-regulating NI activity on sucrose accumulation in greenhouse-grown plants, and compare the results with those of the suspension culture system.

Outcomes: In vivo NI activity was also successfully reduced in the transgenic plants, and sucrose accumulation and purity were significantly increased, particularly in the immature internodes. The decreased rate of sucrose hydrolysis in the transgenic plants reduced the availability of hexoses for respiration, suggesting that a change in NI activity alters the pattern of carbon allocation within a cell/ tissue.

Chapter 5: General discussion.

Aim: To integrate the observations and discussions of the experimental chapters.

Outcomes: The results and findings of previous chapters are integrated and a conclusion regarding the role of NI in sucrose accumulation is presented. The results of the two different experimental systems (namely suspension cultures and whole plants) are compared with one another and conclusions are drawn regarding the potential for suspension cultures as a model system for transgenic studies in sugarcane. The potential focus of future research on this topic is also discussed.

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CHAPTER 2

Sucrose metabolism in sugarcane and the role of neutral invertase

INTRODUCTION

Sugarcane is an extremely important crop plant in many parts of the world and accounts for 60% of the world's sucrose production (Grivet & Aruda 2001). Many plant storage organs that accumulate high levels of sugar accumulate primarily sucrose (Hatch & Glasziou, 1963; Giaquinta, 1977; Schaffer *et al*., 1987; Stommel & Simon, 1989). Sugarcane internodes are no exception, as commercial sugarcane varieties have the capacity to store up to 25% of their fresh weight as sucrose under optimum growth conditions (Moore & Maretzki, 1997).

Research has shown that sucrose accumulation in sugarcane is not limited by photosynthetic rate, but that regulation of sucrose accumulation occurs instead at the translocation and/ or sink level (Moore & Maretzki, 1997). It should also be mentioned that sucrose accumulation in sugarcane is strongly influenced by environmental and nutritional factors: Warm temperatures and abundant water and nitrogen supply, which favour vigorous growth, also tend to produce sugarcane plants with lower sucrose contents (Clements *et al*., 1952; Thomas & Schmidt, 1978). Thus both *in planta* and environmental factors act together to determine the sucrose-accumulating characteristics of sugarcane.

Sucrose accumulation begins in internodes during the stage of elongation (around internodes 4 to 9) and continues long after elongation ceases (internodes 10 onwards). Sucrose storage may contribute to high sugar levels because sucrose has half the osmolarity of the equivalent amounts of glucose and fructose (Steingröver, 1983) and is less metabolically accessible than hexose sugars to respiratory loss (Salerno & Pontis, 1978). The degree to which sucrose can be imported and accumulated by sugarcane internodes is a function of the sink strength of the storage tissues. Sink strength can be considered as the product of sink size and sink activity (Warren-Wilson, 1972). Sink size is a physical constraint that includes cell number and cell size, while sink activity is a physiological constraint that includes multiple factors and key enzymes involved in carbohydrate utilization and storage.

Besides serving as the carbon source for heterotrophic growth, there is increasing evidence that sugars are also involved in the transcriptional regulation of enzymes involved in photosynthesis and heterotrophic metabolism (Farrar, 1991; Williams *et al*., 1992). This type of regulation by glucose, fructose or sucrose has been designated as metabolic regulation (Karrer & Rodriguez, 1992). Consequently, sucrose metabolism in sink organs is also integral to the establishment of sinks and in determining relative sink strength.

The yield of sucrose from sugarcane is a function of both the total plant biomass produced and the sucrose content of the culm (Ebrahim *et al*., 1998). These two opposing pathways (growth and sucrose synthesis) 'compete' for both substrates and energy (Hatch & Glasziou, 1963). Partitioning of carbon thus requires intricately controlled processes to balance the supply of carbon skeletons and energy with the demand from these two pathways, in such a way as to ensure an optimized trade-off between growth and sucrose accumulation.

For decades the improvement of commercial sugarcane varieties in terms of their sucrose yield has been an important focus area in sugarcane research. Until recently, this has been achieved through the lengthy processes of careful breeding and selection for desirable traits. It has become apparent that this laborious approach has reached its limits, and that alternative practices need to be endeavoured in order to advance the performance of sugarcane varieties beyond the current threshold (Grof & Campbell, 2001). To this end, genetic manipulation technologies have been employed to identify and manipulate important reactions in sugar metabolism in order to improve crop yield and performance.

Sucrose production in sugarcane is a widely researched yet poorly understood phenomenon. The wide array of enzymatic reactions and biochemical processes directly and indirectly involved in the accumulation of this important metabolite has made the identification of rate-limiting steps quite problematic. The identification and targeting of crucial reactions remains a priority and could lead to the generation of new genotypes with more desirable characteristics. While both the rates of sucrose synthesis and breakdown will influence the final concentration of accumulated sucrose, the focus here will be on the breakdown reactions.

SUCROSE

Transport

In higher plants sucrose is the major transported form of carbohydrates, as confirmed by ^{14}C-hexose feeding experiments (Hatch & Glasziou, 1964). Sucrose is exported from the source tissues and transported via the phloem to the different sink tissues, driven by mass flow motivated by the turgor potential gradient between the source and sink regions of the phloem (Minchin & Thorpe, 1987). A gradient in sucrose concentration between the phloem cells and the storage parenchyma may be maintained by dilution from incoming water, starch synthesis, respiration, and active sucrose storage in the vacuole.

Metabolism in Sink Tissues

The largest sink for the transported sucrose is the parenchymatous tissues of the core regions of sugarcane internodes (Jacobsen *et al*., 1992). Following transport via the vascular tissues of the core regions, cells in the sink tissues may take up sucrose symplastically or apoplastically (Moore & Maretzki, 1997). During passage via the apoplast, sucrose may be hydrolyzed to glucose and fructose by cell wall invertase (CWI, EC 3.2.1.26). Having entered the cell intact, sucrose may be hydrolyzed by cytosolic neutral invertase (NI, EC 3.2.1.26) or be broken down to UDP-glucose and fructose by the reversible reaction of sucrose synthase (SuSy, EC 2.4.1.13). Alternatively, upon entering the vacuole, sucrose may be hydrolyzed by vacuolar acid invertase (AI).

Sucrose synthesis in the sink tissues occurs exclusively in the cytosolic compartment of the cell via two alternative routes. The first, catalyzed by the enzyme sucrose phosphate synthase (SPS; EC 2.4.1.14), utilizes fructose-6-phosphate and UDP-glucose to synthesize sucrose-6-phospate, which is converted to sucrose by the abundant enzyme sucrose phosphatase (EC 3.1.3.24; Stitt *et al.*, 1987). The second reaction is catalysed by SuSy, which utilizes UDP-glucose and fructose in the synthetic reaction. SPS is regarded as the predominant sucrose synthesizing enzyme in sink tissues (Batta & Singh, 1986; Wendler *et al*., 1990; Botha & Black, 2000). While both SuSy and SPS may contribute to sucrose synthesis in immature tissues, it has been shown that in mature tissues of sugarcane the activity of SPS is up to three times greater than that of SuSy (Botha & Black, 2000). Furthermore, the reaction catalyzed by SuSy is reversible, and the high sucrose concentrations in mature tissues should favour the breakdown activity of SuSy (Lingle & Irvine, 1994; Schäfer *et al*., 2004).

Thus, although the synthesis of sucrose takes place exclusively in the cytosol, stored sucrose is distributed between the symplast, the apoplast and the vacuole to varying degrees and therefore all the hydrolytic and cleavage activities exert an influence on sucrose storage to some extent (Hawker, 1985; Welbaum & Meinzer, 1990). The various sub-cellular locations of these enzymes could together allow for greater control of sucrose metabolism, translocation and storage (Lee & Vattuone, 1996).

Vacuolar Storage

The role of the vacuole as the storage compartment of plant cells is well documented for many soluble compounds in various plants (reviewed in Matile 1978; Boller & Wiemken 1986), particularly for specialized storage tissues like parenchymatous sugarcane culm where close to 90% of the cell volume is occupied by the vacuole (Komor, 1994). Sucrose is virtually the sole sugar transferred from the metabolic compartment to the vacuole. Reducing sugars in this storage compartment are derived from the hydrolysis of accumulated sucrose (Glasziou, 1961). All sugars diffuse slowly from the vacuole, with the hexoses diffusing at a faster rate than sucrose (Glasziou 1960). Sugar stored in the vacuole may be returned for local utilization or may be translocated and used elsewhere (Glasziou, 1960; Hatch & Glasziou, 1963).

It is not entirely clear how sucrose is transferred to the vacuole in storage parenchyma cells. There is, however, some evidence for carrier-mediated or facilitated diffusion transfer of sucrose, which means that the sucrose concentration in the cytosol will have major control on its accumulation in the vacuole (Preisser & Komor, 1991; Preisser *et al*., 1992).

Cycling

An interesting feature of sucrose metabolism in many plant systems is a continuous cycling of sucrose that occurs due to its degradation by the invertases and/or SuSy and subsequent re-synthesis by SPS and/or SuSy. Sucrose content during internode development can thus be roughly correlated with the difference between SPS and invertase activities (Zhu *et al*., 1997). This continuous synthesis and degradation of sucrose has been observed previously in sugarcane cell suspension cultures (Wendler *et al*., 1990) and in sugarcane culm (Glasziou, 1961; Sacher *et al*., 1963). It has been shown in maize root tips that a 'futile cycle' of sucrose turnover consumes 69% of the ATP produced by mitochondrial respiration (Dieuaide-Noubhani *et al*., 1995) which is even lower than that found in banana tissue (Hill & ap Rees,

1994). Both studies emphasize the magnitude of the investment in energy equivalents that plants have to make in order to maintain sucrose turnover in sink tissues in particular.

The continuous cycling of sucrose will, however, allow cells to respond rapidly to both variations in sucrose supply and the cellular demand for carbon for biosynthetic processes (i.e. starch and cell wall synthesis) (Fernie *et al*., 2002). The substrate cycle would allow the rate of sucrose storage to respond in a selective manner to relatively small changes in the rate of synthesis or degradation (Hue, 1980). Furthermore, this apparent futile cycle could have major physiological functions such as control of respiration (Dancer *et al*., 1990), maintenance of osmotic potential (Geigenberger *et al*., 1997), control of sugar accumulation (Rohwer & Botha, 2001), and sugar signaling (Cortès *et al*., 2003).

SUSY

Since its discovery by Leloir and co-workers in 1955 (Cardini *et al*., 1955), SuSy has been assigned pivotal roles in a variety of plant metabolic processes. The enzyme has been ascribed a central function in the determination of sink strength in both storage (Zrenner *et al*., 1995) and developing vegetative tissues (Pak *et al*., 1995; Pfeiffer & Kutschera, 1995). Its functional plasticity is probably due to the enzyme's capacity to utilize several nucleosides, its catalytic reversibility, and its diverse cellular compartmentation.

As mentioned before, SuSy catalyzes the reversible conversion of UDP-glucose and fructose to sucrose and UDP. However, substrate concentrations in most tissues where SuSy is found causes the enzyme to function in the sucrose breakdown direction (Xu *et al*., 1989; Amor *et al*., 1995). The ratio of maximum sucrose breakdown to synthesis activity in sugarcane was found to increase with internode maturity, from internode 3 to internode 9 (Schäfer *et al*., 2004). There is thus a positive correlation between the sucrose breakdown activity of SuSy and total sucrose content.

The reaction catalyzed by SuSy is freely reversible, and has a theoretical equilibrium constant (Keq) of 0.15-0.56 in the direction of sucrose degradation (Geigenberger & Stitt, 1993). The net *in vivo* flux catalyzed by SuSy will, therefore, depend on the concentration of sucrose entering the cell, and on the rate at which UDP-Glc and fructose are used and UDP is

recycled. Most SuSy isoforms characterized to date are subject to either competitive or non-competitive product inhibition by fructose (Wolosiuk & Pontis 1974; Sebkova *et al*., 1995) and UDP-glucose (Wolosiuk & Pontis, 1974). Glucose, on the other hand, acts as an uncompetitive inhibitor with regard to sucrose (Doehlert, 1987; Sebkova *et al*., 1995).

There are several examples of SuSy isoforms that are specifically or predominantly expressed in particular tissues or organs (Yang & Russel, 1990; Martinez *et al*., 1993). SuSy isoforms in various plants are located in the cytosol and/or are membrane-bound (Amor *et al*., 1995; Carlson & Chourey, 1996), or associated with the actin cytoskeleton (Winter *et al*., 1998). In more recent research it was found that at least three different isoforms of SuSy with different kinetic properties (and possibly physiological roles) could be identified in sugarcane sink tissues (Schäfer *et al*., 2005). Depending on the physiological status of the tissue, the ratio between these activities varies.

SuSy is largely localized in the companion cells of vascular bundles (Nolte & Koch, 1993; Rouhier & Usuda, 2001), which generally display higher respiratory rates than the ordinary parenchymatous cells of the culm tissues (Warmbrodt *et al*., 1989). Based on this localization, it has been suggested that SuSy may function to fuel respiration to satisfy the high ATP demand of the plasma membrane H^+-translocating ATPase (H^+/ATPase, Nolte & Koch, 1993). The H^+/ATPase in companion cells is especially important in maintaining an H^+ gradient between the apoplast and the cytosol for the plasmalemma sucrose/H^+ symport system.

Although both the invertases and SuSy are involved in the breakdown of sucrose, SuSy requires only half the net energy of the sucrose metabolic pathway catalyzed by invertase (Black *et al*., 1987). Studies of the distribution of these two enzymes have suggested that high SuSy activity is present in starch and sucrose storage sinks, whereas high invertase activity occurs in tissues in which active cell elongation is occurring (Sung *et al*., 1998). The preference for sucrose cleavage by SuSy, instead of invertase, under low oxygen conditions (Zeng *et al*., 1999) is also consistent with the presence of SuSy in the vascular tissue, which has very low oxygen content compared to other tissues (about 7% versus up to 15%) (Van Dongen *et al*., 2003).

In sugarcane, though, the role of SuSy is still a matter of debate: Significant levels of SuSy activity in sucrose accumulating internodes have been reported (Lingle & Smith, 1991; Buczynski *et al*., 1993; Lingle & Irvine, 1994). It has also been found that SuSy activities remained constant as internodes matured, showing only a minor increase toward the more mature internodes (Botha *et al*., 1996). However, no significant correlations between SuSy activity and sucrose accumulation rate have been found, leaving some uncertainty as to the exact role and importance of this enzyme to sucrose accumulation in the culm (Lingle, 1999).

INVERTASES

General

Several different physiological functions have been proposed for invertases, i.e. to provide growing tissues with hexoses as a source of energy (Ap Rees, 1974), to generate a sucrose concentration gradient and to partition sucrose between source and sink tissues, as well as to aid sucrose transport (Eschrich, 1980). Other proposed functions include regulation of cell turgor for cell expansion (Meyer & Boyer, 1981; Wyse *et al*., 1986, Perry *et al*., 1987), and the control of sugar composition in storage organs (Klann *et al*., 1993). Furthermore, some of the invertases seem to be involved in the responses of plants to environmental factors, such as wounding and infection (Sturm & Chrispeels, 1990; Benhamou *et al*., 1991). Invertases are classified according to their solubility, cellular location and pH optimum.

Soluble Acid Invertase

There are two kinds of acid invertase, both exhibiting optimum activity between pH 5.0 and 5.5. They are located in two separate compartments, the vacuole and the apoplast. The apoplast enzyme or cell wall invertase (CWI) is confined to the cell wall, and controls the flow of sucrose from the conducting tissue to the young growing cells (Hatch & Glasziou, 1963). The vacuolar soluble acid invertase (AI) is concerned with the rate of return of sugar from storage, and is believed to be important in the regulation of hexose levels in certain tissues (Hatch & Glasziou, 1963; Sacher *et al*., 1963).

In sugarcane the AI enzyme is probably the most extensively studied of the invertase classes, mainly because it is located in the storage compartment of sucrose. AI activity shows marked seasonal variation, being high when growth is rapid and low otherwise (Venkataramana *et al*., 1991). AI is absent from mature internodes of sugarcane varieties with a high capacity for sugar storage, which instead contain the cytosolic NI isoform, with optimum activity at pH

7.0 (Sacher *et al*., 1963; Venkataramana *et al*., 1991). AI activities are usually high in tissues that are rapidly growing, such as cell and tissue cultures, root apices and immature stem internodes (Zhu *et al*., 1997). It is not clear whether acid invertase promotes cell expansion energetically (Ricardo & ap Rees, 1970) or through increased osmotically active sugars (Howard & Whitam, 1983).

Conflicting evidence regarding the role of AI in sucrose mobilization has accumulated over the years. It has been reported that this enzyme is not present in tissues of sugarcane, carrot and red beet during sucrose mobilization, suggesting that this enzyme does not mobilize vacuolar sucrose in these species (Echeverria, 1998). However, Zhu *et al.* (1997) reported that the level and timing of sucrose accumulation in the whole stalk and within individual internodes was correlated with the down-regulation of AI activity. In the latter case, high AI activity appeared to prevent most, but not all, sucrose accumulation.

Cell-wall Bound Invertase

The extracellular cell wall-bound isoforms of acid invertase are associated with rapidly growing tissues and are thought to participate in the apoplasmic-symplasmic translocation of sucrose (Sacher *et al*., 1963; Gayler & Glasziou, 1972). A fairly strong correlation between the rate of cell extension and CWI activity has been reported (Gayler & Glasziou, 1972). This is to be expected since this enzyme is the gateway for the entry of sucrose into the cell in juvenile tissue. The cell wall-associated invertases have been implicated in phloem unloading and carbohydrate partitioning between source and sink tissues, as well as in the establishment of metabolic sinks (Roitsch *et al*., 1995).

Transgenic Studies

Although the experimental evidence is largely correlative in nature, a lot of insight regarding the roles of the various invertases is now emerging from studies of transgenic or mutant plants. Several lines of physiological and genetic evidence suggest an important role for cell-wall-bound invertases in carbohydrate partitioning in plants.

Detailed analyses of transgenic tomato (Dickinson *et al*., 1991) and tobacco (von Schaewen *et al*., 1990) plants, over expressing CWI in a constitutive fashion, have been reported. Elevated levels of enzyme activity in these plants caused reduced levels of sucrose transport between sink and source tissues and lead to stunted growth and overall altered plant morphology.

Significant data also emerged from studies in potato where yeast invertase was expressed in both the cytosol and apoplast (Sonnewald *et al*., 1997). Tissue-specific elevated expression of invertase in both cellular locations lead to substantial reductions in the sucrose content of tubers and a corresponding large increase in glucose content. From the above-mentioned studies it becomes apparent that invertase activities exert a significant impact on the growth, development and sugar composition of a wide variety of plants. Moreover, if an increase in invertase activity led to a decrease in the sucrose: hexose ratio, it is reasonable to speculate that a decrease in the activity of any of the invertases could have the opposite effect.

In sugarcane, a reduction in AI activity had no effect on sucrose accumulation (Botha *et al*., 2001). However, Klann *et al*. (1996) reported that several lines of transgenic tomato fruit expressing a constitutive antisense intracellular AI gene had increased sucrose and decreased hexose sugar concentrations. Sucrose-accumulating fruit were significantly reduced in size compared to control fruit, and this differential growth correlated with high rates of sugar accumulation during the last stages of development. Invertase hydrolyzes sucrose to hexose sugars and thus plays a fundamental role in the energy requirements for plant growth and maintenance. It is thus not surprising that transgenic tomatoes with decreased acid invertase activities have highly perturbed growth habits.

NEUTRAL INVERTASE

Molecular and kinetic properties

According to experimental work by Voster & Botha (1998), NI activity in sugarcane was detected in 60 kDa, 120 kDa and 240 kDa forms. NI activity is regulated by feedback inhibition by the products of the hydrolytic reaction, namely glucose and fructose (Lee & Sturm, 1996), although the latter is the more effective inhibitor in sugarcane (Vorster & Botha, 1998). Product inhibition of NI has also been demonstrated in other species (Van den Ende & Van Laere, 1995; Lee & Sturm, 1996; Ross *et al*., 1996). Product inhibition of NI would only be significant at the cytosolic hexose concentrations of internodes 2 to 10, and would have virtually no impact at the symplastic hexose concentrations of internode 20 and greater (Welbaum & Meinzer, 1990).

Activity trends in sugarcane

Both acid and neutral invertases seem to be involved in internode elongation/ expansion activities in immature internodes, but the high activity of neutral invertase on a fresh weight

basis in the maturing internodes suggests an additional role in these tissues (Dendsay *et al*., 1995). Hexose levels have been shown to correlate positively with NI and not AI levels in mature sugarcane stem tissue (Gayler & Glasziou, 1972, Rose & Botha, 2000). However, others have argued that invertase activities are not significantly correlated with hexose levels but positively correlated with sucrose concentration in the sugarcane stem tissue (Singh & Kanwar, 1991).

The cessation of acid and neutral invertase activity has been considered a prerequisite for sucrose storage in sugarcane. Early maturing sugarcane varieties, which show higher sucrose accumulation than the late varieties, exhibit an early decline in invertase levels, with low NI activity levels being maintained throughout the maturation phases (Dendsay *et al*., 1995). In low sucrose-storing varieties, however, NI remained fairly active during the late maturity months (Dendsay *et al*., 1995).

Thus, as indicated by Gayler and Glasziou (1972), Denday *et al*. (1995), and Rose & Botha (2000), both acid and neutral invertase seem to play an important role in sugarcane by regulating the net concentration of sucrose in internodal tissue. It has been suggested that NI is likely to be partially responsible for the cycle of degradation and synthesis of sucrose that has been found in sugarcane suspension cells (Wendler *et al*., 1990), in the immature sugarcane stem (Sacher *et al*., 1963) and in sugarcane tissue discs (Glasziou, 1961; Bindon & Botha, 2002), or that it regulates sucrose movement from vascular to storage tissue in mature internodes (Hatch *et al*., 1963).

The discovery of significant levels of NI activity in mature sugarcane stem tissues (Hatch & Glasziou, 1963; Gayler & Glasziou, 1972; Batta & Singh, 1986; Lingle & Smith, 1991; Dendsay *et al*., 1995) has led to the hypothesis that NI may affect control over the expression of sugar responsive genes in mature internodes by controlling the hexose concentrations of the cytosol (Voster & Botha, 1998). In comparison to SuSy, invertase may be more important as an enhancer of signals to sugar responsive genes, due to the production of two hexoses (Koch, 1996). This is especially relevant in the case of NI due to the repression of AI expression by glucose, which is a product of invertase and not SuSy (Sacher & Glasziou, 1962; Sacher *et al*., 1963; Glasziou & Waldron, 1964).

The most recent work on NI found that the activity of this enzyme (both on a fresh weight and protein basis) increased up to the fifth internode before declining as internode maturity increased, (Hatch & Glasziou, 1963; Lingle, 1997; Ebrahim *et al.*, 1998; Vorster & Botha, 1999). This decline in NI activity with maturation can also not just be attributed to a general down regulation of metabolic activity as some other enzymes, like SPS and PFP, are up regulated (Whittaker & Botha, 1997; Botha & Black, 2000) and PFK remains constant (Whittaker & Botha, 1997).

Rose & Botha (2002) also reported a significant variation in NI expression and sucrose content within sugarcane internodal tissue, but concluded that NI activity definitely does not increase with maturation. A weak inverse correlation was, however, found between NI activity and sucrose, especially in the bottom part of the internode where active growth occurs (Rose & Botha, 2000). These findings support the predictions of a sugarcane metabolic model, which pointed to NI as one of the possible key enzymes regarding its impact on sucrose accumulation (Rohwer & Botha, 2001).

Cycling and carbon allocation

Supplying radiolabelled carbon compounds to plants has long been used to investigate *in vivo* characteristics of various plant metabolic systems. Radiolabelling studies conducted on sugarcane tissue discs or suspension cultures have likewise been applied to study, for example, sucrose accumulation/cycling (Wendler *et al*., 1990; Whittaker & Botha, 1997), sugar transport and translocation, fluxes between the various carbon pools (Botha *et al*., 1996; Bindon & Botha, 2002) and certain enzyme activities (Vorster & Botha, 1999).

In experiments where radiolabelled fructose was fed to both young and mature internodal tissue significant return of label to glucose was found, which demonstrates that invertase-mediated hydrolysis of sucrose occurs (Hawker, 1985; Wendler *et al*., 1990; Whittaker & Botha, 1997). This is because the only pathway of label incorporation into glucose following fructose labelling is via incorporation of the labelled fructose into sucrose and subsequent hydrolysis to glucose of fructose, leading to a randomization of label between these two hydrolytic end-products. It was also evident from these experiments that the net flux of label into sucrose increases sharply between internode 2 and 7 (Hawker, 1985; Whittaker & Botha, 1997) followed by a steady decline towards the most mature internodes (Hawker, 1985).

With regard to 'futile cycling', labelling studies have shown that sucrose cycling decreases with internode maturation as the rate of the hydrolysis reactions decrease (Wendler *et al*., 1990; Whittaker & Botha, 1997). In light of these findings it can be speculated that the converse is true, and that a decrease in the rate of sucrose hydrolysis will diminish the effect of futile cycling on sucrose concentrations in the culm. A lower activity of NI could thus translate to greater steady-state levels of sucrose in the storage tissues.

Radiolabelled hexoses were also used in experiments aimed at investigating changes in carbon allocation during maturation of sugarcane internodes. It was found that maturation coincided with a redirection of carbon from water-insolubles and respiration to sucrose (Whittaker & Botha, 1997; Bindon & Botha, 2002). The reduction in total respiration could be attributed to any number of related factors, including a change in the regulation of a key reaction step in glycolysis, increased gluconeogenesis, inhibition of the TCA-cycle, altered gene expression or substrate availability, to name a few (Lingle & Irvine, 1994).

SUGARCANE CELL SUSPENSION CULTURES

General

Cultures of cells derived from a number of plant species have been used to study physiological and biochemical aspects of carbohydrate metabolism (Huber & Akazawa, 1986; Ashihara & Horikosi; 1987; Spilatro & Anderson, 1988). The storage of sucrose in sugarcane internodes is a developmentally regulated process, during which the parenchyma cells start to accumulate sucrose after a period of growth and expansion. Sugarcane cells in batch suspension seem to exhibit similar characteristics, namely a period of fast growth after transfer to a new medium followed by a period of sucrose accumulation (Thom *et al*., 1982). Heterotrophic cell suspension cultures are also entirely dependent on an externally applied carbon source for growth, thus representing typical sink cells.

Cell suspensions of sugarcane are useful in studies as they resemble rapidly growing apical tissue of intact sugarcane and retain the ability to store up to 25% of their dry weight as sucrose (Thom *et al*., 1982). Sugarcane suspension-cell cultures were originally used as a model system to investigate sugar uptake into cells (Maretzki & Thom, 1972; Komor *et al*., 1981) and transport across the vacuole tonoplast (Thom & Komor, 1985; Thom & Maretzki, 1985). Since then they have also proven useful in studies aimed at investigating metabolism during sucrose storage (Wendler *et al*., 1990).

This system presents several advantages over whole-plant studies. First and foremost, differences attributable to individual plants or tissues are eliminated because the cultivation conditions of the cells are constant and uniform treatments can be carried out. In addition, the cells in a suspension culture are finely dispersed and homogenous, facilitating uniform and repeated sampling. Experiments are not complicated by differentiation processes, and the use of these cultures excludes wounding and stress syndromes observed with cut plant tissues.

However, certain limitations do exist regarding the use of suspension cells as a model system for whole-plant metabolism. Even under strict growth limitation, the culture cells are still more like young parenchymatous cells, resembling storage parenchyma of young internodes and not like mature storage parenchyma. Such an immature developmental status is characterized by the presence of starch and the still relatively low sucrose content, which at a maximum is around half to a third of the concentrations reached in mature internodes (Veith & Komor, 1993). The average size of the vacuole of suspension cultured cells is approximately 62% of the protoplast volume (Thom *et al*., 1981), which is significantly less than the 90% vacuolar volume of culm parenchyma cells in mature internodes. Furthermore, potential problems may arise in experiments with plant cells grown in batch culture, due to potentially complex interactions when multiple factors become limiting for plant growth (Wendler *et al*., 1990).

Nevertheless, the metabolic shifts in culture cells might at least be representative for those processes that proceed in immature internodes during the shift from growth to sucrose storage (Veith & Komor, 1993). It has also been suggested that the extremely high sucrose content of mature internodes might be due to a rise in apoplastic sugar concentrations (Welbaum & Meinzer, 1990). Thus the differences in sugar content between mature suspension cultures and mature internodes may be a reflection of differing transport mechanisms rather than a metabolic feature.

Sucrose metabolism

The sugar uptake system of suspension cells is fairly specific for glucose. Fructose is also taken up, though with a lower affinity (Maretzki & Thom, 1972). The capacity of sugarcane cells to take up hexoses from the external environment, i.e. apoplast or medium, exceeds by far the observed rates of sugar storage. Seeing as CWI is not rate-limiting for sugar storage in

the cells, since sucrose in the medium is largely hydrolyzed within a few days, neither CWI activity nor hexose transport at the plasma membrane are likely to be the rate-limiting steps in sugar storage in suspension cells (Komor *et al*., 1981).

The sugarcane cells in suspension cultures have been found to contain high activities of SuSy and NI, even during the sucrose storage phases. As both enzymes are located in the cytosol, they may compete with each other and vacuole loading for sucrose (Wendler *et al*., 1990). A simultaneous synthesis and degradation of sucrose thus occurs regardless of whether the sugarcane cells are mobilizing their sucrose, actively growing, rapidly accumulating sucrose or simply maintaining high, stable sucrose concentrations towards the end of the culture period. In fact, the slowing down of sucrose storage as the culture reaches maturity is accompanied by a continued high rate of unidirectional sucrose synthesis (Wendler *et al*., 1990). This suggests that an increased rate of sucrose degradation could primarily be responsible for the cessation of sucrose storage in these cells.

Suspension cultures in transgenic research

In light of all the advantages of cell suspension cultures over more conventional greenhouse- or field- grown plants, a novel opportunity presents itself: It is now theoretically possible to carry out rapid, large-scale and cost-effective screening of transgenic cell lines in sugarcane by making use of the suspension culture system. This approach has never been followed in transgenic work in sugarcane. This project is thus intended to test the validity of this system in the context of a specific set of NI antisense transgenic lines which are grown both in suspension cultures and as greenhouse-grown plants. By comparing the results obtained from the two experimental systems the value of suspension cultures as a model system for transgenic research in sugarcane will be confirmed or rejected.

CONCLUSION

It is still not completely clear whether the increase in sucrose content with internode or suspension culture maturity is as a result of an increased accumulation rate of sucrose, or a decreased accumulation rate of other cellular constituents. Just how sucrose accumulation is maintained in an environment where high hydrolytic and cleavage activities are present is also an unresolved issue at present. Further complications arise as data on the expression levels of key enzymes are highly variable (Lingle & Smith, 1991), and depending on whether activity

is expressed on a tissue mass or protein content basis very different activity patterns are evident (Hatch *et al*., 1962; Gayler & Glasziou, 1972; Lingle & Smith, 1991). It is, however, abundantly clear from the data from diverse plant species that invertases play a crucial role in the control of metabolic fluxes, down stream sucrose partitioning, and ultimately, plant development and crop productivity (Cheng *et al*., 1999). Antisense technology presents a novel opportunity for a final assessment of the role of NI in these integral processes.

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CHAPTER 3

Down-regulation of neutral invertase activity in sugarcane cell suspension cultures leads to increased sucrose accumulation.

ABSTRACT

Suspension cultures were used as a model system to investigate sucrose metabolism in four sugarcane (*Saccharum spp.* interspecific hybrids) cell lines transformed with antisense neutral invertase (NI) constructs. Throughout a 14 day growth cycle two cell lines in which the antisense sequence was under the control of a tandem CaMV-35S : maize ubiquitin promoter showed a strong reduction in NI activity, as well as reduced hexose and increased sucrose concentrations in comparison to the control line. In lines where the antisense NI sequence was under the control of the weaker CaMV-35S promoter alone, changes in enzyme activity and sugar concentrations were intermediate to those of the more strongly inhibited lines and the control. In comparison to the control line, a higher sucrose to hexose ratio, i.e. increased purity, was obtained in all the lines with reduced NI activity. These lines also showed a decrease in the *in vivo* rate of sucrose hydrolysis, suggesting that flux through the 'futile cycle' of sucrose breakdown and re-synthesis was minimised in transgenic tissues. Differences between the transgenic cultures and the control were most pronounced during the early stages of the growth cycle and tapered off as the cultures matured. The transgenic cultures displayed severely impaired growth characteristics suggesting that although decreased NI activity may increase sucrose content, the growth rate of the cells was retarded due to a reduced availability of hexoses for respiration.

Keywords: Neutral invertase, sucrose metabolism, sugarcane, suspension cultures, antisense.

INTRODUCTION

Sugarcane accumulates sucrose in the lower, mature internodes of its culm. This accumulation is the net result of a cycle of sucrose synthesis and degradation. The enzymes responsible for the synthesis are sucrose phosphate synthase (SPS, E.C 2.4.1.14) (Botha & Black, 2000) and sucrose synthase (SuSy, E.C 2.4.1.13) (Schäfer *et al*., 2004) and the enzymes that degrade sucrose are classified either as hydrolases (invertases) or glucosyltransferases (SuSy). Invertases (β-D-fructofuranosidase) hydrolyse sucrose to glucose and fructose. Three classes of invertases can be distinguished based on their pH optima and sub-cellular localization. Acid invertases are optimally active between pH 5.0 and 5.5 and exist in a soluble vacuolar form (Sacher *et al*., 1963) as well as an insoluble form bound to the cell wall (Hatch & Glasziou, 1963). It has been suggested that cell wall invertase may control the flow of sucrose from the conducting tissue to young, actively growing cells (Hatch & Glasziou, 1963). The vacuolar acid invertase has been implicated in the mobilization of stored photosynthate to provide substrates necessary for the growth of immature, expanding tissues in sugarcane (Hatch *et al*., 1962; Sacher *et al*., 1963). These two sub-classes of acid invertases are not present in significant amounts in the mature internodes of sugarcane, resulting in neutral invertase (NI, E.C 3.2.1.26) being the predominant sucrose-hydrolysing activity in these tissues (Sacher *et al*., 1963; Venkataramana *et al*., 1991). NI is localised in the cytosolic compartment and in sugarcane exhibits optimum activity at pH 7.0 (Sacher *et al*., 1963; Batta & Singh, 1986; Vorster & Botha, 1999).

Even in mature, sucrose storing, internodal tissue approximately 10% of the total sucrose pool is still turned over (Wendler *et al*., 1990, Whittaker & Botha, 1997). There is also a significant correlation between the levels of hexoses and the levels of NI activity in these tissues, suggesting a possible role for NI in controlling the hexose pool size in mature sugarcane tissues (Gayler & Glasziou, 1972; Rose & Botha, 2000). This could also indicate that NI may have a substantial influence on the regulation of sucrose accumulation in the storage parenchyma of mature sugarcane internodes. Relatively high levels of NI activity in mature internodes have been reported, confirming the integral role of NI as part of the cycle of sucrose accumulation in these tissues (Rose & Botha, 2000; Bosch *et al.,* 2004). In addition, a theoretical kinetic model of sucrose accumulation in sugarcane has identified NI as a possible key regulatory point in the control of flux in the 'futile cycle' between sucrose and the monosaccharide pool (Rohwer & Botha, 2001). The model also predicts that as much as

22% of the sucrose that is synthesized in sucrose accumulating tissues is broken down again by invertase. This suggests that NI activity could be a key determinant of the sucrose accumulating capacity of sugarcane culm.

In contrast, the expression of NI has also shown marked seasonal variation (Batta & Singh, 1986; Lingle & Smith, 1991), and a weak correlation to hexose or sucrose levels in the sugarcane culm (Singh & Kanwar, 1991; Venkataramana *et al.*, 1992; Zhu *et al.*, 1997). This conflicting data could imply that sucrose accumulation is not directly related to the expression of the enzyme, or that the extractable activity does not reflect *in vivo* activity (Vorster & Botha, 1999). Moreover, the variability in the available activity data and the lack of coherent correlations with hexose and/or sucrose levels prevent the elucidation of a potential role of NI in sucrose accumulation. A transgenic approach aimed at down-regulating NI expression in sucrose accumulating tissues could therefore shed more light on the apparent role of this enzyme in sucrose metabolism. In addition, the sucrose yields of sugarcane plants could potentially be increased by reducing the flux through the energetically wasteful process of sucrose cycling in mature internodes.

In the present study we report on the results of a reverse genetic approach, in which sugarcane cell lines with reduced NI activity were generated by transformation with an antisense sugarcane NI sequence under the control of two different promoter sequences. Sugarcane suspension cell cultures were originally used as a model system to investigate sugar uptake into cells (Maretzki and Thom, 1972), and were later adapted to study metabolism during sucrose storage (Wendler *et al.,* 1990). Thom *et al.* (1982) illustrated that sugarcane cells in batch suspension culture exhibit similar growth and metabolic characteristics to the tissues of whole plants, i.e. a period of fast growth and high metabolic activity, followed by a period of sucrose accumulation. Cell suspension cultures were therefore used in the preliminary analyses of these transgenic lines due to their homogeneity and rapid growth rate.

MATERIALS AND METHODS

Chemicals

Restriction enzymes, cofactors and substrates, were obtained from Promega (Madison, Wisconsin, USA). [U-^{14}C] fructose was supplied by AEC-Amersham International (Claremont, South Africa). Enzymes and metabolites for the sugar and activity assays were

from Roche (Mannheim, Germany), and all other chemicals were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, Missouri, USA) unless stated otherwise.

Plant tissues

Sugarcane callus was initiated from the leaf rolls of immature transgenic sugarcane plants (commercial variety NCo310) transformed with two different antisense neutral invertase constructs. Transformation and regeneration procedures were carried out as described by Franks and Birch (1991) and Snyman *et al.* (1996) respectively. Two of the transgenic lines (U1 and U2) were transformed with a construct (pASNI510) in which transgene expression was under the control of a strong, tandem CaMV-35S : maize ubiquitin promoter, while the S1 and S2 lines were transformed with the pASNI10 construct containing only the weaker CaMV-35S promoter (Groenewald *et al.,* 2000). In both these vectors a 1800bp cDNA sequence from sugarcane NI was cloned in the antisense orientation. Untransformed callus of the NCo310 variety was used as the control throughout.

Molecular characterisation

DNA and RNA were extracted from suspension cultured cells 6 days after transfer to fresh media. Genomic DNA and total RNA were extracted as described by Dellaporta *et al.* (1983) and Bugos *et al.* (1995) respectively. For the PCR analyses, the primers SNIF (5'-GCAGCACTCAACTATGACCAGG-3') and SNIR (5'-GATCTTGAGTGGCATCTCACC-3') were used to amplify both the 800 bp fragment from the cDNA antisense construct and the ±1500 bp fragment from the endogenous genomic sequence of the transgenic plants. Southern and northern blot analyses were done as described previously (Bosch *et al.,* 2004). For the Southern blot analysis the genomic DNA was completely digested with Sac I according to the recommended protocols of the manufacturer (Promega). All hybridization and washing steps were done at 42^{o}C, using Ultrahyb^{TM} hybridization buffer (Ambion) according to the manufacturer's recommendations.

Cell suspension cultures

Callus and cell suspension cultures were grown on Murashige-Skoog medium (Murashige and Skoog, 1962) containing 58 mM sucrose, 13.6 µM 2,4-dichlorophenoxyacetic acid and 0.5 g l^{-1} casein. Suspension cultures were initiated from embryonic callus and subcultured at 14 day intervals. Cells were grown at 28^{o}C and swirled on an orbital shaker at 170 rpm. Aliquots

were taken once a week to inoculate yeast and bacterial growth media in order to test for the presence of bacterial or yeast contamination in the cell cultures. Single batch cultures were used to inoculate fresh media (four replicates of each cell line) at the start of the experimental period, in order to closely synchronize the growth cycles of the cultures.

Growth measurement

Cell proliferation, i.e. growth, was determined spectrophotometrically (Powerwave$_X$, Bio-Tek Instruments) by measuring the optical density (at 600 nm) of 200 µl samples of the suspensions over the 14 day experimental period.

Protein extraction and enzyme assays

Cells from suspension cultures were harvested by centrifugation of aliquots taken at 2 day intervals. Per sample, 30 mg of cells were homogenized on ice in 140 µl protein extraction buffer using the Ultra-Turrax T8 (IKA Labortechnik) in 4 × 20 sec bursts. The extraction buffer (100 mM sodium phosphate) contained 1 mM EDTA, 0.5 mM MgCl, 10 mM DTT, 10% (v/v) glycerol, 2% PVPP (v/v) and 0.0016 g/ml Complete^{TM} protease inhibitor (Roche). Insoluble residue was pelleted by centrifugation at 10, 000 × g for 15 min. The supernatant was passed through Sephadex G25 size-exclusion minispin columns (Biopharmacia) by centrifugation at 2500 x G for 1 min, and the eluates used as the desalted protein samples. Protein concentrations in the desalted samples were determined by the method of Bradford (1976) using the BioRad microassay, with BSA as a standard.

Neutral invertase activity was assayed by incubating 80 µl aliquots of the protein extractions in a final reaction volume of 250 µl with 50 mM Hepes (pH 7.5) and 125 mM sucrose at 30^{o}C. At zero, 1 and 2 hrs, 50 µl aliquots of this reaction mixture were transferred to separate wells of a microtitre plate, the reaction was stopped by the addition of 5 µl stop solution (2 M Tris and 22 mM $ZnSO_4$) and kept at 4^{o}C until analysis. Finally, the amount of reducing sugars in these samples was measured using a NAD coupled system (Huber and Akazawa, 1986). Acid invertase activity was assayed in a similar fashion by incubating the protein extracts for 3 hrs in 125 mM sucrose and 50 mM citrate-phosphate buffer (pH 5.5). Reactions were stopped at 1 hr intervals by heat inactivation (2 min incubation at 90^{o}C) and the reducing sugars were measured using a NAD coupled system (Huber and Akazawa, 1986) with a Beckman DU® 7500 spectrophotometer. SuSy activity was determined in the breakdown direction as described by Schäfer *et al*. (2004).

Sugar extraction and analysis

Soluble sugars were extracted at 2 day intervals from 30 mg of suspension cells in 10 volumes (m:v) extraction buffer, as described by Bosch *et al.* (2004). Sucrose, glucose and fructose concentrations were determined using the enzymatic method described by Bergmeyer and Bernt (1974).

Radiolabelling experiments

At days 4 and 8 of the growth cycles, 1-2 g of suspension cultured cells were harvested by centrifugation and washed in a buffer containing 25 mM K-Mes (2-[N-morpholino]ethanesulfonic acid (potassium salt) (pH 5.7), 250 mM mannitol and 1 mM $CaCl_2$. After all excess liquid was removed the cells were incubated for 4 hours at 30^{o}C in 50 ml tubes containing 2 ml incubation buffer (25 mM K-Mes, 250 mM mannitol, 5 mM glucose, fructose and sucrose and [U-^{14}C]-fructose at a specific activity of 37 Bq.nmol^{-1}) with constant agitation on an orbital shaker at 130 rpm. $^{14}CO_2$ released during the incubation period was collected in 500 ul 12% (w/v) KOH and the radioactivity present in the KOH vials was determined in a Beckman Scintillation Counter following the addition of 4 ml Ultima Flo ™ scintillation cocktail.

After the labelling period, unincorporated label was removed from the tissue discs by 8 consecutive washes in 10 ml ice cold wash buffer. Excess liquid was removed by blotting and the tissues were weighed before transfer to 10 ml tubes containing 3 ml sugar extraction buffer (80% ethanol and 30 mM Hepes, pH 7.5). The samples were incubated overnight at 70^{o}C, then centrifuged at 13 000 rpm for 20 min at 4^{o}C. The supernatants were removed and the extraction repeated with another 1 ml of 80% ethanol. Radioactivity in the total 4 ml soluble fraction was determined by counting 10 µl in 4 ml scintillation cocktail in a Beckman LS 1801 scintillation counter.

Following the extraction of the total soluble fraction, the remaining pellets were washed 8 times in 80% ethanol and dried in a vacuum centrifuge, after which 4 ml of the organic solvent Soluene-350 was added to dissolve the insoluble residue. Radioactivity in 200 µl aliquots of these soluene solutions was also determined by scintillation counting as above.

Fractionation of soluble extracts

Seven hundred microlitres of the total soluble extract was passed through strong cation and anion exchangers (Supelco Supelclean columns) in tandem to bind all acidic and aromatic compounds and render an extract containing only the neutral fraction in a volume of 10 ml ddH_2O. This neutral fraction was completely reduced in a vacuum centrifuge and resuspended in 120 ul of 10% (v/v) isopropanol. These neutral samples were prepared for HPLC analysis by passing through 0.45 μm filters (Millipore). The acid and basic fractions were eluted from the columns with 4 ml of 4 M NH_4OH and 4 M formate, respectively. Label recovery in the three fractions was again determined with scintillation counting.

HPLC analysis

Sugars were fractionated by HPLC (Shimadzu SCL-10AVP system) using a Hamilton RCX-10 column. A 30 mM NaOH solution was used as the mobile phase, at a flow rate of 1 ml min^{-1}, and ^{14}C in glucose, fructose and sucrose was determined by inline liquid scintillation spectroscopy (Radiomatic A-500).

RESULTS

Molecular characterisation of transgenic cell lines

PCR and Southern blot analyses confirmed the presence of the transgene sequences in the transgenic lines and also that each of the two lines containing the same vector construct were independent lines (data not shown). Northern blot analyses confirmed the stable integration of the transgene at transcriptionally active loci within the genome of each independent cell line (Fig. 1). While both the endogenous (2.2 kb) and the smaller (1.8 kb) antisense transcripts were detectable in the transgenic lines, only the endogenous transcript was detectable in the control line (Fig. 1). Densitometric analysis revealed that the endogenous transcript levels were significantly reduced in both the S and U transgenic lines, with greater reduction in the latter.

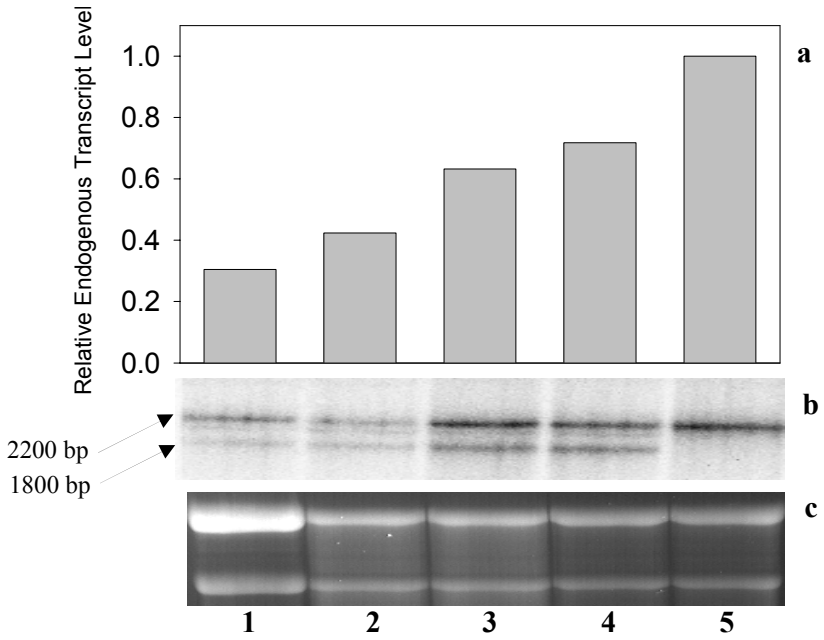

Figure 1. Northern blot analysis confirming the expression of the NI transgene and changes in the levels of the endogenous transcript (panel b). Lanes (1) U1, (2) U2, (3) S1, (4) S2, (5) NCo310 (wild type control). Panel c represents the ethidium bromide stained ribosomal subunits that were used to equalize differences in loading, and the relative expression levels of the endogenous transcript are depicted in panel a.

Neutral invertase activity

NI activity in the suspension cultures exhibited a similar profile to that of maturing sugarcane internodal tissue. Activity increased to a maximum at day eight, then decreased to its lowest levels at day 14 (Fig. 2). In the U1 and U2 lines, activity declined to levels as low as 33.8% of control activity at day 14 (Table 1). NI activity was reduced to a lesser extent in the S1 and S2, although activity did decrease appreciably to approximately 66% of the wild type at days 1 and 14 (Table 1).

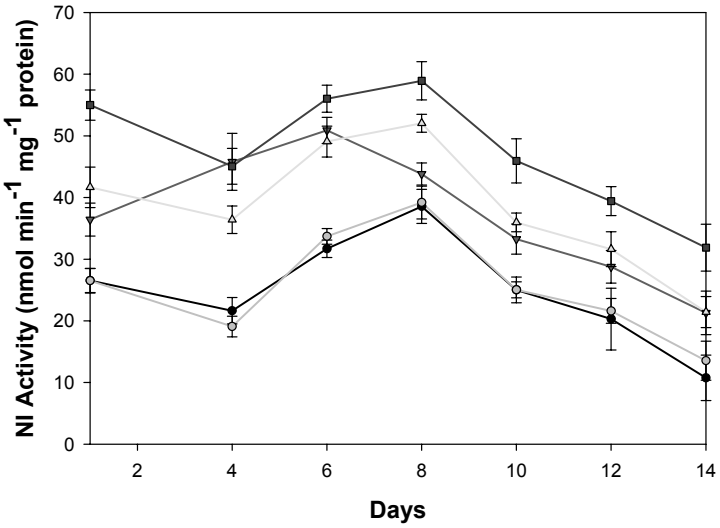

Figure 2. NI activity of suspension cell cultures. Ubiquitin-35S promoter transgenic lines U1 (●) and U2 (○), 35S promoter transgenic lines S1 (▼) and S2 (▽) and NCo310 cells (■) were cultured over a 14 day period. Data points are the average of 4 replicates ± standard deviation.

Table 1. NI activity in the transgenic lines expressed as a percentage of the activity of the NCo310 control line.

Day	Promoter	NI activity as a % control			
		CaMV-35S : Ubiquitin		**CaMV-35S**	
	Line	**U1**	**U2**	**S1**	**S2**
1		48.2	48.2	66.2	75.8
4		48.0	42.3	101.6	80.8
6		56.6	60.2	90.9	87.6
8		65.4	66.5	74.3	88.3
10		54.5	54.5	72.4	78.3
12		51.5	54.8	73.0	80.2
14		33.8	42.5	66.8	67.2

Activities of other sucrolytic enzymes

Because the U1 and U2 lines showed the biggest change in NI activity, possible induced changes in the complimentary activities of vacuolar acid invertase as well as the cytosolic SuSy were investigated in these two lines. No quantitative differences could be observed between the acid invertase activities of the transformed and untransformed tissues. However, significant variations were observed in the SuSy activities of the two transgenic lines (Fig. 3). SuSy activity (measured in the breakdown direction) in these lines was significantly higher in all the samples analysed throughout the 12 day growth cycle of the cultures. Moreover, the activity trends in the antisense lines were almost identical to each other, but differed considerably from that of the control line (Fig. 3). SuSy activity in the control cultures gradually increased to a maximum at day 8, before declining again as the cultures aged. In the transgenic lines, however, SuSy activity increased gradually to day 6 where after it decreased at day 8 and then increased again rapidly to reach a maximum at day 12. This peculiar decrease in activity in the transgenic lines at day 8 was statistically significant ($p = 0.0036$), suggesting that it was a consistent and reproducible feature of SuSy activity in the transgenic cultures.

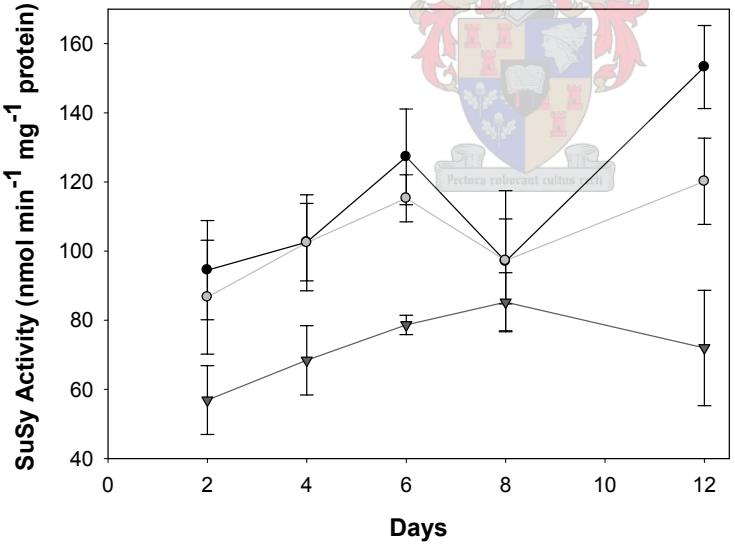

Figure 3. SuSy activity (nmol min^{-1} mg^{-1} protein) in the breakdown direction of the Ubiquitin-35S promoter transgenic lines U1 (●) and U2 (○), and NCo310 cells (▼) during the 14 day culture period. Data points are the average of four replicates ± standard deviation.

Sucrose and hexose levels

To study the possible effects of reduced NI activity on sugar accumulation, sucrose and hexose levels were determined at various developmental stages of the suspension cultures.

Directly after inoculation intracellular sucrose concentrations decreased for an initial 4-day period of internal sucrose mobilization and increased from day 6 onwards as the cells started to accumulate sucrose (Fig. 4a). Sucrose concentrations stabilised after day 12 when maximum internal concentrations were reached. Glucose and fructose concentrations (Figs. 4b and c) showed a pronounced peak during the middle of the growth period (day 8), declining to a minimum in the mature cultures (days 12-14).

Throughout the growth cycle of all the transgenic lines sucrose concentrations were higher, and hexose concentrations lower compared to the control (Figs. 4a-c). Again, these differences were greatest in lines where transgene expression was driven by the tandem CaMV-35S : maize ubiquitin promoter. In these lines sucrose concentrations in the mature cultures were 30% higher than that of the control, while the hexose levels were reduced to the same extent at day 8 where the highest hexose levels were present. Although less pronounced, significant differences of approximately 20% were also evident between the sucrose and hexose concentrations of the lines transformed with the weaker CaMV-35S promoter construct and the untransformed control cultures (Figs. 4a-c).

The purity, i.e. sucrose concentration expressed as a percentage of the total sugar concentration, of all the cultures were also representative of whole sugarcane plants, as purity increased as the cultures mature (Fig. 4d). The purity of the transgenic samples was consistently higher than that of the control, although these differences were most pronounced in immature cultures.

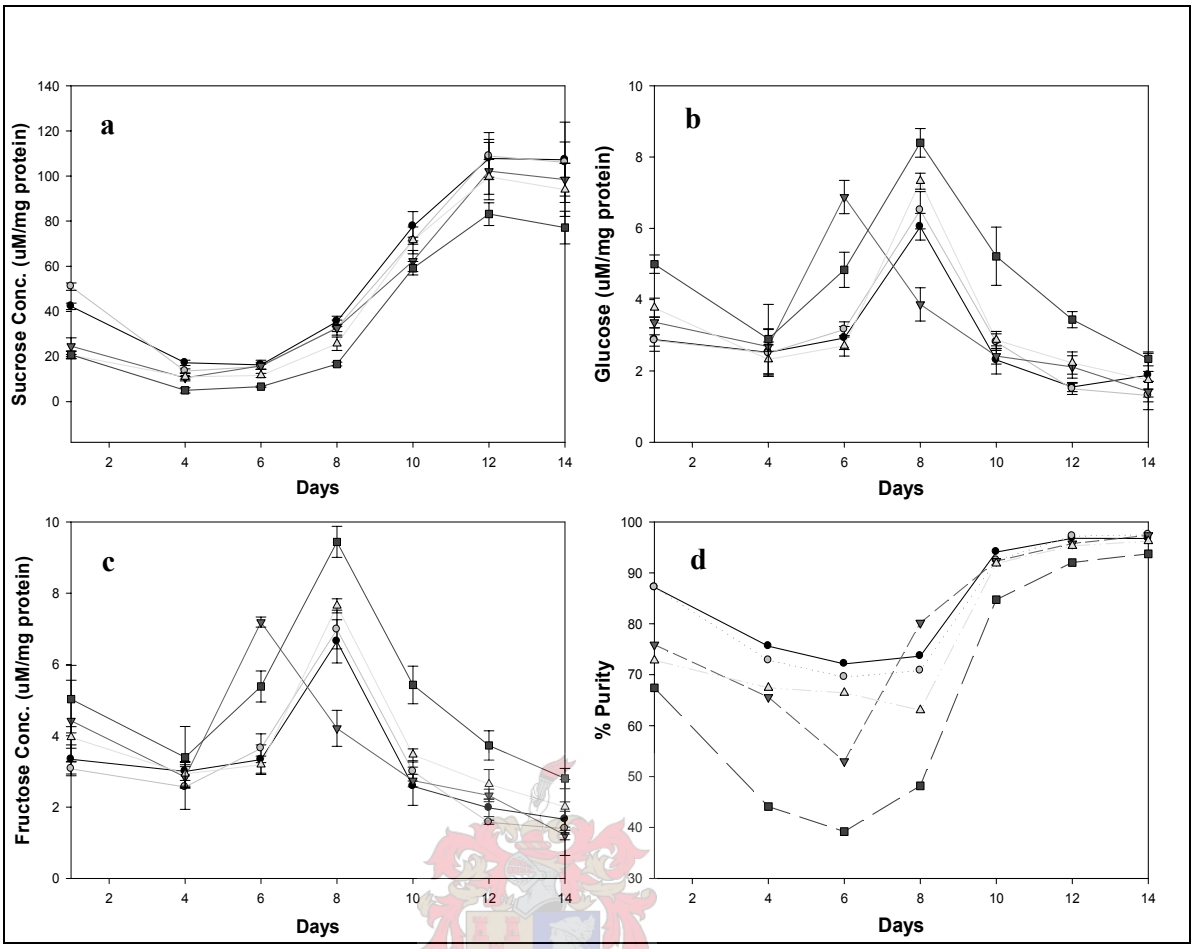

Figure 4. Cellular sucrose (a), glucose (b) and fructose (c) concentrations, as well as percentage purity (sucrose as a percentage of the total sugar pool) (d) during growth of suspension cultures. The curves are representative of the U1 (●), U2 (○), S1 (▼), S2 (∇) and NCo310 (■) lines respectively. These values represent the average of four independent samples and standard deviations are indicated by error bars.

Uptake and partitioning of radio-labelled fructose

To analyse the effects of reduced NI activity on carbon fluxes radio-labelled fructose was fed to the suspension cultures, and its uptake and partitioning was determined in the transgenic and control lines. The radio-labelling studies described were carried out using only U1 and U2 lines, as NI activity was down-regulated most effectively in these lines. Suspension cultured cells were incubated in buffer containing [U-^{14}C]fructose in order to examine the uptake and distribution of radio-label among various soluble carbohydrate pools, and to approximate the *in vivo* rate of NI-mediated sucrose hydrolysis. Days 4 and 8 were selected for the labelling experiments as being representative of immature and maturing cultures, respectively.

At both the days 4 and 8, the total uptake of label was higher (although not significant) in the transgenic lines than in the control. Total label in internal fructose and sucrose in the transgenic lines significantly exceeded that of the control, while glucose showed a reduction in label (Table 2). The total label in the organic and amino acid fractions, as well as the respired CO_2 (together accounting for total respiration) was lower in the transgenic samples compared with the control. Label incorporation to the insoluble fraction, however, was identical between the transgenic lines and the control at both day 4 and day 8. The same trends were evident from the data depicting the distribution of label between the various fractions as a percentage of the total label (Fig. 5).

Table 2. Distribution of ^{14}C in suspension cultured cells supplied with [U-^{14}C]fructose for four hours. The total activity of each component is expressed as kBq mg^{-1} protein. Each value is the average ± standard deviation of four separate samples. *Indicates values that differ significantly from the control ($p < 0.05$).

Culture age	Cell line	Total Uptake	Insoluble Fraction	Sucrose	Glucose	Fructose	Organic Acids	Amino Acids	CO_2	Total Respiration
Day 4	NCo310	13.36 ± 0.87	4.04 ± 0.19	1.80 ± 0.62	0.76 ± 0.23	1.22 ± 0.30	3.00 ± 0.29	2.03 ± 0.17	1.23 ± 0.21	7.26 ± 0.38
	U1	15.48 ± 1.71	4.14 ± 0.55	6.01 ± 0.51*	0.34 ± 0.05*	1.57 ± 0.38	3.23 ± 0.35*	1.43 ± 0.23*	0.80 ± 0.06*	5.46 ± 0.51*
	U2	14.68 ± 1.11	4.38 ± 0.55	3.65 ± 0.63*	0.43 ± 0.08*	1.65 ± 0.50	3.43 ± 0.25*	1.62 ± 0.19*	0.82 ± 0.10*	5.86 ± 0.25*
Day 8	NCo310	11.31 ± 1.50	1.93 ± 0.38	2.45 ± 0.47	0.71 ± 0.20	1.83 ± 0.23	3.00 ± 0.10	1.91 ± 0.16	0.88 ± 0.11	5.78 ± 0.24
	U1	13.60 ± 1.14	2.05 ± 0.20	6.78 ± 0.84*	0.28 ± 0.04*	3.08 ± 0.21*	2.38 ± 0.40*	1.41 ± 0.10*	0.58 ± 0.13*	4.37 ± 0.45*
	U2	12.98 ± 0.69	1.93 ± 0.22	4.32 ± 0.96*	0.35 ± 0.14*	2.77 ± 0.99	2.48 ± 0.18*	1.31 ± 0.23*	0.66 ± 0.10*	4.44 ± 0.28*

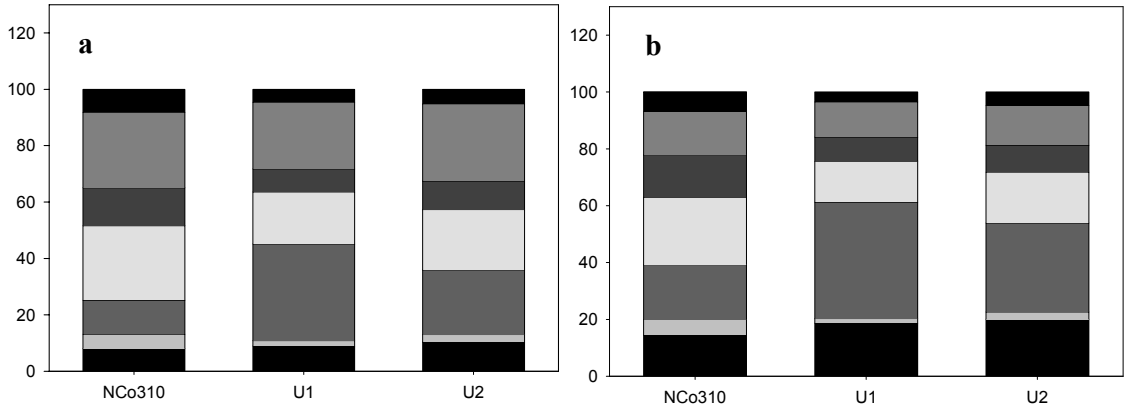

Figure 5. Percentage allocation of ^{14}C to the various pools after a four hour [U-^{14}C]fructose labelling period in (a) 4 day and (b) day 8 old cultures. The fractions represented from the bottom to the top are as follows: fructose ( ), glucose ( ), sucrose ( ), organic acids ( ) amino acids ( ), insoluble ( ) and CO_2 ( ).

Estimated Metabolic Flux

The specific activities of the fructose and sucrose pools were significantly greater in the two transgenic lines, both at day 4 and day 8, while the specific activities of glucose in these two lines were reduced relative to the control (Table 3).

Table 3. Specific activities of glucose, fructose and sucrose in extracts from young and maturing suspension cells of the control and two transgenic lines. Specific activities are expressed as Bq μmol^{-1} sugar. Values are the average of four replicates ± standard deviation. *Indicates values that differ significantly from the control ($p < 0.05$).

Culture age	Cell line	Fructose	Glucose	Sucrose
Day 4	NCo310	234.5 ± 52.2	137.0 ± 31.9	165.3 ± 44.3
	U1	487.4 ± 123.9	101.4 ± 21.2	202.3 ± 18.2*
	U2	519.2 ± 165.9	101.7 ± 17.9	182.1 ± 52.3*
Day 8	NCo310	182.5 ± 11.2	68.1 ± 8.5	65.6 ± 5.2
	U1	459.1 ± 52.0*	49.1 ± 14.3	116.5 ± 6.5*
	U2	345.7 ± 117.3*	51.0 ± 4.9*	92.5 ± 18.1*

The *in vivo* rate of neutral invertase mediated hydrolysis was estimated by calculating the flux of ^{14}C into glucose from the labelled sucrose pool, as only invertase-mediated hydrolysis can

account for label recovery in glucose after feeding [U-^{14}C] fructose. Again, flux into glucose was considerably reduced in the transgenic lines, particularly in 8 day old cultures (Fig. 6).

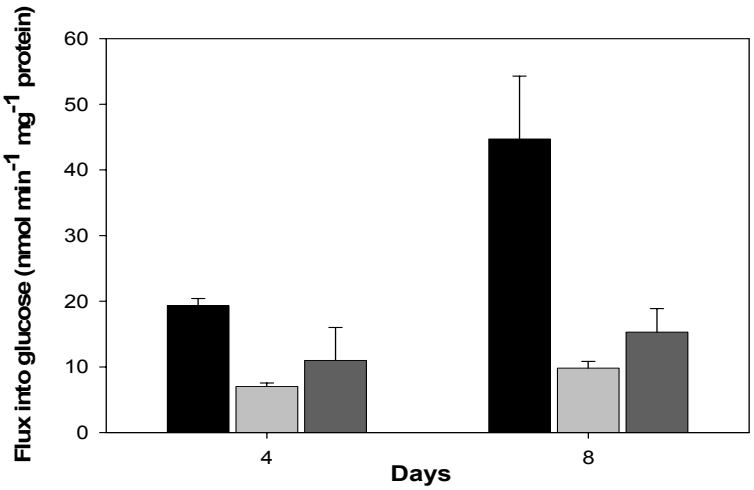

Figure 6. Net flux of label into glucose after a 4 hr labelling period. The bars represent, from left to right, NCo310 control ( ), U1 ( ) and U2 ( ). Values are the average of four replicates ± standard deviation.

Growth of transgenic cell lines

In order to analyse whether the reduction in NI activity exerted any effects on the growth of the suspension cells, their growth rates were determined. All cell lines displayed a typical sigmoidal growth cycle over a 14 day growth period (Fig. 7). Relative to the untransformed NCo310 control, the growth rates of the transgenic lines were considerably reduced (Fig 7). Moreover, the U1 and U2 transgenic lines in which NI activity was reduced the most (Table 1) displayed the biggest decline in their growth rates.

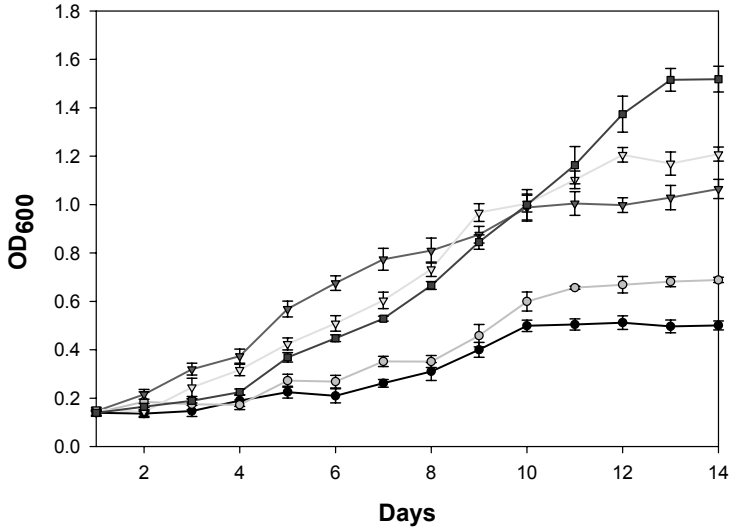

Figure 7. Growth of suspension cultured cells over a 14 day period. The curves are representative of the NCo310 control (■), U1 (●), U2 (○), S1 (▼) and S2 (∇) lines respectively. Data points are the average of 4 replicates ± standard deviation.

DISCUSSION

Northern blot analyses did not only confirm the expression of the antisense sequences in all the transgenic cell lines but also that the endogenous transcript levels were significantly reduced in both the S and U transgenic lines (Fig. 1). The NI activity patterns throughout the growth cycle of the control and transgenic suspension cultures (Fig. 2) were similar to that reported earlier for sugarcane suspension cells (Wendler *et al.*, 1990) and also to the trend observed in maturing sugarcane internodes (Vorster and Botha, 1999). The peak in activity coincided with the exponential growth phase of the cultures (Figs. 2 and 7), corroborating the theory that NI contributes to the provision of monosaccharide substrates for respiration and cell growth during this period (Hatch & Glasziou, 1963; Sacher *et al.*, 1963). The observed reduction in the growth rates of the transgenic lines in comparison to the control correlates with the level of NI repression and is consistent with the hypothesis that cells with the greatest reduction in NI activity will also have the greatest difficulty in mobilizing sucrose during the active growth stages of the cultures. This is confirmed by the reduced glucose and fructose concentrations in the transgenic cultures (Figs 4b and c). Furthermore, the concurrence of the hexose concentration peaks and the maximum NI activity in all the cultures points to the importance of NI in sucrose hydrolysis in cell suspension cultures. This could be extrapolated

to sugarcane internodal tissue where a correlation between NI activity and hexose concentrations has also been reported (Bosch *et al*., 2004).

Although the pattern of SuSy activity in the NCo310 control line was similar to that reported earlier for suspension cells (Wendler *et al*., 1990), the transgenic lines had a distinct activity pattern characterised by a reduction in activity at day 8 and a subsequent increase (Fig 3). The decrease in activity in the transgenic lines was statistically significant ($p = 0.0036$), suggesting that it was a consistent and reproducible feature of SuSy activity in the transgenic cultures. This also meant that although the NCo310 control line's NI and SuSy activities followed similar patterns (both peaking at day 8), this relationship was not valid for the transgenic lines. These similar patterns of activity for NI and SuSy and the relative amounts of the individual activities for the control line were also analogous to that reported by Wendler *et al*. (1990). The reduction in SuSy activity in the transgenic lines corresponds with the peak in hexose concentrations as well as the start of rapid sucrose accumulation (Fig 4a-c). In addition, over time both the reduction in NI activity and corresponding reduction in hexose concentrations are consistent between the control and the transgenic lines, i.e. they change in parallel. This is not true for SuSy activity and if the total cytoplasmic sucrolytic activity is calculated as the sum of the NI and SuSy activities, the SuSy activity plays a determining role in the total activity pattern, i.e. the total cytoplasmic sucrolytic activity shows the same decrease in activity on day 8 followed by an increase on day 12. This seems to indicate that although SuSy is the predominant sucrolytic activity in the cytosol and although its activity is induced to supplement the reduction in NI activity it is not able to regulate the size of the hexose pool.

In addition, pulse labelling experiments with [U-^{14}C]-glucose and unlabelled fructose have shown that although suspension cells contain high SuSy levels, SuSy does not contribute significantly to the synthesis of sucrose (Wendler *et al*., 1990). It can thus be concluded that the increased sucrose concentrations reported here are not related to the observed increase in SuSy activity. Rather, in the U1 and U2 transgenic lines, the increased SuSy activities throughout the growth cycle could be explained as part of a mechanism to mobilise sucrose in the cells. This would allow the supply of hexoses to essential pathways, and could compensate for the decline in invertase-mediated sucrose hydrolysis. In experiments conducted on bean suspension cells (Botha *et al*., 1992), the lowest NI activity was twice as high as the maximum SuSy activity, suggesting that the cytosolic-located invertase is the

predominant sucrose hydrolyzing enzyme in bean suspension culture cells. The same could be said for sugarcane cells in batch suspension, as even comparatively small alterations (± 10 – 30% decrease) in NI activity had significant effects on the growth and metabolism of these cells in spite of an increase in SuSy activity. Although the increase in SuSy activity could compensate for the reduced NI to still allow for a certain measure of normal functioning of the plant, these data suggests that this compensatory mechanism is still insufficient to restore the wild-type growth characteristics and metabolite levels.

Compared to the untransformed NCo310 control line the transgenic cultures accumulated significantly more sucrose at their mature stage (day 12) (Fig 4a). The increased sucrose content of the transgenic cells is not the result of the higher residual sugar concentrations of the growth media of the mature cultures (due to the reduced growth rate of the transgenic cells) as the process of sucrose accumulation in mature sugarcane suspension cultures was shown to be independent of the hexose concentration in the medium (Wendler *et al.*, 1990).

High purity levels (sucrose expressed as a percentage of the total sugars) were obtained earlier in the growth cycle of all the transgenic cultures, but the differences between these cultures and the control declined in the mature stages (Fig. 5d). This suggests that a decrease in NI activity may promote the 'early ripening' of immature sugarcane tissues. NI activity in the mature day 12 cultures also showed a significant correlation ($R^2 = 0.82$, $p \ll 0.05$) to the purity of the sugar samples, which further underlines the impact of NI activity on the sucrose-accumulating capacity of mature sugarcane cell cultures.

Data obtained from ^{14}C labelling experiments also support the hypothesis that the increase in the sucrose content of the transgenic lines is due to a decrease in invertase-mediated sucrose hydrolysis and a concomitant decline of the futile cycling between sucrose and hexoses. The increased accumulation of the imported [U-^{14}C]-fructose in sucrose in the transgenic cultures relative to the controls is apparently at the expense of label allocation to the pool of respiratory intermediates in these cultured cells. In previous sugarcane cell suspension culture experiments, sucrose accumulation commenced after about 5 days (Wendler *et al*., 1990), which is in agreement with our data (Fig. 4a). Respiration reportedly increased to a maximum at day 5 and declined as the cultures aged (Wendler *et al*., 1990), again corroborating the respiratory activity measured at days 4 and 8 in these experiments (Table 3). Glucose and fructose levels in our cultures increased up to day 8, and then decreased sharply towards the

end of the growth cycle (Fig. 4b & c). In contrast, glucose and fructose concentrations increased steadily throughout the growth of the cell cultures in the experiments of Wendler *et al*. (1990). This could be due to expression of the hexoses on a per gram FW basis, which does not represent cellular metabolism as accurately as the expression of metabolites on a per mg protein basis (Bindon & Botha, 2001).

As concluded by Wendler *et al*. (1990) and Botha *et al*. (1996) there is a simultaneous synthesis and degradation of sucrose irrespective of whether the sugarcane cells are mobilizing their internal sucrose stores, actively growing or rapidly accumulating sucrose. The net rate of sucrose accumulation should therefore depend on the relative balance of enzyme activities and fluxes in the two opposing directions of sucrose synthesis and degradation. Even small changes in either of these unidirectional fluxes will result in large changes in the net rate of sucrose storage (Wendler *et al*., 1990). This was confirmed by our findings, even though it is clear that the control of sucrose accumulation is regulated by a multitude of inter-related factors (Moore, 1995).

It will be interesting to see whether the transgenic suspension cultures are useful as a model for studying sucrose accumulation in the culm of regenerated transgenic plants. If the findings reported here are mirrored in mature plants, suspension cultures could be established as a valuable research tool for manipulating and analysing sucrose metabolism in sugarcane.

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CHAPTER 4

Down-Regulation of Neutral Invertase Activity in Transgenic Sugarcane Plants Reduces Respiration and Futile Sucrose Cycling in the Internodes

ABSTRACT

Transgenic sugarcane plants were regenerated from previously described cell lines with reduced neutral invertase (NI) activity, which had been metabolically characterized as suspension cultures. The effects that were observed in the differentiated internodal tissues at different stages of maturity resembled those that were observed in suspension cultures. Reduced NI activity correlated with an increase in sucrose and a decrease in hexose levels. However, the magnitude of the reduction in enzyme activity and the accompanying changes in carbohydrate metabolism were not as pronounced as in the suspension cultures. Feeding experiments with radio-labelled fructose provided evidence that the futile cycling of sucrose as well as the total respiration rate were reduced in the transgenic plants with reduced NI activity. Sucrose synthase was up-regulated in the transgenic plants, probably to compensate for the reduction in invertase activity. This study clearly demonstrates the importance of NI in directing carbon towards the respiratory processes in the sugarcane culm. In addition, the correlation of the data with that obtained from suspension cells supports the use of cell cultures as a model system for metabolic engineering studies in sugarcane.

Keywords: Neutral invertase, antisense, sugarcane, carbon flux.

INTRODUCTION

Sugarcane (*Saccharum officianarum* interspecific hybrids) is a commercial crop plant of great economic significance to various countries. In an attempt to improve its productivity, various plant breeding approaches have been used in the past to create fast-growing, high sucrose-accumulating varieties. While sugarcane breeding programs have been responsible for some great improvements in sucrose yield in the past, the same techniques have met with limited success in more recent years (Grof & Campbell, 2001). With the advent of genetic

engineering of plants the potential has arisen for molecular genetic approaches to play an important role in increasing the capacity of sugarcane plants to accumulate sucrose.

Sucrose accumulation in sugarcane is regulated by a cycle of synthesis and degradation and the subsequent transfer to the vacuole (Glasziou, 1961; Sacher *et al*., 1963; Wendler *et al*., 1990). It is a carefully controlled process that balances the supply of carbon substrates from the apoplast with the demand of competing pathways for carbon skeletons. This is accomplished by the metabolic regulation of the various enzymes in the synthetic and degradation pathways. Although the 'futile cycling' of sucrose is an energetically demanding process (Hill & ap Rees, 1994; Dieuaide-Noubhani *et al*., 1995), it could have major physiological functions such as control of respiration (Dancer *et al*., 1990), maintenance of osmotic potential (Geigenberger *et al*., 1997), control of sugar accumulation (Rohwer & Botha, 2001), sugar signalling (Cortès *et al*., 2002) and control of carbon allocation for biosynthetic processes (Fernie *et al*., 2002).

Various studies have shown that significant effects are exerted on the sucrose cycle by the enzymes sucrose phosphate synthase (SPS; EC 2.4.1.14; Grof *et al*., 1998; Botha & Black, 2000), the invertases (EC 3.2.1.26; Zhu *et al*., 1997; Echeverria 1998, Vorster & Botha 1999; Rose & Botha 2000; Bosch *et al*., 2004) and sucrose synthase (SuSy; EC 2.4.1.13; Lingle & Irvine, 1994; Schäfer *et al*., 2004 & 2005), which are responsible for the synthesis and breakdown of sucrose in the various cellular compartments. While sucrose can only be synthesized in the cytosol via SPS or SuSy, it is distributed to various degrees between the apoplast, the vacuole and the cytosol of the storage parenchyma (Hawker, 1985; Welbaum & Meinzer, 1990). All the hydrolytic and/or cleavage activities of the enzymes in these sub-cellular compartments can therefore, to some extent, exert an influence on sucrose metabolism, translocation and storage (Lee & Vattuone, 1996).

Invertases are a class of enzyme that hydrolyse sucrose to glucose and fructose. It has been suggested that invertases play a crucial role in the control of metabolic fluxes, sucrose partitioning, and ultimately, plant development and crop productivity (Sonnewald *et* al., 1991; Klann *et al*., 1996; Tang *et al*., 1999). The degree to which this statement is true for each of the three invertase isoforms remains an unresolved issue. Other possible functions of the invertases include the regulation of cell turgor for cell expansion, (Meyer & Boyer, 1981; Wyse *et al*., 1986, Perry *et al*., 1987), and the control of sugar composition in storage organs

(Klann *et al*., 1993). Furthermore, some of the invertases seem to be involved in the responses of plants to environmental factors, such as wounding and infection (Sturm & Chrispeels, 1990; Benhamou *et al*., 1991).

Various isozymes of invertase with differing pH optima and subcellular localisations exist. There are two kinds of acid invertase, both exhibiting optimum activity between pH 5.0 and 5.5. They are located in two separate compartments, the vacuole and the apoplast. Cell wall invertase (CWI) is confined to the apoplast, where it controls the flow of sucrose from the conducting tissue to the young growing cells (Hatch & Glasziou, 1963). Vacuolar invertase or soluble acid invertase (AI) may play a role in the remobilization of stored sucrose from the vacuole (Sacher *et al*., 1963) and is believed to be important in the regulation of hexose levels in certain tissues (Singh & Kanwar, 1991). Mature sucrose-storing internodes of sugarcane contain negligible AI levels (Hatch & Glasziou, 1963), but do, however, exhibit significant SuSy and neutral invertase (NI) activity (Hawker & Hatch, 1965). NI is located in the cytosolic compartment, where it functions optimally at pH 7.0.

Early studies found that NI activity (expressed on a FW basis) increases with internode maturity and its activity level is thus positively correlated with sucrose concentrations in internodal tissues (Hatch *et al*., 1963; Gayler & Glasziou, 1972; Batta & Singh, 1986; Singh & Kanwar, 1991). However, conflicting data have also been reported where NI activity (also on a FW basis) decreases with internode maturation (Dendsay *et al*., 1995). The most recent studies reported that NI activity (on a per mg protein basis) increases up to the fifth internode before declining as the internodes mature further (Ebrahim *et al*., 1998; Vorster & Botha, 1999). In addition, NI was reported to be the only sucrolytic enzyme that shows any correlation to sugar concentrations in mature sugarcane stem tissue, where its activity level is positively correlated to hexose levels. (Gayler & Glasziou, 1972; Bosch & Botha, 2004) and negatively correlated to sucrose levels (Rose *et al*., 2000).

It was initially reported that NI activity expressed on a fresh weight basis showed no particular distribution pattern related to varietal differences (Hatch & Glasziou, 1963). However, subsequent research showed that NI activity was low or absent in mature storage tissues of many high-sugar storing, early-maturing varieties of sugarcane (Dendsay *et al*., 1995). This differential expression of NI therefore suggests that it may be an important determinant of the sucrose-accumulating characteristics of sugarcane.

As NI is a cytosolic enzyme, it is the only invertase that functions in the compartment where sucrose is actively synthesized. When looking at the findings of NI research over the past decade it becomes apparent that this enzyme might play a key role in the process of sucrose accumulation in sugarcane. Of particular interest is the role that NI might play in controlling the 'futile cycle' of sucrose breakdown and resynthesis. In addition, a sugarcane kinetic model also pointed to NI as one of the possible key enzymes in terms of its impact on flux through this cycle (Rohwer & Botha, 2001). While only theoretical and speculative in nature, this *in silico* model suggests that NI is a suitable target for a reverse genetic approach aimed at elucidating the precise function and significance of this enzyme to central carbon metabolism in sugarcane.

In a recent study we have described the establishment and characterization of transgenic sugarcane suspension cultures with reduced NI activity (Chapter 3). These suspension cultures showed reduced sucrose cycling and increased sucrose concentrations as well as increased sucrose to hexose ratios. Here we describe the characterization of the transgenic plants that were regenerated from these respective cell lines. We also demonstrate that sugarcane suspension cultures can serve as a valuable model to predict the effects of genetic manipulations in carbohydrate metabolism in whole plants.

MATERIALS & METHODS

Biochemicals

Chemicals were purchased from Sigma-Aldrich SA. All coupling enzymes and cofactors used in the sugar and enzyme assays were obtained from Roche Biochemicals. The [U-^{14}C]fructose was from Amersham International (Claremont, South Africa). All other solvents and biochemicals were of analytical grade.

Plant material

Callus of the NCo310 variety was transformed as described in the previous chapter with neutral invertase antisense constructs containing a strong ubiquitin-based promoter in tandem with the 35S promoter (pASNI510). After regeneration from tissue culture, the stalks were planted and the new sugarcane plants grown in greenhouses for 12 months before characterization. The transgenic plants were stunted in their growth in comparison to control plants.

Two of the transgenic lines, U1 and U2, as well as control NCo310 plants were harvested in Stellenbosch between 09:00 and 10:00 towards the end of summer. Internodes were numbered according to the system whereby internode 1 is defined as the internode from which the leaf with the first exposed dewlap originates. Internodes 3 and 4 were grouped together to represent immature tissues and internodes 7 and 8 were grouped as representative of maturing to mature tissues.

Samples were taken for protein and enzyme analysis from these tissues, and one mm tissue disc slices were sectioned with a hand microtome from core internodal tissue 6 mm in diameter. The tissue discs were immediately placed in 50 ml ice-cold buffer containing 25 mM K-MES (pH 5.7), 250 mM mannitol and 1 mM $CaCl_2$ (Whittaker & Botha 1997) for at least 15 mins.

Protein extractions

Samples were ground in liquid nitrogen and 150 mg tissue extracted in 500 ul extraction buffer (100 mM sodium phosphate) containing 1mM EDTA, 10 mM DTT, 10% ($^{v}/_{v}$) glycerol, 2% PVPP ($^{v}/_{v}$) and 0.0016 g/ml Complete protease inhibitor.

Insoluble residue was pelleted by centrifugation at 10, 000 × g for 15 min. The supernatant was passed through Sephadex G25 size-exclusion minispin columns (Biopharmacia) by centrifugation at 2500 × g for 1 min, and the eluates used as the desalted protein samples. Protein concentrations in the crude and desalted samples were determined by the method of Bradford (1976) using the BioRad microassay, with BSA as a standard.

The pellet was washed 3 times in extraction buffer (without PVPP) to remove any residual soluble invertase activity.

Enzyme assays

Cell-wall bound invertase activity was measured in the insoluble pellets according to the method of Albertson *et al*. (2001).

Acid invertase, neutral invertase and SuSy activity was determined in the desalted enzyme extracts: SuSy activity was determined in the breakdown direction as described by Schäfer *et*

al. (2004). Acid invertase activity was assayed by incubation of extracts for 3 hrs in 125 mM sucrose and 50 mM citrate-phosphate buffer (pH5.5). Reactions were stopped at 1 hr intervals by a 2 min incubation at 90^{o}C. Neutral invertase activity was similarly assayed by a 2 hr incubation in 50 mM Hepes (pH 7.5) and 125 mM sucrose. At zero, 1 and 2 hrs, aliquots were transferred to a second microtiter plate (kept at 4^{o}C), and the reaction terminated by the addition of 5 µl stop solution (2M Tris, 22 mM $ZnSO_4$). For both the acid and neutral invertase assay the reducing sugars were measured using a system coupled to NAD^+ (Huber & Akazawa, 1986) with a Beckman DU® 7500 spectrophotometer.

Radiolabelling experiments

For ^{14}C metabolic studies, the tissue discs were labelled as described by Bindon and Botha (2002). Tissue discs were incubated on a rotary shaker at 102 rpm for 4 hrs in buffer containing 5 mM glucose, fructose and sucrose and [U-^{14}C] fructose at a specific activity of 37 nmol^{-1}. $^{14}CO_2$ released during the incubation period was collected in 500 µl 12% (w/v) KOH contained in central wells of the 250 ml Erlenmeyer flasks. The radioactivity present in the KOH vials was determined by a Beckman Scintillation Counter following the addition of 4 ml Ultima Flo ™ scintillation cocktail.

After the labelling period, unincorporated label was removed from the tissue discs by two consecutive washes in 50 ml ice cold wash buffer. The discs were then blotted dry and ground in liquid nitrogen. The ground tissues were weighed before transfer to 2 ml eppindorf tubes containing 1 ml 80% EtOH and 30 mM Hepes (pH 7.5). The samples were incubated for 2 hrs at 80^{o}C, then centrifuged at 13 000 rpm for 20 mins at 4^{o}C. The supernatants were removed and the extraction repeated with another 1 ml of 80% EtOH. The supernatant from this step was added to that of the first extraction. Radioactivity in the total soluble fraction was determined by counting 10 µl in 4 ml scintillation cocktail in a Beckman LS 1801 scintillation counter.

The remaining pellet was washed 5 times in 80% EtOH and dried completely in a vacuum centrifuge, after which 2 ml of the organic solvent Soluene-350 was added to dissolve the insoluble residue. Radioactivity in 200 µl aliquots of the cell pellet suspension were also determined with a scintillation counter as described for the KOH solutions.

Fractionation of cellular constituents

One ml of the total solubles were passed through strong cation and anion exchangers (Supelco Supelclean columns) in tandem to bind all acidic and aromatic compounds and render an extract containing only the neutral fraction in a volume of 10 ml ddH_2O. This neutral fraction was completely reduced in a vacuum centrifuge and resuspended in 10% (v/v) isopropanol. The acid and basic fractions were eluted from the columns with 2 ml of 4 M NH_4OH and 4 M formate, respectively. Label recovery in the three fractions was again determined with scintillation counting.

The remaining soluble extracts were dried under vacuum and resuspended in 10% isopropanol. Samples were prepared for HPLC analysis by passing through 0.45 μm filters (Millipore).

Enzymatic assay of total sugar content

Glucose, fructose and sucrose were assayed according to the method of Bergmeyer and Bernt (1974).

Analysis of sugars by HPLC

Sugars were fractionated by HPLC (Shimadzu SCL-10AVP system) using a Hamilton RCX-10 column. A 30 mM NaOH solution was used as the mobile phase, at a flow rate of 1 ml per min, and ^{14}C in glucose, fructose and sucrose was determined by inline liquid scintillation spectroscopy (Radiomatic A-500).

RESULTS

The transgenic plants in this study were transformed with an antisense NI sequence under the control of a tandem 35S-ubiquitin promoter (Chapter 3). It was difficult to regenerate plants from the transformed callus and the survival and growth rates of the regenerated plants were severely impaired. Only two transgenic lines were therefore successfully regenerated for characterisation, most probably because NI activity was down regulated less efficiently in these two particular lines (U1 and U2).

A reduction in NI activity leads to an increase in sucrolytic SuSy activity

For the initial characterization of the transgenic lines all enzymes that contribute to the breakdown of sucrose were determined in young and maturing internodes. All sucrose-hydrolyzing activities decreased from young to mature internodal tissues (Table 1). When comparing the data of the control and transgenic lines it is clear that NI activity was reduced by up to 40% in the U1 and U2 lines. The opposite pattern was evident in the case of SuSy, as activity in the breakdown direction increased in the transgenic lines relative to the control. No significant differences were evident in the activities of the acid and cell-wall bound invertase isoforms.

Table 1. Activity of the sucrolytic enzymes in young (internodes 3-4) and maturing (internodes 7-8) internodes of the control (NCo310) and two transgenic sugarcane lines (U1 and U2). Enzyme activity is expressed as nmol.min^{-1} mg^{-1} protein.Values are the average of 4 replicates ± standard deviation.
* Indicates values that differ significantly from the control ($p < 0.05$).

	Young internodes			Maturing internodes		
	NCo310	U1	U2	NCo310	U1	U2
Neutral Invertase	**30.6** ± 2.4	**19.2** ± 2.3*	**20.5** ± 2.9*	**22.5** ± 3.6	**13.8** ± 0.9*	**14.6** ± 2.7*
Sucrose Synthase	50.6 ± 5.8	76.8 ± 1.8*	65.8 ±11.2*	24.3 ± 4.8	45.3 ± 3.9*	36.5 ± 19.8
Acid Invertase	32.6 ± 2.2	32.1 ± 4.2	30.4 ± 5.1	15.6 ± 3.2	14.2 ± 1.3	15.6 ± 2.1
Cell Wall Invertase	20.8 ± 3.2	23.3 ± 3.6	22.4 ± 2.2	11.8 ± 1.6	12.0 ± 2.4	12.1 ± 1.5

A reduction in NI activity leads to an increase in sucrose concentrations

Clear alterations were found in the sugar compositions of the transgenic tissues in comparison to the control. Glucose and fructose concentrations were substantially lower in the transgenic tissues, with the differences being more pronounced in the young internodes (Table 2). Although not significant, the average sucrose concentrations were consistently higher in the young and maturing internodes of both the U1 and U2 lines. The purity (the amount of sucrose expressed as a percentage of the total sugars, i.e. sucrose plus glucose plus fructose) of the transgenic samples was 23% and 6% higher in the young (total 70% purity) and maturing (total 85%) tissues respectively in comparison to the control samples.

Table 2. Concentrations of glucose, fructose and sucrose in extracts from young and mature tissues of the control and two transgenic lines (U1 and U2). Sugar concentrations are expressed as μM mg^{-1} protein. Values are the average of 4 replicates ± standard deviation.
* Indicates values that differ significantly from the control ($p < 0.05$).

	Young internodes			Maturing internodes		
	NCo310	U1	U2	NCo310	U1	U2
Glucose	23.9 ± 7.0	17.7 ± 4.4	17.6 ± 5.4	22.5 ± 3.2	17.4 ± 1.4*	19.3 ± 2.6
Fructose	19.9 ± 8.2	13.1 ± 2.1	15.9 ± 7.3	23.7 ± 49.3	17.0 ± 2.3	17.9 ± 7.0
Sucrose	57.3 ± 15.9	72.1 ± 10.8	77.3 ± 16.8	173.6 ± 28.8	198.1 ± 13.7	199.4 ± 22.1
% Purity	56.8 ± 6.7	70.1 ± 3.9*	70.2 ± 3.9*	79.2 ± 3.0	85.1 ± 1.2*	84.4 ± 1.6*

A reduction in NI activity leads to an increased flux into sucrose

To investigate changes in the *in vivo* partitioning of carbon to various key metabolite pools radio-labelled fructose was fed to excised internodal tissue discs. It appeared that the labelled fructose was taken up with greater efficiency by the transgenic tissue discs (Table 3). The label allocation to the insoluble fraction decreased considerably with internode maturity, and the labelled fructose was partitioned to the insoluble fraction to the same degree in both transformed and untransformed tissues. Label allocation to sucrose increased with internode maturity in all the stalks sampled and label incorporation into sucrose was on average consistently greater in both young and maturing internodes of the transgenic plants compared to the control. The pattern was reverse for glucose, as the total radiolabelled carbon incorporated in the glucose pool was greatly reduced in the transgenic samples relative to the control.

In both the young and maturing transgenic internodes, slightly less label was incorporated in the amino acid and organic acid fractions, as well as in released CO_2. Collectively these data indicate a decline in label allocation to the respiratory intermediates and end-products in the transgenic plants in comparison to the control.

Table 3. Distribution of ^{14}C in internodal tissue discs supplied with [U-^{14}C]fructose for 4 hours. The total activity of each component is expressed as kBq mg^{-1} protein. Each value is the average ± standard deviation of 4 separate samples. * Indicates values that differ significantly from the control ($p < 0.05$).

		Total Uptake	Insoluble Fraction	Sucrose	Glucose	Fructose	Organic Acids	Amino Acids	CO_2	Total Respiration
		20.85 ±	7.21 ±	**6.73** ±	1.83 ±	1.49 ±	2.95 ±	0.96 ±	0.41 ±	
Young Internodes	NCo310	3.88	0.70	2.02	0.66	0.63	0.56	0.40	0.08	**4.32** ± 0.89
		30.80 ±	7.48 ±	**11.58** ±	1.00 ±	2.08 ±	2.79 ±	0.69 ±	0.22 ±	
	U1	4.56*	1.11	2.96*	0.31	0.52	0.13	0.10	0.03*	**3.69** ± 0.17
		26.06 ±	6.85 ±	**9.89** ±	0.76 ±	1.30 ±	2.99 ±	0.73 ±	0.23 ±	
	U2	6.96	1.89	2.30*	0.05*	0.51	0.93	0.07	0.07*	**3.94** ± 0.97
		21.88 ±	2.67 ±	**8.94** ±	1.12 ±	1.64 ±	2.36 ±	1.06 ±	0.20 ±	
Maturing Internodes	NCo310	8.83	1.80	1.89	0.32	0.77	0.67	0.33	0.07	**3.62** ± 0.94
		29.47 ±	2.52 ±	**21.39** ±	0.63 ±	2.59 ±	2.20 ±	1.08 ±	0.09 ±	
	U1	2.96	1.21	2.91*	0.18	0.37	0.63	0.05	0.03*	**3.37** ± 0.65
		24.62 ±	2.50 ±	**13.26** ±	0.61 ±	2.13 ±	2.07 ±	0.94 ±	0.15 ±	
	U2	2.62	1.11	3.38	0.18*	0.50	0.57	0.17	0.03	**3.16** ± 0.70

The percentage contribution of the label in sucrose to the total label in the transgenic lines is clearly greater than that of the control (Fig. 1), particularly in U2, which coincides with the greatest reduction in NI activity (Table 1). Corresponding to this increase in labelled sucrose the percentage label of some of the other fractions, including glucose, the organic and amino acids, and respired CO_2 declined.

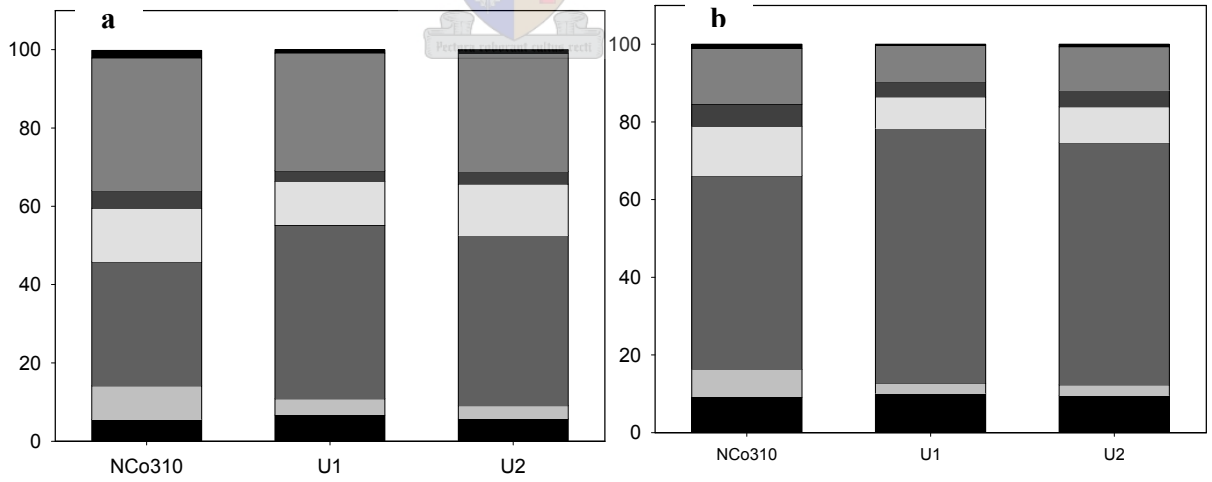

Figure 1. Percentage allocation of ^{14}C to the various pools after a 4 hr [U- ^{14}C]fructose labelling period in (a) young internodes and (b) maturing internodes. The fractions represented from bottom to top are as follows: fructose ( ), glucose ( ), sucrose ( ), organic acids ( ), amino acids ( ), insoluble ( ) and CO_2 ( ).

Specific activity was determined in order to quantify the flux into sucrose as well as the flux into glucose via invertase-mediated hydrolysis of sucrose. The specific activity of sucrose and fructose was higher, and that of glucose lower in at least some of the transgenic samples, relative to the control (Table 4). The net flux into sucrose was greater in the U1 and U2 lines compared to the control (Fig. 2), while the converse was true for the flux into glucose (Fig. 3). In the young internodes, the flux into glucose was reduced by around 60% in the transgenic lines, while in the mature internodes the reduction was between 50 and 75%.

Table 4. Specific activities of glucose, fructose and sucrose in the tissue disc extracts from young and mature tissues of the control and two transgenic lines. Specific activities are expressed as Bq μmol^{-1} sugar. Values are the average of 4 replicates ± standard deviation.
* Indicates values that differ significantly from the control ($p < 0.05$).

		FRUCTOSE	GLUCOSE	SUCROSE
Young Internodes	NCo310	60.8 ± 11.2	77.6 ± 24.8	117.7 ± 12.7
	U1	124.9 ± 23.0*	57.7 ± 6.6	153.1 ± 24.3*
	U2	84.4 ± 11.3*	46.4 ± 13.5	128.1 ± 11.9
Maturing Internodes	NCo310	71.6 ± 9.7	57.8 ± 15.5	53.4 ± 10.4
	U1	159.2 ± 31.6*	40.0 ± 12.3	90.0 ± 33.8
	U2	129.7 ± 42.3*	31.3 ± 6.4*	69.8 ± 20.0

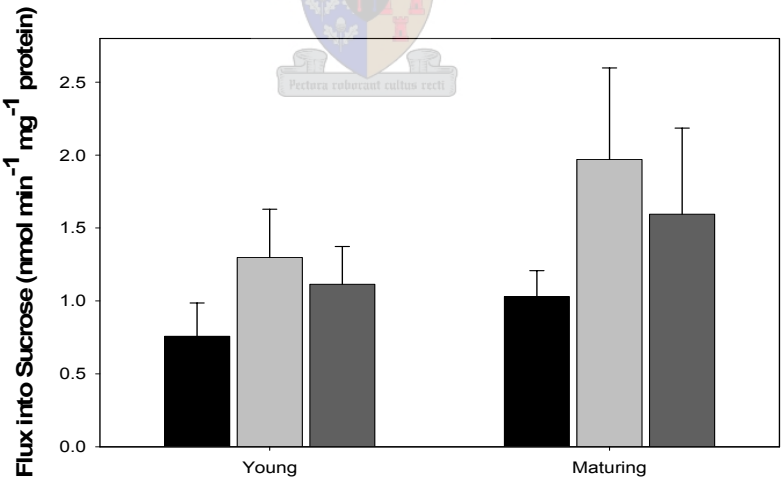

Figure 2. Net flux of [U-^{14}C]fructose into sucrose after a 4 hr labelling period. Values are the average of 4 replicates ± standard deviation. The three lines in each group are represented from left to right as NCo310 ( ), U1 ( ) and U2 ( ).

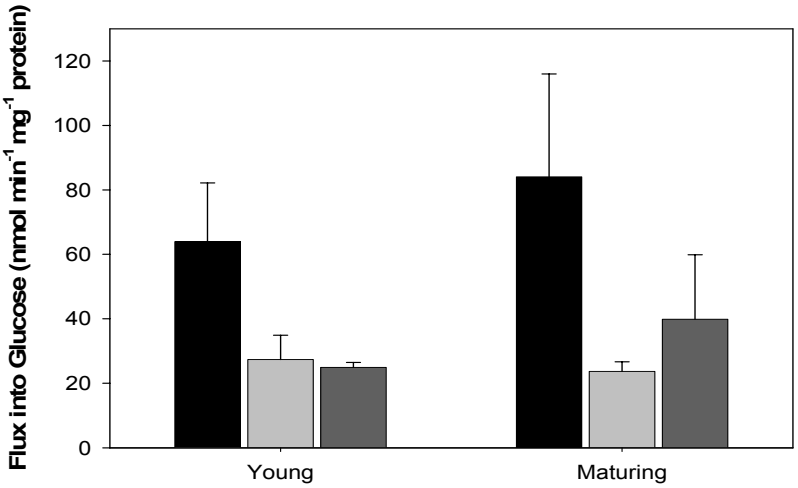

Figure 3. Net flux of label into glucose after a 4 hr labelling period. Values are the average of 4 replicates ± standard deviation. The three lines in each group are represented from left to right as NCo310 ( ), U1 ( ) and U2 ( ).

DISCUSSION

Transgenic sugarcane plants that contained only approximately 60% of the NI activity found in control plants, both in immature and maturing internodes, were regenerated and characterized. No changes in CWI or AI activity were evident, although the activity of SuSy increased quite substantially in the transgenic plants. This possibly occurred in order to compensate for the decline in NI activity, which especially in younger internodes will interfere with the normal growth and developmental processes if there is a shortage in hexoses.

Glucose, fructose and sucrose concentrations, percentage purity and the respective specific enzyme activities in the control samples were similar to the reported values (Whittaker & Botha, 1997; Vorster & Botha, 1999; Bindon & Botha, 2002). Sucrose concentration and percentage purity in the transgenic lines were consistently higher, and the hexose (glucose and fructose) concentrations lower, than that of the control lines in all the tissues sampled (Table 2). The sucrose concentrations in the immature and maturing internodes of the transgenic plants were respectively up to 35% and 15% higher than control levels. The percentage purity of the transgenic samples was approximately 13% and 6% higher than in the control in the young and maturing internodes respectively. In general, purity increases in the maturing internodes as the demand for glycolytic intermediates decreases (Botha *et al*., 1996). It

appears therefore that the down regulation of NI has a smaller influence in the more mature internodes where glycolytic flux is smaller, i.e. the remaining activity and complementary activity of SuSy can cope more aptly with the demand for hexoses in these tissues. This explains the relatively lower increase in the percentage purity of the transgenic samples in the older internodes compared to the increase measured in the immature transgenic tissues.

In terms of the allocation of label to the various carbon pools in the tissue discs, some interesting differences were evident in the transgenic lines: Firstly, although not significant, it appears as though the majority of the transgenic tissue sections took up a greater quantity of the [U-^{14}C]fructose from the incubation buffer during the labelling period (Table 3). This is supported by the significant increase in the specific activity of fructose in all the transgenic tissues (Table 4). A possible explanation could be that the lower glucose and fructose concentrations of the transgenic cells established a greater concentration gradient between the medium and the intracellular hexoses. It is also possible that the change in metabolite concentrations in the transgenic tissues altered some aspects of the hexose uptake and translocation system in such a way to increase the uptake of hexoses from the incubation buffer.

The amount of label in the insoluble pool decreased by approximately 60% from young to maturing internodes in all the plants sampled, which is in agreement with trends as reported by Bindon and Botha (2002). No differences between the control and transgenic lines were evident for the total incorporation of label in the insoluble pool, suggesting that this branch of carbon metabolism remained relatively unaffected by the changes in the sucrolytic activities in the transgenic tissues.

The increase in label allocation to sucrose between the young and maturing internodes can be seen in terms of both the amount of label per mg protein and as a percentage of the total label taken up (Tabel 3 and Fig. 1). In the control plants, the percentage allocation of label to sucrose increased from approximately 30% to 50% between the immature and maturing internodes respectively, whereas in the transgenic plants the label allocation increased from 45% to 65% (Fig. 1). The control values for label allocation to sucrose as well as the other fractions are similar to those reported by others (Whittaker & Botha, 1997; Bindon & Botha, 2002).

Label recovery in glucose was greatly reduced in both the young and maturing internodes of the U2 transgenic line (Table 3). The only pathway of label incorporation into glucose after feeding labelled fructose is via hexose phosphorylation, followed by incorporation into sucrose (leading to a randomization of label in the hexose moieties of sucrose) and subsequent hydrolysis by invertase. The reduction in the flux of label into glucose in the transgenic tissues (Fig 3) is thus a clear indicator of a lower *in vivo* rate of invertase-mediated sucrose hydrolysis. The apparent increase in the net flux into sucrose in the transgenic lines (Fig. 2) is therefore probably due to the decreased hydrolysis rates, which would result in an accumulation of label in the sucrose pool, as opposed to a distribution of label to other pathways and competing carbon pools. In combination the data therefore suggests that the decrease in sucrose hydrolysis did not lead to a corresponding decrease in the rate of sucrose synthesis. Thus the enzymes in the synthetic branch of the pathway do not appear to be significantly influenced by the increased sucrose concentration in the cells.

One pathway that is clearly affected by the altered metabolism of the transgenic cells is respiration. In nearly all the transgenic tissues sampled the CO_2 released (the end-product of oxidative respiration) contained less labelled carbon than the control samples. This points to a decrease in the respiration rate of these tissues, which could explain the slow-growing phenotype of the transgenic plants (data not shown). The same trends with regards to respiration and growth rate were also reported for the suspension cells (Chapter 3). In experiments conducted in tomato plants transgenic lines were created with greatly reduced levels of acid invertase in ripe tomato fruit (Klann *et al*., 1996). These antisense lines had increased sucrose concentrations, and were approximately 30% smaller than control fruit. Thus, similar to our findings, correlations between reduced sucrolytic rates and growth inhibition have been reported. If it is envisioned to genetically engineer sugarcane for enhanced sucrose accumulation through reducing NI activity, this would have to be done using a developmentally regulated promoter that is only active in mature sugarcane culm.

The % decrease in NI activity in the internodes is not as substantial as was found in the suspension culture experiments conducted with the same transgenic lines (Chapter 3). In the transgenic suspension cultures NI activity accounted for only 33% of the NI activity found in control cultures, whereas the NI activity in the regenerated plants amounted to 60% of wild-type levels in the internodes. This is to be expected, as the metabolic regulation of undifferentiated meristematic cells is bound to differ from differentiated storage parenchyma

cells (Veith & Komor, 1993). The fact that no sink-source relationships exist for suspension cultured cells means that this facet of the sucrose accumulation system cannot be emulated in suspension cultures. Also, vacuolization is 40% lower in cell suspensions than in culm tissue (Komor, 1994) therefore the physical characteristics of the sucrose storage system are somewhat different in suspension cells compared to storage parenchyma cells. As such, the relative contribution to sucrose accumulation of the various sucrose-hydrolyzing enzymes in the different sub-cellular compartments will be different in the less vacuolated suspension cells compared to internodal tissues. For example, the relatively larger cytosolic compartment of the suspension culture cells could account for the more significant effect of NI activity on total sucrose levels.

However, the same trends in terms of decreased *in vivo* NI activity, increased sucrose concentrations and decreased respiratory flux were apparent in the transgenic lines in suspension culture (Chapter 3). The similarities between the results of the suspension culture studies and the whole plant studies in terms of overall trends and correlations are thus fairly encouraging. In light of these findings it would seem as though suspension cultures could represent an alternative approach to the characterization of transgenic sugarcane in future research.

In conclusion, our data suggests that NI plays an important role in controlling the rate of sucrose hydrolysis and therefore in the provision of hexose substrates to the subsequent cellular processes, particularly in immature internodes. Moreover, sucrose synthesis and storage do not seemed to be influenced by a decrease in NI activity but the reduced availability of hexoses impacts negatively upon respiration resulting in the reduced vitality of the transgenic plants.

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CHAPTER 5

General Discussion

Between the young and maturing internodes of the greenhouse-grown sugarcane plants the activity of NI showed a general decline in both control and antisense plants. This is similar to the trends reported by others (Dendsay *et al.*, 1995; Ebrahim *et al.*, 1998; Hatch & Glasziou, 1963; Lingle, 1997; Vorster & Botha, 1999). In the suspension cultures the activity of NI increased up day 8, coinciding with the exponential growth phase of the cultures. Activity then declined as maturation continued, reaching a minimum in the mature cultures at the end of the growth cycle. It would seem as if NI activity in both the suspension cultures and sugarcane plants followed similar general trends, namely a decline in activity as metabolic activity decreased.

Transgenic sugarcane transformed with antisense NI constructs showed a reduction in NI transcript levels and a resulting decrease in the activity of the enzyme, both in undifferentiated suspension cultured cells and in internodal storage tissues of sugarcane plants. In the suspension cultured cells, the extent of the decrease in NI activity varied depending on the strength of the promoter used. Interestingly, even the lines transformed with the stronger ubiquitin promoter construct never showed levels of NI activity below 34% of control activity. Compared to modern RNAi approaches, the levels of residual NI activity in the transgenic plants/suspension cells are still fairly high. Yet, even just this relatively moderate reduction in NI activity seems to have far-reaching effects on the metabolism and physiology of the transgenic systems. This, combined with the low transformation efficiency and regenerability of callus during the transformation process suggests that a 'minimum, basal level' of NI activity is required to sustain essential metabolic processes and maintain the viability of sugarcane cells/tissues; i.e. if NI activity is reduced below this minimum level it is lethal to the transformed cells.

Of primary importance in the context of our focus area is the effect of reduced NI activity on the sucrose:hexose ratio (i.e. % purity) in sugar-storing tissues. Transgenic tissues were characterised by an increase in sucrose concentrations and a concomitant reduction in glucose and fructose levels. The ^{14}C flux data confirmed this decrease in the *in vivo* rate of invertase-

mediated sucrose hydrolysis and the resultant increase in the overall sucrose:hexose ratio. This effect was most pronounced in the younger internodes of the transgenic plants and in the suspension cultures at the immature stages of the growth cycle. In the more mature tissues of both systems, the rate of sucrose hydrolysis was not decreased to such a great extent in the transgenic samples relative to their respective controls. This was reflected by the metabolite levels in mature tissues, as the % purity of these samples was only nominally increased. This suggests that the role of NI in sucrose degradation may be more critical in immature tissues, where the demand for glycolytic precursors is greater due to the higher metabolic activity of these tissues. In addition, possible NI induced changes in the cytosolic sugar pool could be obscured in mature tissues by the increased relative contribution of the vacuolar sugar pool to total cellular sucrose levels.

The most obvious phenotypic effect of reduced NI activity was the reduction in the growth rate of the transgenic lines. This was first observed in the suspension cell cultures but later also in the transgenic plants. Labelling studies in both systems revealed a decreased in the amount of label in the respiratory intermediates and end-products, suggesting a change in the allocation of carbon to these pathways. This would account for the slow-growing phenotype of the transgenic plants and suspension cultures, and supports the view that NI plays a fundamental role in sustaining the necessary energy requirements for plant growth and maintenance (Sacher *et al.,* 1963; Dendsay *et al.,* 1995). Moreover, NI is involved in internode elongation/expansion activities, and is an important growth-regulating enzyme, especially in immature tissues (Dendsay *et al.,* 1995). Our data also suggests that the younger the internode, the more pronounced the impact of NI activity on the respiratory pathway. The same trend was evident in the suspension cultures, where the percentage reduction in label allocation to the respiratory pathway was greater in the younger cultures. In the more mature tissues where the demand for glycolytic precursors is less, the remaining NI and SuSy activities may be sufficient to sustain the required flux into glycolysis.

In the control suspension cultures SuSy activity showed a gradual increase up to day 8 of the growth cycle, after which the activity seemed to slowly decline. Similarly, SuSy activity in the breakdown direction showed a decrease between the young and maturing internodes of the control plants. In the transgenic suspension cultures SuSy activity was consistently higher at all stages of the growth cycle in comparison to the control cultures. SuSy activities in the transgenic internodal tissues also showed interesting trends, as activity was significantly

increased in the breakdown direction in both immature and maturing internodes. This suggests that SuSy activity could be up-regulated in order to compensate for the decline in NI activity. This plasticity in sucrose breakdown would help to maintain the flux of carbon to the pathways fuelling cellular respiration and growth. This would counteract some of the negative consequences of a reduced hexose availability incurred by the down-regulation of one of the enzymes involved in sucrose breakdown in the cytosol. The fact that metabolite concentrations in the more mature transgenic internodes and suspension cultures are not altered to the same degree as in the immature stages suggests that perhaps the required flux into glycolysis in these tissues decreases to the point where the increase in SuSy activity is sufficient to restore equilibrium. However, even large increases in SuSy activity still do not compensate entirely for NI activity reductions at the order of magnitude that we have reported, especially in the younger, more metabolically active tissues. This is evidenced by the significant changes in various metabolite levels (most notably sucrose) in the immature tissues, both in suspension culture and *in planta*.

The cytosolic compartment only occupies a small percentage of the total cell volume, so the fact that changes in NI activity in the cytosol exert such a pronounced effect on the levels of total cellular metabolites highlights its importance in central carbon metabolism. However, even though % purity is increased (particularly in immature internodes), the increased sucrose content of the transgenic sugarcane comes at the expense of growth. The practical implication of this may therefore be that the total yield of sucrose per plant or per unit area may be reduced because the growth and development of the transgenic plants is negatively impacted upon – an aspect which should be investigated under field conditions.

Another outcome of the work has been the establishment of suspension cultures as a viable model system for metabolic engineering studies in sugarcane. Although differing in their cellular architecture and in various metabolic features, suspension cultured cells are fairly representative of sugar-storing parenchyma cells in certain respects, e.g. their pattern of sucrose accumulation during the growth cycle and the activity trends of the enzymes involved in this process approximates to the trends and patterns observed in internodal tissues during maturation (ref; Chapters 3 and 4). This, in addition to their homogeneity and rapid growth characteristics, makes suspension cultures useful in terms of the initial screening of possible metabolic targets and large numbers of transgenic lines without the time and costs involved in regenerating mature plants.

In conclusion, both the primary goals presented in this dissertation were achieved in this study, namely the confirmation of suspension cultures as a suitable model system for transgenic research and the elucidation of the influence of NI on sucrose metabolism. Our data clearly show a significant effect of NI on sucrose concentrations in sink tissues. *In vivo* hydrolysis rates were decreased and the purity of yielded sugars (sucrose as percentage of the total sugars) was increased in all tissues sampled, particularly in the immature tissues. Our findings also point to a change in the carbon allocation patterns in both the transgenic plants and suspension cells, as the flux from the central hexose pool to the respiratory pathways is apparently reduced. From an agricultural perspective, the potential definitely exists to increase the yield of sucrose, although careful consideration should be made of the growth penalty incurred in the transgenic plants. Future prospects would include the use of inducible or tissue-specific promoters in transformation constructs for a more controlled decrease in NI activity in specific tissues.

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