SUCROSE ACCUMULATION AND THE EXPRESSION OF NEUTRAL INVERTASE 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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ABSTRACT

The goals of this project were to (i) determine maximum extractable neutral invertase (NI) activity in the sugarcane culm, (ii) sequence a cDNA encoding for the sugarcane NI (SNI), (iii) determine SNI copy number in the genome, (iv) describe SNI transcript and protein expression patterns throughout the plant, and (v) attempt to determine the contribution of hydrolysis to sucrose accumulation.

SNI and sugars were extracted from the developing culm tissues of sugarcane, commercial variety N19. Tissues were divided according to developmental stage (internodes 3, 6 and 9) and anatomical differentiation (enriching for elongating, vascular or storage tissues). The lowest sucrose content was found in the core of the bottom of each of the internodes. The ratio between hexoses and sucrose was highest in the young internodes. In these internodes hexose content was higher in the bottom than the top. There was a significant correlation between sucrose content and NI. Fluxes involved in sucrose synthesis and hydrolysis were investigated. The hexoses glucose and fructose were supplied as a carbon source for tissue discs of young and maturing internodal tissues of sugarcane, varieties N19 and US6656-15. Sucrose content was 10-fold higher in maturing internodes of N19 than US6656-15. Calculated sucrose hydrolysis rates via invertase were higher in maturing internodes of US6656-15 than N19. Taking metabolic compartmentation into account, hydrolysis of sucrose via invertase made a significant contribution to the net turnover of sucrose. Along with this, it would appear that the ability to partition sucrose between the vacuole and cytosol causes a significant difference in sucrose content between varieties.

A full-length cDNA for SNI was sequenced. This expressed gene showed significant homology to known NI sequences on both nucleic and amino acid levels. The SNI sequence did not contain the putative invertase catalytic amino acid sequence, suggesting it developed separately from the other classes of invertases. Approximately 1.8 kb of the SNI cDNA was incorporated into a vector suitable for direct bombardment into sugarcane tissue. Southern blot analysis showed the enzyme has a low copy number. SNI transcript expression was observed in all tissues of the sugarcane plant: roots, internodes, leaf roll and leaves. In culm tissues where sucrose content was low and hexose contents were high, SNI transcript and protein levels were high. This suggests that SNI is involved in growth metabolism.

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OPSOMMING

Die doel van die projek was om (i) maksimum ekstaheerbare neutrale invertase (NI) aktiwiteit in die suikerriet stingel te bepaal, (ii) die volgorde van 'n cDNA wat vir suikerriet NI (SNI) kodeer te bepaal, (iii) die SNI kopie-getal in die genoom te bepaal, (iv) SNI m-RNA en proteïenuitdrukkingspatrone deur die plant te beskryf, en (v) te poog om die bydrae van hidrolise op sukrose akkumulering te bepaal.

SNI en suikers is geëkstraheer uit 'n kommersiële varieteit, N19. Weefsels was volgens ontwikkelingstadiums (internodes 3, 6 en 9) en anatomiese verskille (verryking vir groeiende, vaat- en bergings-weefsels) verdeel. Die laagste sukrose inhoud is in die middel van die onderste helfte van elke internode gevind. Die verhouding van heksoses tot sukrose was die hoogste in die jong internodes. Die inhoud heksoses was hoër in die onderste deel van die internode as die boonste deel. 'n Betekenisvolle korrelasie tussen sukrose inhoud en SNI is gevind. Flukse betrokke by sukrose sintese en hidrolise is ondersoek. Glukose en fruktose is as koolstofbron aan stingelweefsel van twee variëteite (US6656-15 and N19) toegedien. Sukrose-inhoud het tienvoudig tussen volwasse weefsels van die twee variëteite verskil. Hidrolise via invertase was hoër in ouer weefsels van US6656-15 as N19, en het 'n noemenswaardige bydrae tot sukrose-omset gemaak. Die verdeling van sukrose tussen die vakuool en die sitosol kan moontlik 'n groot rol speel in die vermoë van die sel om sukrose te akkumuleer.

Die volgorde van 'n volledige SNI cDNA is bepaal. The uitgedrukte geen het, op beide 'n nukleïen- en aminosuur vlak, betekenisvolle ooreenkoms getoon met ander bekende plant NI volgordes. Die SNI volgorde bevat nie die kenmerkende invertase katalitiese setel nie, wat daarop kan dui dat dit onafhanklik van ander klasse invertases ontwikkel het. Min of meer 1.8 kb van die SNI cDNA is in 'n vektor geskik vir bioliestiese transformering van suikerrietweefsel, geïnkorporeer. Southern klad analise het gewys dat die ensiem 'n lae kopiegetal op geen vlak het. SNI mRNA uitdrukking is waargeneem in elke weefseltipe van die suikerriet plant: wortels, internodes, blaarrol en blare. In stingelweefsels met lae sukrose- en hoë heksose-inhoud, was die vlakke van beide SNI-mRNA en -proteïen hoog. Dit dui daarop dat SNI moontlik betrokke is by groei-metabolisme.

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LIST OF ABBREVIATIONS

°C	degrees centigrade
AI	acid invertase
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt
Bq	Bequerel
bp	nucleic acid base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
^{14}C	radio-labeled carbon
$^{14}CO_2$	radio-labeled carbon dioxide
CB	core bottom of the sugarcane internode
CT	core top of the sugarcane internode
CWI	cell wall invertase
Da	Dalton
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribo nucleic acid
DTT	1,4-dithiothreitol
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
edn.	edition
excl.	excluding
FW	fresh weight
g	gram
xg	gravitational force
G6PDH	glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
gDNA	genomic DNA
h	hour
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1)
HPLC	high performance liquid chromatography
IgG	immunoglobulin G

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J	Joule
K_m	substrate concentration producing half maximal velocity
L	litre
m	meter
M	molar
min	minute
MES	2-[N-morpholino] ethanesulfonic acid
NAD^+	oxidised nicotinamide adenine dinucleotide
NADP	reduced nicotinamide-adenine phosphate dinucleotide
NBT	nitro blue tetrazolium chloride
NI	neutral invertase (β-fructofuranosidase, EC 3.2.1.26)
npt II	gene coding for neomycin phosphotransferase
PAGE	polyacrylamide gel electrophoresis
Pa	Pascal
PB	periphery bottom of the sugarcane internode
PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependant phosphofructokinase (pyrophosphate:D-fructose-6-phophate 1-phosphotransferase, EC 2.7.1.90)
PGI	phosphoglucoisomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)
PT	periphery top of the sugarcane internode
PVPP	polyvinylpolypyrrolidine
RNA	ribonucleic acid
RNaseA	ribonuclease A
rpm	revolutions per minute
SAI	soluble acid invertase (β-fructofuranosidase, EC 3.2.1.26)
SDS	sodium dodecyl sulphate
se	standard error
SNI	sugarcane neutral invertase (β-fructofuranosidase, EC 3.2.1.26)
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)
TBE	tris-borate/EDTA electrophoresis buffer

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TBST	tris-buffered saline containing Tween
TE	tris/EDTA buffer
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate
UV	ultra violet
v	volume
V	Volt
w	weight

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CHAPTER 1

General introduction

Sugarcane is a C4 grass that has the unique ability to accumulate significant quantities of sucrose in the culm tissues (Moore and Maretzki, 1997). This phenomenon makes sugarcane a highly productive crop plant viable for commercial exploitation. In 1999/2000 it was estimated that the South African sugar industry contributed R1.7 billion to the country's foreign exchange earnings (www.sasa.org.za). With approximately 412 000 hectares under cane, South Africa's sugar industry ranked as the world's 13th largest producer and 10th largest exporter of processed sugar (South African Sugar Association Annual report 1997/1998). From these facts, it is evident that the utilisation of sugarcane for sucrose production is advantageous to both the local consumer and export markets.

The improvement of the commercial sugarcane plant (interspecific hybrid of *Saccharum officianarum* and *S. spontaneum*) with regards to pathogen resistance, environmental tolerance and sucrose yield is the focus of many industry-supported research groups worldwide. For many years these goals have been reached by traditional breeding methods. Although these processes have rendered improved plants, the selection procedure is very lengthy and laborious. The natural potential of hybrids produced by manual crossing, with regards to desired traits such as sucrose yield, appears to have reached a threshold. It has also been speculated that the maximum concentration of sucrose in the cell solution has been reached. However, genotypes displaying higher sucrose contents than current commercial varieties are known, leading us to believe that the sucrose concentration has not reached its limit. With this in mind, technologies utilising genetic manipulation can be employed to improve crop yield and performance by introducing traits not obtainable through conventional breeding practices, or improving current traits beyond their present genetic limit.

Correct targeting of areas for genetic manipulation is crucial for the effective production of new plant lines and also for minimisation of nonessential research efforts.

Although sugarcane is widely cultivated for its sucrose yield, many of the biochemical processes involved in the production of sugars (and especially sucrose) are not well understood. Understanding the basis for these processes could lead to further targeted

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improvement of the crop and its sucrose yielding potential. Net sucrose accumulation is determined by synthesis, intracellular partitioning and breakdown rates. Here we will focus on the biochemical aspects of sucrose accumulation, with specific reference to the processes of sucrose breakdown.

Two pathways facilitate the degradation of sucrose. The first cleaves sucrose resulting in UDP-glucose and fructose via the enzyme sucrose synthase (SuSy, UDP-glucose:D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13). The second group of enzymes (invertase, β-fructofuranosidase, EC 3.2.1.26) hydrolyse the disaccharide producing free glucose and fructose. The invertases are classed according to pH optima and include the acid invertases, both soluble and cell wall bound, and neutral invertase (NI).

NI is located in the cytosol. In sugarcane its distribution between tissues has been inconsistently reported in different studies. Little is known of its gene and transcript expression and its absolute function in the plant cell. This lack of knowledge of an enzyme present in the cellular compartment where sucrose is synthesised necessitates further investigation.

In order to resolve the variance observed in metabolic activity, a detailed study of maximum extractable sugarcane neutral invertase (SNI) activities and its correlation with sucrose content was carried out (*chapter 3*). A cDNA coding for the enzyme was sequenced (*chapter 4*) and used to elucidate gene and transcript expression throughout the sugarcane plant (*chapter 5*). The contribution of hydrolysis to net sucrose accumulation was determined in order to further elucidate the mechanisms of sucrose accumulation in commercial sugarcane varieties (*chapter 6*).

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CHAPTER 2

Neutral invertase and sucrose

2.1 Introduction

2.1.1 Primary site of sucrose synthesis and transport

In sugarcane, before assimilated carbon is transported from the source (leaves) to the sink tissues (meristems and culm) it is synthesised to sucrose. This process was confirmed by feeding [^{14}C]-labelled hexoses and observing that the translocated sugar was symmetrically labelled sucrose (Hatch and Glasziou, 1964). Feeding sucrose labelled in the fructose moiety to leaves, resulted in the translocated sugar retaining its asymmetry (Hatch and Glasziou, 1964). This confirmed the theory that sugars are transported as sucrose. A study in 1963 (Hartt et al.) showed that most of the sucrose produced during the day arrived in the stem where a large proportion was stored in rapidly accumulating internodes, with a smaller proportion reaching the more mature internodes. The currently accepted model of sucrose translocation via the phloem is that of mass flow motivated by the turgor potential gradient between the source and sink ends of the phloem route (see Minchin and Thorpe, 1987).

2.2 Sink tissues

Internodal tissue of the sugarcane culm acts as the plant's largest sink site for sucrose (as compared with the growing meristems of leaves and culm). These internodal tissues are comprised of parenchyma tissue interspersed with vascular bundles. The peripheral tissues contain more vascular tissue than the core regions. According to Jacobsen et al. (1992) 75% of the vascular tissue is present in the outer 3% of the internode cross-section. The vascular bundles in the peripheral tissues contain little or no phloem and form a schlerenchymatous boundary around the ground tissue. The vascular tissue in the core regions contain large xylem vessels and phloem. Sucrose is translocated in the phloem to the sink tissues. Once the sucrose has reached the sink via the phloem it can be degraded to its hexose moeities and transported into the storage parenchyma (see Moore and Maretzki, 1997).

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2.2.1 Sucrose synthesis in sink tissues

Sucrose synthesis occurs exclusively in the cytosolic compartment of the cell. There are two routes for the synthesis of sucrose. The first is catalysed by the enzyme sucrose synthase (SuSy; UDP-glucose:D-fructose 2-α-D-glycosyl-transferase, EC 2.4.1.13), the second by sucrose phosphate synthase (SPS; UDP-glucose:D-frucotse-6-P 2-α-D-glucotransferase, EC 2.4.1.14). SPS is regarded as the enzyme that carries the bulk of the responsibility for the synthesis of sucrose in sink tissues (Batta and Singh, 1986; Wendler et al.; 1990, Botha and Black, 2000). In immature internodes both SuSy and SPS contribute to sucrose synthesis, however in mature tissues of sugarcane, SPS activity exceeds SuSy more than three-fold (Botha and Black, 2000). This could be explained by various mechanisms, including that high concentrations of sucrose in mature tissues could favour sucrose cleavage by SuSy. Incubation of internodal tissue slices in a solution containing [^{14}C]-glucose for 3 h, and thereafter extracting and hydrolysing sucrose, showed that in young internodes 70% of the label was present in the glucose moiety of the disaccharide, and in mature internodes this decreased to 50% (Botha and Black, 2000). This could only imply that the relative contribution of SuSy to sucrose synthesis decreases with internode maturity, once again supporting the theory that SPS is the enzyme responsible for the majority of sucrose synthesis in accumulating tissues. Although SPS activity is so high in the mature internodes, it is not the only factor involved in the accumulation of sucrose. It is crucial to note the distinction between the synthesis and accumulation of sucrose, the latter being the difference between synthesis and degradation.

2.2.2 Sucrose degradation

Sucrose can be degraded in the apoplastic space, cytosol or vacuole. Degradation of sucrose renders fructose and either glucose (via invertase hydrolysis) or UDP-glucose (via SuSy). The invertases (β-D-fructofuranosidase, EC 3.2.1.26) are divided into two groups defined by their pH optima. Acid invertases (AI) display maximum activity at pH's ranging from 4 to 5.5. The neutral/alkaline invertases (NI) exhibit maximal activity between pH 7 and 8 (Tymowska-Lalanne and Kreis, 1998).

SuSy has been detected in all tissues of sugarcane (Buczynski et al., 1993). There are two forms of SuSy (SS1 and SS2) coded for by different genes generating proteins whose antibodies do not recognise the other isoform. SuSy is highest at a neutral pH and both isoforms display similar K_m (substrate concentration producing half-maximal

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velocity) values for sucrose and UDP (uridine 5'-diphosphate). Determined K_m's for sucrose were between 3- and 19-fold higher than those for fructose for both isoforms in SS1 and SS2, respectively. Even though SuSy kinetics favour its synthetic activity, in sugarcane where sucrose contents are high, the equilibrium could favour the cleavage reaction.

2.3 Invertase

It is generally accepted that the principal function of invertases is to supply carbohydrates to sink tissues (see Tymowska-lalanne and Kreis, 1998). They have also been attributed with generating a sucrose gradient to aid transport, regulating cell turgor, promoting cell expansion, controlling sugar composition in storage organs and participating in stimulus induced responses.

2.3.1 Metabolic characteristics

The sensitivity of the balance of biochemical reactions in the plant cell is so fine, that when yeast invertases were targeted to either the apoplast, cytoplasm and vacuole the plant could not function normally (Sonnewald et al., 1991). This highlighted the metabolic imbalance brought about by the inclusion of invertase in the wrong cell compartment. Although invertases have been extensively studied (especially AI) there is still a lack of knowledge concerning their absolute function and regulation.

2.3.3.1 Acid invertase

The acid invertases are further divided into soluble and insoluble classes. The soluble form is located in the vacuole and free in the apoplastic space (SAI), the insoluble form bound to the cell wall (CWI)(Tymowska-lalanne and Kreiss, 1998).

Acid invertases have been extensively studied in many plants including (*Ricinus communis* (Vattuone et al., 1983), potato tubers (Trethewey et al., 1998), sugar beet suspension cultures (Masuda et al., 1988), faba bean (Ross et al., 1996) and sugarcane (Batta and Singh, 1986; Zhu et al., 1997, Vorster and Botha, 1999).
Acid invertases are involved in sucrose metabolism (ap Rees, 1984), phloem unloading (Giaquinta, 1979), control of sugar composition in storage organs (Sturm et al., 1995), osmoregulation (Wyse et al., 1986), gravitropism (Wu et al., 1993) and response to pathogens and wounding (Sturm and Chrispeels, 1990). Further it has been suggested

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that the soluble invertases participate in the regulation of hexose levels in mature tissues (Ricardo, 1974).

What seems to be a futile cycle of sucrose synthesis and degradation between cell compartments, facilitated by enzymes including invertases, involves some "wastage" of energy, but allows the net flux to respond very sensitively to factors which modulate the rates of synthesis and degradation (Dancer et al., 1990).

Exploiting genetic manipulation techniques, transgenic carrot lines were generated with varying activities of CWI and SAI. This work showed that invertases were important in the partitioning of carbon into sucrose in early plant development (Tang et al., 1999).

In many plants SAI dominates in unripe storage tissues (Hubbard et al., 1989) and activity decreases as sucrose content increases. It would thus seem apparent that relative acid invertase activity would give us an indication of sucrose content.

In sugarcane, SAI has been the subject of many studies, perhaps because of its location in the storage compartment of sucrose. As seen between studies, reported activities vary in both order of magnitude and distribution through the cane (*table 2.1*). Despite these differences, acid invertase activity has been correlated negatively with sucrose content in internodal tissues (Ebrahim et al., 1998), implicating it as an important determinant in the cycle of sucrose accumulation.

2.3.1.2 Neutral invertase

Neutral/alkaline invertases are located in the cytosol (Masuda et al., 1988). The distinction between neutral and alkaline invertase is also based on pH optima. In many studies they are viewed as the same enzyme. However, the purification of two soluble invertases with pH optima of 6.8 (neutral invertase) and 8 (alkaline invertase) from carrot was observed by Lee and Sturm (1996). It was concluded that the two isoforms may be encoded by the same gene and generated by differential splicing or proteolytic cleavage. To date only one isozyme has been identified in sugarcane. Sugarcane neutral invertase (SNI) has been the focus of this thesis.

NI has been studied but not as extensively as its acid counterparts. The relationship between the two types of invertases is not clear. However, their different cellular

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compartmentation and particular biochemical properties they both potentially contribute to regulation of sucrose metabolism, transport and storage (Chen and Black, 1992).

It has been suggested that NI activity compensates for low AI activity in certain tissues (Ricardo and ap Rees, 1970). The distribution of NI between stages of ripening tissues differs from species to species. In faba bean (Ross et al., 1996) SuSy is the predominant sucrose degrading enzyme in young tissues. As the tissue develops SuSy activity declines and NI activity plays an ever-increasing role. In fruit tissues of Musk melon (Hubbard et al., 1989) and root nodules of soy beans (Morell and Copeland, 1984) NI activity remains the same as the tissues develop.

So the pressing questions are what the relative importance of neutral and acid invertases are, and how crucial a role does NI play in sucrose metabolism?

Physical attributes

NI isolated from carrot (Lee and Sturm, 1996) had a molecular mass of 456 kDa with subunits of 57 kDa. Ross et al. (1996) isolated a homotetramer of 238±45 kDa from Faba bean.

The pH optimum for NI is within the range of 6.8 and 8. The carrot NI has a pH optimum of 8 (Lee and Sturm, 1996), that of Soy bean nodules has highest activity at pH 7.6 (Morell and Copeland, 1984) and from Faba bean is optimal at pH 7.4 (Ross et al., 1996).

Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) and fructose are reported as major inhibitors (Lee and Sturm, 1996; Ross et al., 1996; Morell and Copeland, 1984). In carrots (Lee and Sturm, 1996) $CuSO_4$ acts as an inhibitor, and in Soybean nodules (Morell and Copeland, 1984) $CaCl_2$ and $MgCl_2$.

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2.3.1.3 *Sugarcane neutral invertase*

According to experimental work by Del Rosario and Santisopasri (1977), NI in sugarcane is present as a monomer (15kDa), dimer (35 kDa), tetramer (66 kDa) and decamer (160 kDa) (table 2). Sugarcane neutral invertase (SNI) activity has been detected in 240 kDa, 120 kDa and 60 kDa forms (Vorster and Botha, 1998). In a further study by Vorster and Botha (1999), SNI was purified and molecular weights of subunits determined with the use of specific antibodies on denatured protein blots rendering a single cross-reacting polypeptide of approximately 58 kDa. The smaller polypeptides reported by Del Rosario and Santisopasri (1977) could just be hydrolysis products of the 58 kDa monomer isolated by Vorster and Botha (1998 and 1999).

Various pH optima have been reported in recent literature with reference to SNI. The range (pH 7 to pH 7.5) is narrower than that reported for other plants. In literature reviewed here, the following pH optima were used in SNI experimental assays: 7.0 (Del Rosario and Santisopasri, 1977; Vorster and Botha, 1999), 7.1 (Batta and Singh, 1986), 7.2 (Vorster and Botha, 1998) and 7.5 (Lingle, 1997; Zhu et al., 1997).

In the study of Del Rosario and Santisopasri (1977), lauryl sulphate and metasilicate were observed as invertase inhibitors, possibly useful for addition to crusher juice to avoid sucrose breakdown after milling. As in studies with other plants, Tris was an effective inhibitor of SNI (Vorster and Botha, 1998). SNI is also affected by feedback inhibition from its products glucose and fructose, although fructose is a more effective inhibitor. The metal ions Hg^{2+}, Zn^{2+}, Ag^{+} and Cu^{2+} exercised significant inhibition on SNI and partial inhibition by Co^{2+}(Vorster and Botha, 1998).

Successful partial purification of SNI has been achieved by sequential ammonium sulphate precipitation (the majority of SNI precipitated between 20 and 60%) and anion exchange chromatography (Vorster and Botha, 1998). SNI displayed typical Michaelis-Menton kinetics (Vorster and Botha, 1998). Other reported kinetic properties include K_m and V_{max} (maximum reaction rate) values (*table 2.2*).

As in other plants, SNI is a soluble enzyme located in the cytosol (Vorster and Botha, 1998). In sugarcane varieties with high sucrose storing ability, SNI activities were reported as being low in meristematic tissues and increasing in the sucrose storing tissues (Hatch and Glasziou, 1963; Batta and Singh, 1986). In contrast, Lingle and Smith (1991) found that the highest SNI activities were found in youngest internodes.

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This profile of SNI activity has been confirmed in subsequent publications (Zhu et al., 1997; Ebrahim et al., 1998; Vorster and Botha, 1999, Rose and Botha, 2000) *(table 2.1)*. It was also found that SNI activity was highly variable among internodes of the same age and between sampling dates (Lingle, 1997). Within the same internode SAI activities were higher in early development, but as the plant matured the SNI took a slightly more dominant role.

What has come of Lingle's studies is that a high AI activity is correlated with rapid growth, perhaps as AI frees substrates for energy producing biochemical pathways as well as for increasing cell turgor. It is reported that there is a negative relationship between sucrose concentration and AI activity levels (Zhu et al., 1997; Ebrahim et al., 1998). High sucrose accumulation, is however not a direct result of low AI levels. Additional factors play a role in the eventual accumulation of sucrose in the sugarcane culm (Zhu et al., 1997).

In contrast, there are conflicting reports of SNI activity levels in relationship with any free sugar in sugarcane storage tissue. It has been suggested that the fragmented data could be because SNI activity actually has nothing to do with sucrose levels, or that the extractable activity does not reflect the *in vivo* activity (Vorster and Botha, 1999). In recent years, extraction and assay procedures seem to have been standardised which makes data generated more comparable. Early extraction protocols did not always include protease inhibitors (Hatch and Glasziou, 1963) and used lengthy dialysis steps for desalting extracts (Batta and Singh, 1986). These could have contributed greatly to a loss, and therefore an incorrect calculation of maximum potential activity. SNI activity was variable between individual plants (Rose and Botha, 2000), and if sampling methods are not correctly chosen, erroneous data can be produced.

In light of the differences in reported data, it was necessary to further confirm SNI activity patterns, and attempt to determine the function of NI in the accumulation of sucrose in sugarcane. In this study the expression of SNI activity is reported within the sugarcane culm (*chapters 3 and 5*). The impact of hydrolysis on total sucrose accumulation was also determined (*chapter 6*).

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Table 2.1. Sugarcane internodal soluble invertase and sucrose profiles

Variety and internode	*In vitro* NI activity		*In vitro* SAI activity		Sucrose		Reference and comments
	nmol.min^{-1}.g FW^{-1}	nmol.min^{-1}.mg protein	nmol.min^{-1}.g FW^{-1}	nmol.min^{-1}.mg protein	µmol.g FW^{-1}	µmol. mg protein^{-1}	
Pindar							Hatch and Glasziou (1963)
2	-	0	21.67	-	-	-	
3	-	0.17	0	-	-	-	
6	-	1.5	0	-	-	-	
CoJ 64							Batta and Singh (1986)
immature	(0.6) 1.3	-	(1.0) -28	-	(36) -500	-	Values in parenthesis are
maturing	(2.0) 3	-	(0.5)-2.4	-	(80) -550	-	from the beginning of the
internode	(3.0) -9	-	(0.3)-2.1	-	(200) -551	-	growing season and those in maturing stage
CP70-321							Lingle and Smith (1991)
1	-	0-417	-	167-417	-	0	Values represent bracket of
2	-	67-250	-	125-383	-	0	variation between three
3	-	167-167	-	190-480	-	0	seasons. Plant material was
4	-	50-167	-	150-400	-	15-220	glasshouse grown.
5	-	33-167	-	10-40	-	85-460	BSA (bovine serum albumin)
6	-	83-250	-	10-400	-	160-720	protein standard
7	-	17-333	-	10-440	-	280-930	
8	-	83-300	-	130-200	-	490-1000	
Mol 5829 (LA Purple)							Zhu et al. (1997) Mol 5829 is a low-sucrose
2	-	4 (4)	-	340 (40)	10 (40)	-	variety, LA purple is a high-
3	-	13 (13)	-	130 (10)	25 (150)	-	sucrose. BSA protein
5	-	17 (17)	-	0 (0)	20 (200)	-	standard
7		21 (21)	-	0 (0)	22 (290)	-	
9	-	4 (25)	-	0 (0)	50 (350)	-	
11	-	9 (20)	-	0 (0)	40 (480)	-	

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Variety and internode	*In vitro* NI activity		*In vitro* SAI activity		Sucrose		Reference and comments
	nmol.min^{-1}.g FW^{-1}	nmol.min^{-1}.mg protein	nmol.min^{-1}.g FW^{-1}	nmol.min^{-1}.mg protein	µmol.g FW^{-1}	µmol. mg protein^{-1}	
Nco 376							Vorster and Botha (1999)
3	30	14	12	6	-	8.2	
4	31	22	13	9	-	15.8	
5	27	21	14	10	-	25.9	
6	25	20	10	8	-	51.8	
7	22	17	9.5	7	-	73.1	
9	25	16	9	6	-	102.8	
10	20	15	10	7	-	106.5	
N19							Rose and Botha (2000)
3	-	28 (23)	-	-	-	30 (35)	Sampled from bottom and top
6	-	26 (14)	-	-	-	180 (160)	(in parenthesis) of high-
9	-	16 (16)	-	-	-	200 (200)	sucrose variety internodes.

(-) not reported

Table 2.2. Sugarcane invertase kinetic properties as determined in various studies

Cane Variety	Invertase type	K_m (suc) MM	V_{max} (suc) μmol.min^{-1}.mg protein^{-1}	M_r (KDa)	pH optimum	Reference
CoJ 64	Soluble Acid	45	0.065		5.5	Batta et al. (1991)
CAC 57-11	Acid	2.78	0.044	380		Del Rosario and Santisopasri (1977)
CAC 57-50	Neutral	0.32	0.0365	dimer-35		
Phil 56-226				tetramer-66		
				decamer-160		
Nco 376	Neutral	9.8	0.439	Monomer-60	7.2	Vorster and Botha (1998)
				Dimer- 120		
				Tetramer-240		

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2.3.2 Molecular characteristics

2.3.2.1 Acid invertase

Until recently, all studies examining the role and distribution of invertases have been done using metabolic techniques (e.g. enzyme activity and protein blotting assays). In recent years, many genes encoding SAI and CWI, and to a lesser degree NI, have been isolated from various plants making studies of these enzymes' gene and transcript expression possible.

In plant species, many small, multigene families encoding for AI have been identified. By 1998, AI genes (both soluble and cell wall bound) had been identified in at least 12 plant species including Arabidopsis, carrot, tomato, maize, mung bean, potato, wheat, tobacco, pea, fava bean and rice. These sequences contain approximately seven exons and six introns (Tymowska-Lalanne and Kreis, 1998). Comparison of SAI (or CWI) amino acid sequences between species shows a higher degree of homology than comparing SAI and CWI sequences in the same species. This indicates that these two acid invertases belong to different classes.

AI genes contain short regulatory sequences upstream of the promoter region. Some of these regions show homology to auxin-inducible, ethylene-responsive, wound-response, stress-response and bZIP-binding elements (Tymowska-lalanne and Kreis, 1998). This supports previous metabolic work indicating stimulus response in AI enzyme activity levels.

Distinguishing features of AI amino acid sequences include an N-terminus located NDPN$^{G}/_{A}$ motif. Enzymes containing this motif are glycosyl hydrolases (Sturm and Chrispeels,1990). Another highly conserved motif occurs in the region thought to be the catalytic site and is the cysteine residue in the motif WEC$^{V}/_{P}$DF (Martin et al., 1987). A third motif conserved between plants and yeast is a FRDPTT sequence. AI is synthesised as a pre-protein with a cell compartment directed signal peptide and a short N-terminal leader sequence (Sturm and Chrispeels, 1990).

The isolation of specific gene sequences allows for investigation of differential expression patterns of transcripts throughout a plant. In carrot, a detailed analysis of transcript expression of AI in different tissues was facilitated by using three genes encoding for CWI and two for SAI (Unger et al., 1994; Lorenz et al., 1995). In young

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growing plantlets, one of the CWI clones was primarily expressed in growing meristematic tissues. In plants with developing tap roots this gene's expression was only detected in low levels in the leaves. The other two CWI transcripts were only detected in vegetative organs, one of which was flower specific. Both SAI transcripts were identified in root tissues in high levels. Other identified sequences used for similar studies have been isolated from potato, tomato and fava bean (Tymowska-lalanne and Kreis, 1998). All of these studies show how the various forms of AI are expressed in different parts of the plant, allowing for their different functions to be fulfilled effectively.

Sugarcane SAI sequences and use in manipulation of invertase levels

In sugarcane two invertase cDNAs coding for SAI were cloned from two different varieties (Zhu et al., 2000). Both showed homology to other plant AI sequences and were 98% similar to each other. It was therefore hypothesised, as seen in other plant species, that SAI in sugarcane are coded for by a multigene family. Steady state levels of SAI transcripts decreased with an increase in internode maturity. In a low-sucrose storing variety the absolute transcript levels of SAI were higher than a high-sucrose storing variety, even though their expression patterns across tissues of different maturity were the same. In culm tissues, transcript levels were mimicked in SAI protein contents determined by protein blots. SAI enzyme activities have been negatively correlated with sucrose content in sugarcane internodal tissues (Zhu et al., 1997; Ebrahim et al. 1998) and therefore their significance as targets for manipulation was established.

After cloning these two SAI genes sugarcane was transformed with antisense constructs. Simultaneously they transformed sugarcane with a yeast invertase targeted to the cytoplasm, apoplast and and vacuole (Ma et al., 2000). Changes in metabolism were observed in a callus/liquid culture system. As expected, an increase in apoplastic invertase caused a concurrent increase in hexoses in the medium and a decrease of sucrose. Increasing cytoplasmic invertase activity caused a significant decrease in sucrose in the cell. The cell line with decreased SAI showed a doubling in sucrose levels. The researchers concluded that sugar composition both inside and outside the cell are dependent on invertase levels. Most important was the decrease in SAI causing an increase in sucrose content.

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2.3.2.2 Neutral invertase

Two full-length cDNA sequences have been identified for cytoplasmic (alkaline/neutral) invertases in plants. The first was a clone isolated from poison rye grass (*Lolium temulentum,* EMBL database accession no AJ003114, Gallagher and Pollock, 1998), the second from carrot (*Daucus carota,* EMBL database accession no. Y16262, Sturm et al., 1999).

Partial sequences, reported on international databases, showing homology to these two sequences were only retrieved from plants and photosynthesising bacteria (Sturm et al., 1999). Deduced amino acid sequences from carrot and poison rye grass shared a 71% similarity. And although there is high homology between different isoforms of AI, there is very little between classes of acid and neutral sequences. The pentapeptide NDPNG, identified as a critically conserved sequence for activity in AI, was not found in the carrot clone. However, the first four peptides of this motif were identified close to the N-terminus of the poison rye grass clone. It was therefore hypothesised that this sequence is not essential for the function of NI. One other major difference between the *D. carota* and *L. temulentum* neutral invertases was an additional 49 amino acid sequence present in the former after the initiation residue. This variation may be because the carrot ORF does not begin at the first methionine residue, or that its mature protein is produced by post-translational processing.

The functionality of proteins coded for by these sequences were confirmed by expression in *Escherichia coli* and subsequent metabolic analyses. Both displayed sucrose hydrolytic activity in a neutral pH range. Their specificity for sucrose as a substrate along with their novel sequence (when compared to AI) has led to the hypothesis that NI developed independently of other sucrose degrading enzymes.

To date there is no sequence available for a cytoplasmic invertase from sugarcane. The nature of the enzyme, in view of its involvement in sucrose metabolism, demands further investigation into its molecular characteristics. In this thesis we report the first full-length cDNA sequence for SNI (*chapter 4*) and its gene and transcript expression throughout the plant (*chapter 5*).

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CHAPTER 3

Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues

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Abstract

Neutral invertase (NI, EC 3.2.1.26) and sugars were extracted from the developing culm tissues of sugarcane. Tissues were divided according to developmental stage (internodes 3, 6 and 9) and anatomical differentiation (enriching for elongating, vascular or storage tissues). The lowest sucrose content was found in the core of the bottom of each of the internodes. The ratio between the two hexoses, glucose and fructose, and sucrose was highest in the young internodes and was also significantly different between the top and bottom parts of the young internodes. There was a significant negative correlation between sucrose content and NI and this was largely due to a tighter association between the two components in the bottom of the internodes.

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3.1 Introduction

Sucrose yield from the culm of sugarcane is dependent on two interlinked processes; plant biomass production and sucrose concentration (Ebrahim et al, 1998). Growth and storage compete for substrates and energy (Hatch and Glasziou, 1963) requiring carefully controlled partitioning into these pathways. Sucrose accumulation begins in internodes during the stage of elongation and continues long after elongation ceases. Under the right conditions commercial sugarcane varieties have the capacity to store up to 25 % of their fresh weight as sucrose (Moore and Maretzki, 1997).

The ability to accumulate such high concentrations of sucrose in storage parenchyma is the net result of sucrose synthesis and breakdown. Two separate enzymatic pathways in the cytosol (catalysed either by sucrose phosphate synthase (UDP-glucose: D-fructose-6-P 2-α-D-glucotransferase, EC 2.4.1.14) or sucrose synthase (UDP-glucose: D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13)) facilitate sucrose synthesis. Although sucrose synthesis is exclusively a cytosolic phenomenon, the degradation of sucrose can occur in the vacuole, on the cell wall, in the apoplast or in the cytosol. Vacuolar acid invertase (β-D-fructofuranosidase, E.C. 3.2.1.26), cell wall acid invertase, apoplastic space acid invertase and neutral invertase (NI), or SuSy facilitate these catabolic reactions respectively. The various sub-cellular locations of these enzymes could allow for greater control of sucrose metabolism, translocation and storage (Lee and Sturm, 1996).

Sucrose is constitutively present in the cytosol due to this being the site of synthesis and the compartment mediating inter-organellar and inter-cellular transport. Thus enzymes involved in sucrose breakdown in this compartment, NI and SuSy will be of importance in the control of sucrose accumulation and utilisation. The role of neither of these enzymes in sucrose accumulation in sugarcane is well understood.

Early work on sugarcane (Hatch et al., 1963; Batta and Singh, 1986) reported an increase in SNI activity (on a fresh weight basis) with increased maturity of internode (i.e. older internodes on a single culm will have higher SNI activity). Based on this it has been suggested that SNI regulates sucrose movement from vascular to storage tissue in mature internodes (Hatch et al., 1963), or that it is involved in the turnover of hexoses in mature tissues (Gayler and Glasziou, 1972). However, subsequent studies have reported an almost opposite distribution of activity (both on a fresh weight and protein basis) with SNI increasing slightly up to the fifth internode followed by a steady

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decline in activity as internode maturity increases (Hatch et al., 1963; Lingle, 1997; Ebrahim et al., 1998; Vorster and Botha, 1999)

These discrepancies could be a result of enzyme inactivation during extraction or measurement, or due to sampling techniques. In the past, most studies in sugarcane culm sucrose metabolism have focussed on trends formed by data collected from entire internodal tissue. The sugarcane internode is made up of differentiated cell types (Deerr, 1921). In young, actively growing internodes, biomass production occurs in the bottom region of the internode. Apart from the differentiation between top and bottom of the internode, striking differences are evident between core and peripheral tissues. The peripheral tissues consist of a high number of vascular bundles interspersed in thick-walled ground tissue. This is contrasted by a larger proportion of storage parenchyma tissue in the core with less vascular tissue. It is therefore conceivable that the metabolism in these different parts of the internodes will be adapted to specific functions.

To investigate the potential role of SNI in more detail we examined the SNI activity profiles and SNI content, and sugar levels in these differing tissue types. For this purpose we distinguished four sections of the internode: core bottom and top, periphery bottom and top. All four sections were analysed in three internodes of increasing maturity.

Here we report a significant variation in SNI expression and sucrose content within sugarcane internodal tissue. In addition, we find a negative correlation between NI activity and sucrose, especially in the bottom part of the internode where active growth is occurring.

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3.2 Materials and methods

3.2.1 Plant materials

Plant material of 18-month-old field grown sugarcane of the commercial variety N19 was used. Culms were harvested in late winter at 10:00 h. Three internodes were sampled to represent immature (internode 3), maturing (internode 6) and mature (internode 9) culm tissue. Internode one is defined as the internode from which the leaf with the first exposed dewlap originates. Each internode was divided into four samples according to tissue type: core bottom, core top, periphery bottom and periphery top. In cross-section the core is defined as an inner-circle with a 5mm diameter. The peripheral section is the outer 3mm of tissue inside the rind. Longitudinally the internode was divided in half, rendering the top and bottom sections. All tissue was frozen in liquid nitrogen and stored at –80 °C within an hour of harvest.

3.2.2 Neutral invertase extraction

SNI was extracted using a modification of a method previously described (Vorster and Botha, 1999). Immediately after harvest the specific tissue samples were ground in liquid nitrogen and stored at –80 °C until extraction. The frozen tissue powder was weighed off and ice-cold extraction buffer was added in a 2:1 (v/w) ratio. The extraction buffer contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA (ethylenediaminetetraacetic acid), 10 mM dithiothreitol (DTT), 0.5 mM Pefabloc SC (4-(2-Aminoethyl)-benzenesulfonyl fluoride, hydrochloride), 10 % (v/v) glycerol and 2 % (v/v) insoluble polyvinylpolypyrrolidine (PVPP). After vortexing, the slurry centrifuged at 10 000 xg for 15 min at 4 °C. The supernatant was immediately desalted on a Sephadex G-25 (Pharmacia PD-10) column equilibrated with desalting buffer (100 mM sodium phosphate buffer (pH 7), 1 mM EDTA, 10 % (v/v) glycerol). Aliquots of the eluate containing proteins were rapidly frozen in liquid nitrogen and stored at –80 °C for protein determinations and protein blots.

3.3.3 Neutral invertase assay

Desalted protein extracts were assayed at 30 °C in a final volume of 1 mL. The final assay contained 50 mM Hepes (pH 7.0), 125 mM sucrose (Vorster and Botha, 1999). Preliminary experiments indicated that the reaction velocity under these conditions was linear for at least 6 h. The assay was stopped after a 2 h incubation period by boiling

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for 2 min. All aliquots were then stored at –20 °C for determination of reducing sugars. An enzyme coupled reaction based on the method described by Huber and Akazawa (1986) was used. The assay was scaled down for use in microtiter plate format in a final volume of 250 μL. Reduction of NAD^+ was measured using a plate reader (PowerwaveX, Biotek Instruments Inc.).

3.3.4 Sugar determinations

Sugars were extracted from aliquots of frozen tissues in 70 % (v/v) ethanol (1/10, w/v) by incubating at 65 °C overnight. The samples were then centrifuged at 10 000 xg, for 10 min at room temperature. The supernatant was dried under vacuum and resuspended in 500 μL 10 % (v/v) isopropanol. For sucrose determinations all samples were diluted 10 times. Sucrose, glucose and fructose concentrations were determined using the enzymatic method described by Bergmeyer and Bernt (1974).

3.3.5 SDS PAGE and protein blotting

Polypeptides in the desalted protein extracts were separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The samples were resolved on discontinuous 12 % (m/v) polyacrylamide (acrylamide/bis-acrylamide, 37.5/1) gels with 4 % (m/v) stacking gel (Laemmli, 1970). Samples were loaded such that each lane contained the proteins equivalent to 1.3 mg FW. Preliminary experiments revealed that a linear response between protein loaded and specific SNI signal on the protein blots was obtained in a range of 0 to 5 mg FW. The resolved polypeptides were transferred to a nitrocellulose membrane, Hybond™-C Extra (Amersham Life Science Ltd., Buckinghamshire) using the Trans-Blot® SD system (Bio-Rad). The proteins were transferred at 12 V for 1 h, using ice-cold transfer buffer consisting of 48 mM Tris, 39 mM glycine , 0.0375 % (w/v) SDS and 20 % (v/v) methanol. All subsequent steps were performed at room temperature. The membranes were blocked in TBST (Tris-buffered saline buffer containing Tween, 137 mM NaCl, 20 mM Tris, 0.1 % (v/v) Tween, pH 7.6) containing 3 % (w/v) BSA for 2 h. Primary antibody (*Beta vulgaris* neutral invertase rabbit polyclonal antibody) was added to the blocking buffer to a final concentration of 1:1000, and binding proceeded overnight at room temperature. Blots were washed three times for 10 min in TBST. The secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG) was bound to the blots in a 1: 2 500 dilution in TBST for 1 h. The blots were rinsed once, and washed twice for 10 min in TBST, followed by a 10 min wash with TBST containing 0.1 % (w/v) SDS and two further 10 min washes with TBST. Cross-reacting polypeptides stained after reaction with alkaline

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phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (BCIP). The dephosphorylation reaction product reacted further with Nitro blue tetrazolium chloride (NBT) giving a dark-blue indigo-dye product. NBT/BCIP ready-to-use tablets (Boehringer Mannheim) were used for this stain. Protein concentrations were determined by using gamma-globulin as a standard (Bradford, 1976).

3.4 Results

We have previously shown that there is no significant difference between protein content expressed on a cell basis between internodes (Bindon and Botha, 2000). This is also consistent with previous findings that the protein content per internode remains constant (Botha et al., 1996). All data in this study were therefore compared on a soluble protein basis to compensate for changes in the volume of the cytosolic (metabolic) compartment.

3.4.1 Sugars

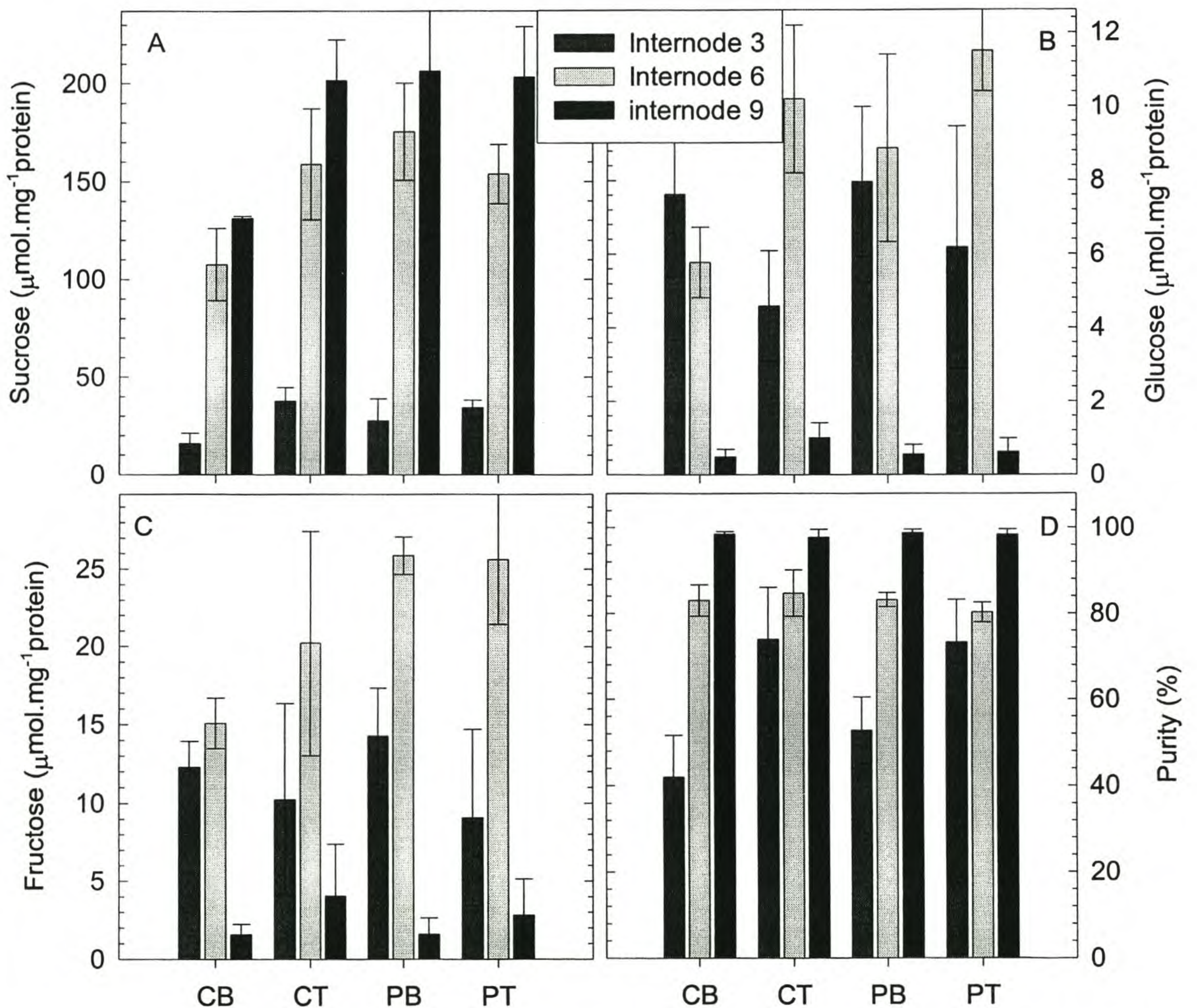

Figure 3.1 Sugar content was determined from sugarcane internodal tissue. Three internodes representing young (3), maturing (6) and mature (9) tissues were sampled. Internodes were sectioned into core bottom (CB), core top (CT), periphery bottom (PB) and periphery top (PT). Purity is the ratio between sucrose and reducing sugars. Data points are the mean of at least three samples ± se.

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There is a sharp gradient in sucrose content between internodes 3 and 6 in all four the tissue types examined (*figure 1A*). Although the sucrose content further increases between internodes 6 and 9 this change is only significant in the peripheral top tissue. It is also evident that the sucrose content of the core bottom tissue is consistently lower than in the other three tissue types. Fructose increases between internodes 3 and 6 in all the tissues (*figure 1B*), and glucose only in the top tissue from both the core and periphery (*figure 1C*). Both the hexoses sharply decrease between internodes 6 and 9 (*figure 1B and C*). Both reducing sugars are present in lower amounts in the core bottom tissue in internode 6. The purity (molar ratio between sucrose and the total sugar pool (including glucose, fructose and sucrose)) sharply increases in the bottom of the internode between internodes 3 and 9. The increase in purity in the top of the internodes is much less, as the purity in the young internode 3 tissue is already significantly higher than that in the bottom of the internode (*figure 1D*).

3.4.2 Neutral invertase

There are major differences in the neutral invertase activity in the four tissues as well as in expression patterns within each tissue (*figure 2A*). In the bottom of the internode the specific activity of NI decreases by 43 % between internodes 3 and 9. This is largely due to a sharp drop in the NI activity between internode 6 and 9. A similar decrease activity (approximately 30 %) is evident in the periphery of the internode except that in this region the large decrease occurs between internodes 3 and 6.

To determine whether the measured NI activity truly reflects the amount of NI protein a range of extracts were blotted and probed with a specific NI antibody (Vorster and Botha, 1999). In all four the tissue types a single cross-reacting polypeptide of 58 kDa was detected (*figure 2C*). Linearity of the immunological reaction was verified by loading a range of activities (0.048 $nmol.min^{-1}$ to 2.317 $nmol.min^{-1}$) and probing with the antibody. Under the conditions used a linear response was evident 0.048 $nmol.min^{-1}$ and 0.386 $nmol.min^{-1}$ of NI activity (not shown). In all the extracts tested a highly significant (P=0.001) linear correlation between enzyme activity and amount of NI activity was found (*figure 2B*). In addition, we could find no evidence for the presence of any invertase activators or inhibitors (not shown).

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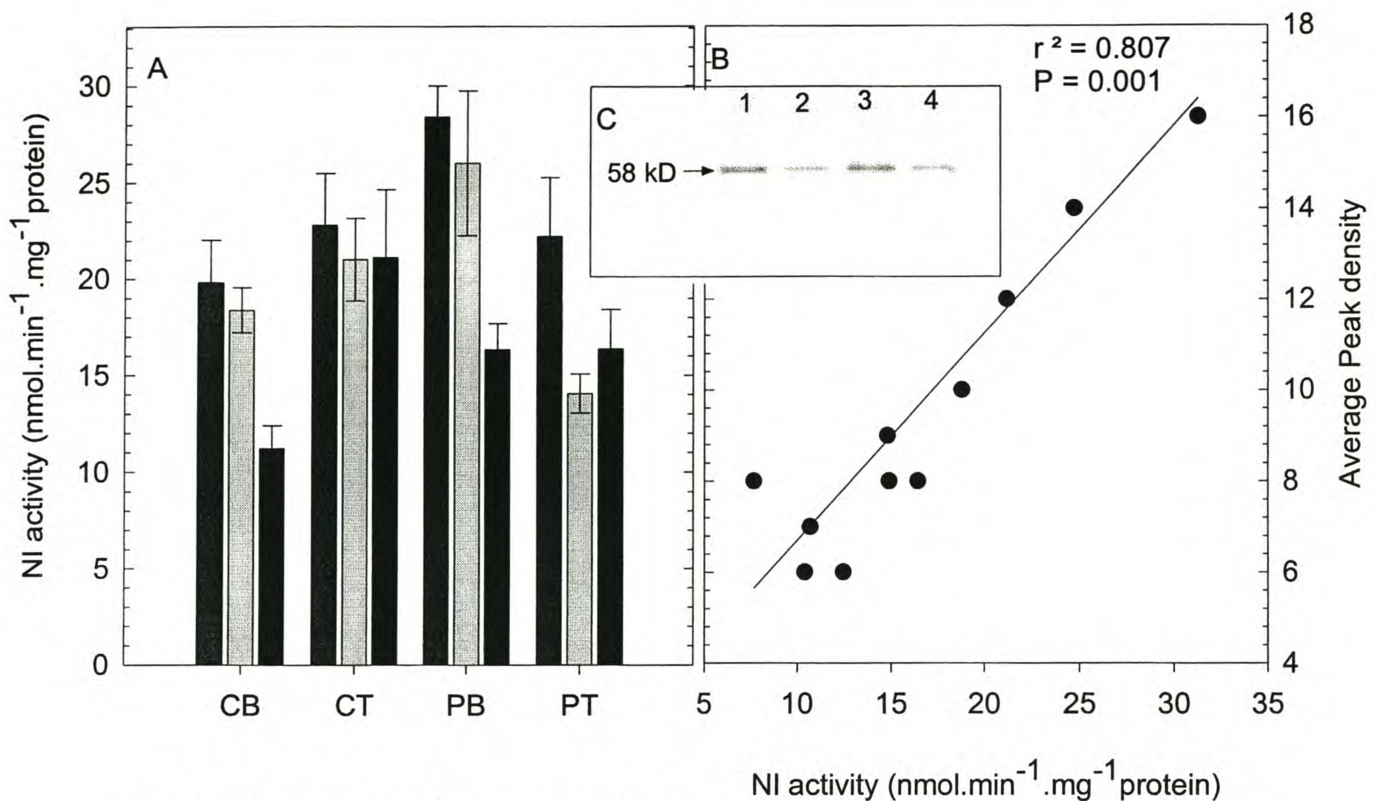

Figure 3.2 Neutral invertase activity from internodes 3, 6 and 9, and four tissue sections (core bottom and top, and periphery bottom and top) (A). Relationship between the NI activity and NI concentration (B) and the specificity of the NI antibody on a protein blot of total soluble proteins extracted from the four sections of internode 3(C). Data points are the mean of at least three samples ± se.

Over all the data points collected there was a significant correlation (P=0.0109) between NI activity and sucrose content (*figure 3A*). However, it is evident that this is a very weak association and that within a very narrow range of sucrose content large variation in NI activity can be found. This is largely due to the differences observed in the correlation within each tissue type. The correlation is much tighter in the bottom of the internodes, both core and periphery tissue (*figure 3B and D*), than in the top of the internodes (*figure 3C and E*). It is important to note that the largest change in purity occurs in the bottom of the internode during maturation (*figure 1D*). The tightest correlation between NI activity and purity therefore exists in the periphery of the bottom of the internodes (P=0.062).

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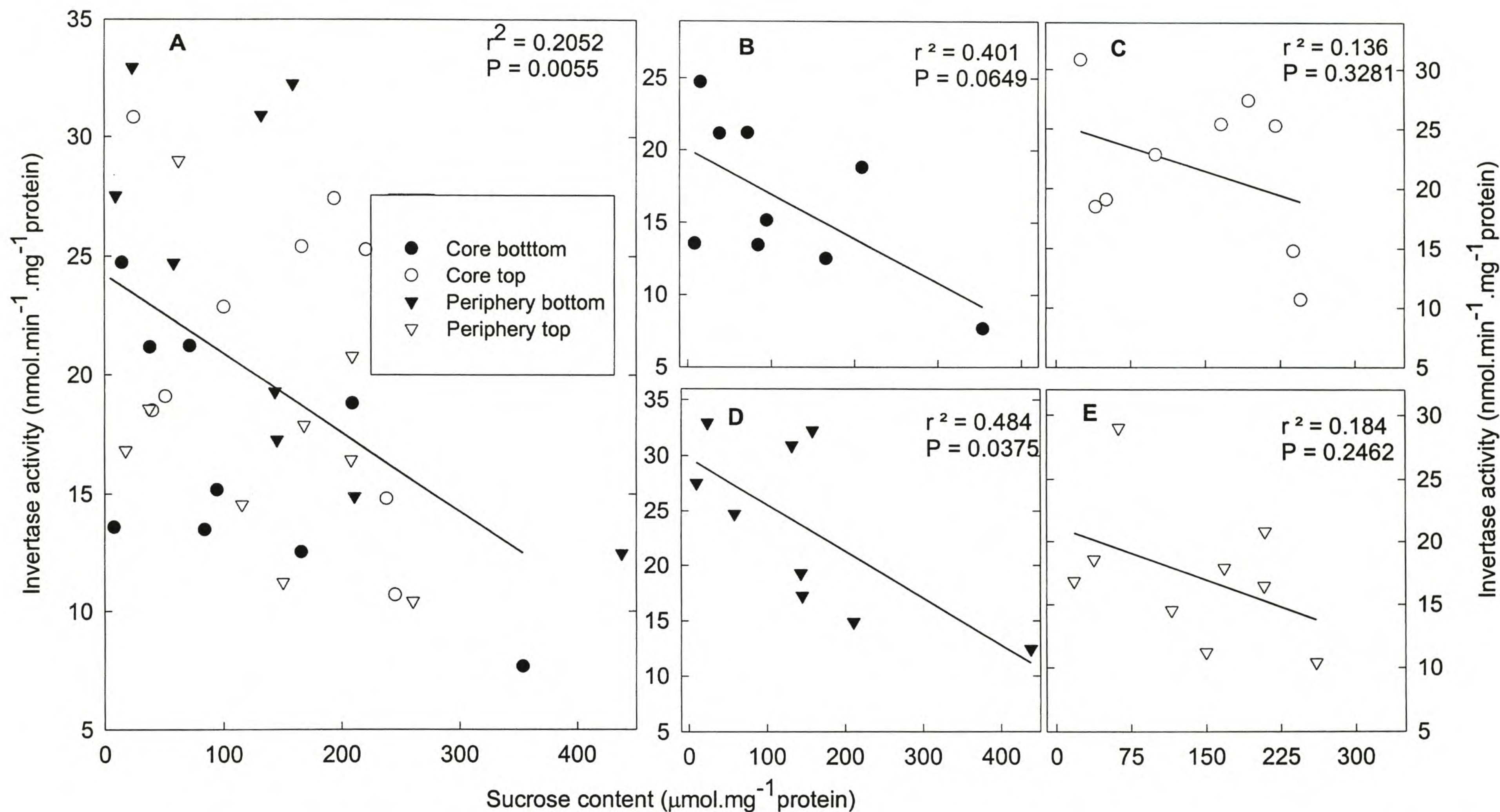

Figure 3.3 Correlation between neutral invertase activity (nmol.min^{-1}.mg^{-1}protein) and sucrose content (µmol.mg^{-1}protein). (A) Data collected from three stools, three internodes and four tissue types. Correlation between NI activity and sucrose for core bottom (B), core top (C), periphery bottom (D) and periphery top (E), respectively. Data points are the mean of at least three samples ± se.

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3.4 Discussion

The data presented here clearly emphasise the variation that occurs in sugar content, sugar composition and NI activity within sugarcane internodes. This variation highlights the difficulties involved in attempting to obtain representative samples of internode tissue for biochemical studies.

The low sucrose content in the core of the bottom of the internode is consistent with the hypothesis that this region of the internode is metabolically more active in non-sucrose storing metabolism (e.g. processes involving respiration and growth). In fact, this is evident even in the more mature internode 9 tissue that already accumulates high sucrose levels (>11 % on a fresh mass basis) but still growing. In support of this the purity in the very young internodal tissue is also significantly lower in the bottom of the internode exhibiting a high hexose content.

NI activity does not increase with maturation in any of the tissues studied. The tight linear correlation between NI activity and the invertase protein concentration illustrates that we have not inactivated or underestimated the enzyme activity in any of the tissues studied. Based on this result, as well as the data from other studies (Lingle, 1997; Zhu et al., 1997; Vorster and Botha, 1999) the increase that was reported by others (Hatch et al., 1963; Batta and Singh, 1986) must be questioned. The activity reported in the latter two papers are at least an order of magnitude lower than that found by others. The method of pressing the juice out followed by dialysis could be largely responsible for the low values reported by Hatch and Glasziou (Hatch and Glasziou, 1963). In our studies we have found that for a number of enzymes protection against oxidation and proteolytic degradation is required during extraction in especially the young internodes.

The NI activity in all the tissues of N19 largely exceed the sucrose accumulation rate previously observed in this variety (Botha and Black, 2000). The specific NI activity in internodes 3 and 6 is also higher than that of SPS and similar to SuSy (Botha and Black, 2000). However, sugarcane NI could be regulated by metabolites such as fructose (Vorster and Botha, 1998) and *in vitro* activities might therefore largely overestimate the activity of the enzyme *in vivo*.

The very weak correlation between sucrose content, and even purity, and NI activity is not surprising. Sucrose accumulation is a complex process and reflects the difference between the synthesis and breakdown activities. NI is but one of the two possible rates

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of breakdown activities in the cytosol. In addition the rate at which sucrose is removed from the cytosolic compartment into the vacuole could also be important. Similarly, free hexoses in the cytosol form part of a very active metabolic pool that turns over rapidly (Whittaker and Botha, 1997) and therefore does not accurately reflect production. In view of this complexity one would at best expect the contribution of one enzyme to sucrose accumulation to be small.

However, the data clearly show that invertase is not expressed to the same level in all tissues. The fact that the expression of NI activity in all the tissue types decreases with maturation is also not a mere reflection of a general down regulation of metabolic activity as some other enzymes, like SPS and PFP (Whittaker and Botha, 1999; Botha and Black, 2000), are up regulated and PFK (Whittaker and Botha, 1999) remains constant. We are currently investigating *in vivo* invertase activity in the same tissues as used for this investigation to gain better insight into the contribution of the invertases to total sucrose hydrolysis in an intact cell environment.

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CHAPTER 4

Characterisation of a sugarcane neutral invertase cDNA and the potential for genetic manipulation

Abstract

The sugarcane neutral invertase (SNI) gene was sequenced and characterised to determine its suitability as a target for genetic manipulation. A full-length cDNA for SNI was sequenced. This expressed gene showed significant homology to known NI sequences on both nucleic and amino acid levels. The SNI sequence did not contain the putative invertase catalytic amino acid sequence, suggesting it developed separately from the other classes of invertases. Approximately 1.8 kb of the SNI cDNA was incorporated into a vector suited for direct bombardment into sugarcane tissue. An antisense NI sequence from a related grass *(Lolium temulentum)* was used for transformation of sugarcane, however only transgenics containing the selection gene were hardened off.

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4.1 Introduction

Until recently, the distribution and function of plant invertases have been primarily determined by enzyme and protein studies (Tymowska-lalanne and Kreis, 1998). Studying the response of invertase gene expression to factors that influence metabolic characteristics has only been made possible by identifying and sequencing invertase genes.

In 1993 only three gene sequences of invertase isoenzymes had been cloned, but by July 2000 more than 100 full or partial sequences had been submitted to electronic databases. These sequences represented up to 70 different invertase genes cloned from 28 plant species (Goetz and Roitsch, 2000). The majority of these sequences code for soluble acid (SAI) and cell wall (CWI) bound forms of invertase. SAI and CWI show a high degree of homology between species and to each other (see Tymowska-lalanne and Kreis, 1998). Two motifs have been identified as important for β-fructofuranosidase activity. The first is a sequence of amino acids near the N-terminus of the peptide: NDPN. This sequence is hypothesised to be the catalytic site. A second sequence is implicated as a sequence that interacts with the catalytic site and essential for activity. This sequence in CWI is WECPD, and WECVD in SAI.

Expression patterns observed in AI enzyme studies, regulated by developmental stage and stimulus response, have been confirmed by observing differences in transcript levels. Not only have endogenous wild type expressions been examined, but antisense technology using sequenced AI clones have further highlighted the importance of invertases in metabolism. This was most clearly seen in the transgenic carrot lines with altered CWI and SAI levels whose phenotypes were significantly different from the wild-type control. Storage organs, leaves and roots were all affected in the process (Tang et al., 1999).

To date, full-length cDNA clones have only been reported for neutral invertase (NI) from *Lolium temulentum* (Gallagher and Pollock, 1998) and *Daucus carota (*Sturm et al., 1999). Various incomplete sequences are also available from international databases. These include partial sequences from *Glycine max, Oryza sativa, Lycopersicon esculentum* and a number of clones from *Arabidopsis thaliana*.

Although the *L. temulentum* sequence contains the putative invertase catalytic site: NDPN, it is not present in the *D. carota* NI clone. No further conserved domains

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deemed important for activity have been identified as yet. With this in mind, as well as the fact that NI has only been detected in plants and photosynthesising bacteria it has been suggested NI is a novel sucrose hydrolysing enzyme having developed separately from the other classes of invertases (Sturm et al., 1999).

In plants, including sugarcane, the role of NI in metabolism (including sucrose metabolism) has not been clearly defined. SNI activity has been detected in all tissues of the storage organ of sugarcane (Ebrahim et al., 1998; Vorster and Botha, 1999; Rose and Botha, 2000). Even though SNI activity has been measured in many studies along with other enzymes involved in sucrose accumulation, its function has not been conclusively determined. Considering this, further studies aimed at elucidating the role of SNI are necessary. Preliminary *in vitro* studies of the SNI enzyme have been reported (Rose and Botha, 2000; *chapter 3*). The goal of this chapter was to create a platform for further studies of NI endogenous gene expression (*chapter 5*) and potential genetic manipulation of the enzyme in sugarcane.

The aim of this component of the project was two-fold. Firstly, to transform sugarcane with a construct carrying the antisense NI from a related grass (*Lolium temulentum)* in an attempt to down-regulate the endogenous activity. Secondly, to produce antisense SNI constructs suited for direct bombardment into sugarcane tissue with the same aim as the transformation using the *L. temulentum* fragment.

At the outset of the project we had obtained the cDNA for the full-length *L. temulentum* cDNA and cloned it into transformation vectors in both expression (pENI 510) and antisense (pANI 510) directions, under regulation by the maize ubiquitin and cauliflower mosaioc virus 35S promoters (see *Appendix A* for plasmid maps). Later in the project, a cDNA coding for the entire SNI gene was obtained from the CSIRO (Australia). The sequence was isolated in routine screening of cDNA libraries. Its identity was confirmed after sequencing the first 600 bp of the 5' region and comparing it with known sequences on an international database.

In this chapter we discuss the verification of the *L. temulentum* NI transformation constructs and their use in transformation of sugarcane. We report the full-length SNI cDNA sequence and its construction into an antisense plant transformation vector.

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4.2 Materials and methods

4.2.1 General

All restriction enzymes were obtained from Promega. *Escherichia coli,* strain MC1061, was used to maintain all plasmids. Oligonucleotide primers were synthesised by IDT (Integrated DNA Technologies, Inc.).

4.2.2 Transformation of sugarcane with L. temulentum NI antisense and expression vectors (pENI 510 and pANI 510)

Tungsten particles were prepared to a final concentration of 60ng.μL^{-1} in sterile H_2O. The preparation began by suspending 30 mg 1.1μm tungsten particles in 500 μL absolute ethanol and vortexing for one min. The suspension was centrifuged at 13 000 rpm for one min and the ethanol removed. The wash step was repeated and the tungsten resuspended in 500 μL sterile ddH_2O, followed by a one min vortex step and a one min centrifugation at 13 000 rpm. The supernatant was removed and the water wash repeated. The precipitated tungsten was resuspended in sterile ddH_2O to a final concentration of 60ng.μL^{-1}. Coating of tungsten particles with DNA for one transformation event was prepared by adding 500 ng trait plasmid (pENI 510 or pANI 510, *Appendix A, figure 4.10*), 500 ng selection plasmid (pNPT 500, *Appendix A, figure 4.11*), 500 ng prewashed and suspended tungsten, spermidine (final concentration of 80 mM), and $CaCl_2$ (final concentration 5 M) in a volume of 28 μL. The suspension was sonicated and vortexed for 2 min, centrifuged for 2 min at 13 000 rpm, the supernatant removed and the pellet washed with 1 mL absolute ethanol. The suspension was then sonicated and vortexed for one min, centrifuged at 13 000 rpm and the supernatant removed. The pellet was washed with 1 mL absolute ethanol and centrifuged at 13 000 rpm for one min. The DNA coated tungsten was then resuspended in 5.5 μL absolute ethanol and sonicated before bombardment.

Pre-embryogenic sugarcane callus (variety Nco 310) was bombarded under a vacuum of 90 kPa using discharged helium. After bombardment the callus was transferred to recovery medium (30 mg.L^{-1} sucrose, 1 X Murashige and Skoog basal salts, 2g.L^{-1} gelrite, 0.1 g.L^{-1} casein acid hydrolysate, pH 5.8, 3 mg.L^{-1} 2-4-dichloro-phenoxyacetic acid) in the dark for 2 weeks. Thereafter calli were subcultured (still in the dark) every two weeks to new medium (recovery + 30 mg.L^{-1} geneticin) until putative transgenic

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embryos proliferated. Thereafter embryos were transferred to the light on the same medium. Once embryos had germinated, plantlets were separated and subcultured on medium containing 1X Murashige and Skoog basal salts, 30 $mg.L^{-1}$ geneticin and $2g.L^{-1}$ gelrite until roots were well established. From the light room plantlets were transferred to trays (2:1 sand:potting soil) and sealed in plastic bags for two weeks. Thereafter coverings were removed and the plants allowed to acclimatise for a month in humid glasshouse conditions. Plants were then transferred to larger pots and moved to a less humid environment in the glasshouse.

4.2.3 Sequencing of the full length SNI cDNA

The SNI cDNA was harboured in the pZL1 Excision Vector from Lambda ZIPLOX® (Life Technologies, http://www.lifetech.com/Content/Tech online/molecular_biology manuals _pps/15397029.pdf) in a plasmid called pZI-NINV7. All sequences were determined using automated procedures with the ABI PRISM Dye Terminator kit (Perkin Elmer) and desalted primers.

The 5' end of the fragment (*figure 4.1A.*) was sequenced out of the pZL1 Excision Vector using the T7 primer (5'-AAT ACG ACT CAC TAT AGG-3'). Primer SNIF was designed on the 3'- end of A (5'-GCA GCA CTC AAC TAT GAC CAG G-3') and used to obtain the sequence of fragment B. Fragment D was sequenced after doing a random restriction enzyme digestion (*Eco*R I) of the SNI cDNA and cloning into pUBI 510 (*Appendix A, figure 4.12)* and sequencing in the same manner as before out of the vector. Primers on both the 5' and 3'-ends of D were designed in order to amplify fragments C (SNIR- 5'-GAT CTT GAG TGG CAT CTC ACC-3') and E (SNI3- 5'-AGA CTA CGA GTA CAG CCA CAA AG-3') for sequencing. All five sequenced fragments were aligned using the software package DNASIS (version 2.5, Hitachi Software Engineering Co., ltd.) and the BLAST program (Altschul, 1990).

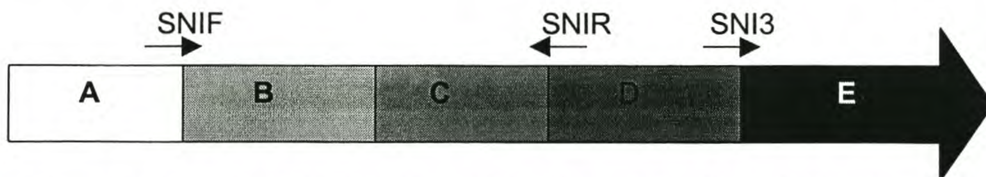

Figure 4.1 Schematic representation of the full-length SNI cDNA with primer and restriction endonuclease sites used to facilitate sequences as indicated.

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4.2.4 Construction of vectors for plant transformation

The SNI cDNA was used for construction of vectors suitable for sugarcane transformation via particle bombardment. 1814 bp of the 2228 bp SNI cDNA was restricted from pZL-NINV7 using restriction endonuclease *Eco*R I, and ligated in both sense (pESNI 510) and antisense (pASNI 510) directions into *Eco*R I linearised pUBI 510 (*Appendix A, figure 4.12*). Orientation of the insert was determined both by restriction endonuclease mapping and sequencing using the primer Ubi-ex that amplifies out of the promoter region into the insert.

4.2.5 Genomic DNA extraction

Genomic DNA was extracted according to a modified method described by Dellaporta et al. (1983). Tissue was finely ground in liquid nitrogen and extracted in a ratio of 1:6 (w/v) in a buffer containing 100mM Tris-HCl (pH8.0), 500mM NaCl, 50mM EDTA (pH 8.0) and 1% (v/v) β-mercaptoethanol. After vortexing, SDS was added to a final concentration of 1.67 % (w/v). The emulsion was incubated at 70°C for 20 min. After adding potassium acetate (final concentration of 0.7 M) the mixture was incubated on ice for 20 min. Cell debris was removed by centrifugation at 8 000 rpm , for 10 min at 4°C. The supernatant was filtered through muslin (pre-wet in extraction buffer). One volume of ice-cold isopropanol was added and the tubes incubated on ice for 10 min. Precipitated nucleic acids were pelleted by centrifugation at 8 000 rpm, for 10 min at 4°C. Pellets were air-dried and resuspended in TE (Tris-EDTA buffer) (6 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). RNA was degraded using RNaseA in a final concentration of 10 $\mu g.\mu l^{-1}$, at 37°C for at least 2 h. Remaining protein was removed from the DNA preparation using a 1:1 ratio of sample:Chloroform /iso-amyl alcohol (24:1) until no interface was visible. DNA was concentrated by precipitating (using 5mM $MgCl_2$ in 70% (v/v) ethanol, for 10 min on ice) and pelleting by centrifugation at 13 000 rpm, 25 min, 4°C. Excess salts were removed by washing pellets twice with 70% (v/v) ethanol. The clean DNA was resuspended in TE buffer and quantified fluorometrically (Dynaquant, Hoefer).

4.2.6 DNA amplification

Polymer chain reactions were carried out with biochemicals from Promega. Sixty ng gDNA template was used per reaction with 1X Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs,

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0.2 μM of each primer, 1U Taq DNA polymerase. Reactions were done in a Perkin Elmer cycler. The first step was one min at 94°C, followed by 35 cycles of three steps: 45 s at 94°C, 45 s at 55°C, 2 min at 72°C; followed by 7 min at 72°C and 5 min at 15°C.

4.2.7 Southern blot analysis

Membrane preparation

Genomic DNA (gDNA) was fully digested according to recommended protocols of the restriction enzyme company (Promega). Restriction enzymes *Eco*R I, *Hinc* II, *Hind* III, *Sac* I and *Eco*R V were used for preparation of 10 μg gDNA for Southern blot analysis in a final volume of 250 μL. Fully digested DNA was separated on a 0.8% (w/v) agarose gel at 120 V. DNA was then denatured by incubating the gel in a buffer containing 1.5M NaCl and 0.5M NaOH for 15 min, followed by a 15 min neutralising step in a buffer containing 1M Tris-HCl (pH 7.4) and 1.5M NaCl. The gel was equilibrated in 10 X SSC (1.5M NaCl and 0.3M $Na_3C_6H_5O_7$, pH 6.8) at room temperature for 20 min. DNA was transferred from the gel onto pre-equilibrated positively charged membrane (Boehringher Mannheim) by downward blot method overnight. Once transfer was completed the DNA was cross-linked to the membrane under UV light (1200 $mJ.cm^{-2}$, 2 min).

Probe preparation and hybridisation

Probes were prepared by random primer incorporation of α^{32}P-dCTP (Prime it II Random Labelling kit, Stratagene). After preparation, unincorporated label was removed using Qiagen quick spin columns. Specific activity of all probes were determined using a Beckmann liquid scintillation counter. The membrane was prehybridised in 15 mL hybridisation buffer (50 % (v/v) deionised formamide, 5X SSC, 5X Denhardt's solution, 0,1% (w/v) SDS and 200 $\mu g.mL^{-1}$ herring sperm DNA) for 6 h at 42°C. The probe was boiled for 5 min, added to the hybridisation bottle and incubated overnight at 42°C. Wash steps were as follows: Rinsed once in 2X SSC, 0.1% (w/v) SDS; two 10 min washes in 2X SSC, 0.1% (w/v) SDS; two 10 min washes in 1X SSC, 0.1% (w/v) SDS; one 15 min wash with 0.2X SSC, 0.1% (w/v) SDS. Hybridisation was visualised using a Phosphor imager (Packard Cyclone).

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4.3 Results

4.3.1 SNI cDNA sequencing and verification

The complete cDNA sequence for SNI *(figure 4.2)* was generated from multiple alignments of fragments (*section 4.3.3*). The clone's identity was verified by submitting to GenBank. The results showed significant homology with other published NI sequences on both a nucleotide and amino acid level (*table 4.1*).

```
3 ACGCGTCCGCAAAAACTAGCAAAGATGGTGCAATGTACTCAACCTCCTCCCCAGTTAAAG 62
1                         M  V  Q  C  T  Q  P  P  P  Q  L  K   12

63 CTCCCAGAGAGTAAGATCACAGAACTGACAGATGATGAGAACCATGATTCGCCGCCAAAA 122
13  L  P  E  S  K  I  T  E  L  T  D  D  E  N  H  D  S  P  P  K   32

123 CCTGAGAAAAGGACAAGGATGCACCACATTGAGAGGCACAGATCTTGTGTTGTGACCTTA 182
 33  P  E  K  R  T  R  M  H  H  I  E  R  H  R  S  C  V  V  T  L   52

183 TCTGACATAGAACTTAATGGTCTGCAATCTCGGCGTCTGCTCCAGACCATTGAGAAAAGC 242
 53  S  D  I  E  L  N  G  L  Q  S  R  R  L  L  Q  T  I  E  K  S   72

243 CCAGGAGGAGGATCACAGTCTTCTCTCCATGAAGAAACACCTACAGATACTAATGCATCA 302
 73  P  G  G  G  S  Q  S  S  L  H  E  E  T  P  T  D  T  N  A  S   92

303 CACAGGCATGCAATTGCAGATGCTGCTTGGGAAGCCCTCAAAGGGTCAATAGTTTACTTC 362
 93  H  R  H  A  I  A  D  A  A  W  E  A  L  K  G  S  I  V  Y  F  112

363 AGAGGCCAGCCAATTGGGACTGTTGCCACAATAGACAAGTCTCAGGGGGCAGCACTCAAC 422
113  R  G  Q  P  I  G  T  V  A  T  I  D  K  S  Q  G  A  A  L  N  132

423 TATGACCAGGTTTTCATGAGGGATTTCATTCCTAGTGCATTGGCTTTTCTTATGAAAGGA 482
133  Y  D  Q  V  F  M  R  D  F  I  P  S  A  L  A  F  L  M  K  G  152

483 GAACACTTGATAGTGAAGAATTTTCTGGTAGAAACTGCACGCCTTCAGTCAAGGGAGAAG 542
153  E  H  L  I  V  K  N  F  L  V  E  T  A  R  L  Q  S  R  E  K  172

543 ATGGTTGACCTTTTCAAGCTTGGTCAGGGTGTGATGCCTGCAAGCTTCAAGGTGCATCAT 602
173  M  V  D  L  F  K  L  G  Q  G  V  M  P  A  S  F  K  V  H  H  192

603 CGCAACCCTACCCAGAAGACAGAAAGCTTACTGGCTGATTTTGGTGAAACTGCCATTGGG 662
193  R  N  P  T  Q  K  T  E  S  L  L  A  D  F  G  E  T  A  I  G  212

663 AGGGTTGCTCCTGTAGATTCTGGCTTATGGTGGATTATTCTCCTTCGTGCTTACACCAAA 722
213  R  V  A  P  V  D  S  G  L  W  W  I  I  L  L  R  A  Y  T  K  232

723 TGGACAGGGGACAATTCTCTGGCTGAAAGTCCTAACTGCCAAAGGGCCATGCACCTTATT 782
233  W  T  G  D  N  S  L  A  E  S  P  N  C  Q  R  A  M  H  L  I  252

783 CTCAGGTTGTGTCTCTCAGAGGGATGTGATACTTCTCCAGCCTTGCTTTGTGCTGATGGG 842
253  L  R  L  C  L  S  E  G  C  D  T  S  P  A  L  L  C  A  D  G  272

843 TGCTCCATGATAGACCGAAGAATGGGCATATATGGCTACCCAATTGAAATCCAGGCTCTC 902
273  C  S  M  I  D  R  R  M  G  I  Y  G  Y  P  I  E  I  Q  A  L  292
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903  TTTTTCATGGCTATGAGATGTGGCCTAAGCTTGTTGAAACAAGACTCTGATGCTGACTTT   962
293   F  F  M  A  M  R  C  G  L  S  L  L  K  Q  D  S  D  A  D  F    312

963  GTGAACCACATCACAAAACGAATCCAAGCTTTGAGCTACCATTTGCACAGTTACTACTGG  1022
313   V  N  H  I  T  K  R  I  Q  A  L  S  Y  H  L  H  S  Y  Y  W    332

1023 TTAGACTTCCAAAGACTTAATGACATATACCGCTACAAGACTGAAGAATACTCACAGACA  1082
333   L  D  F  Q  R  L  N  D  I  Y  R  Y  K  T  E  E  Y  S  Q  T    352

1083 GCTTTGAACAAGTTCAATGTGATACCTGAATCAATACCTGATTGGATATTTGACTTCATG  1142
353   A  L  N  K  F  N  V  I  P  E  S  I  P  D  W  I  F  D  F  M    372

1143 CCTAGCCGGGGTGGATACTTCATTGGCAACGTTAGTCCTGCAAGGATGGATTTCCGCTGG  1202
373   P  S  R  G  G  Y  F  I  G  N  V  S  P  A  R  M  D  F  R  W    392

1203 TTTTGCTTGGGCAACTTCATTGCAATTCTGTCATCATTGGGACCGGAGAGCAGGCTGAAG  1262
393   F  C  L  G  N  F  I  A  I  L  S  S  L  G  P  E  S  R  L  K    412

1263 CAATACTGGATCTTGTGGAAGGAGCGCTGGCAAGAACTCATTGGTGAGATGCCACTCAAG  1322
413   Q  Y  W  I  L  W  K  E  R  W  Q  E  L  I  G  E  M  P  L  K    432

1323 ATCTGTTACCCTGCAATGGAAAATCAGGAATGGCAGATAGTCACTGGATGCGACCCAAAG  1382
433   I  C  Y  P  A  M  E  N  Q  E  W  Q  I  V  T  G  C  D  P  K    452

1383 AACACCAGGTGGAGCTACCACAACGGAGGCTCATGGCCAGTGCTGCTGTGGCTGCTGGTG  1442
453   N  T  R  W  S  Y  H  N  G  G  S  W  P  V  L  L  W  L  L  V    472

1443 GCGGTGAGCGTGAAGCTGGGGCGCCCGCACCTGGCGCGGAGAGCCGTGGAGCTGATGGAG  1502
473   A  V  S  V  K  L  G  R  P  H  L  A  R  R  A  V  E  L  M  E    472

1503 CAGCGTCTGGCGAAGGATGACTTCCCCGAGTACTACGACGGCAAGGCGGGGCGGTACGTG  1562
493   Q  R  L  A  K  D  D  F  P  E  Y  Y  D  G  K  A  G  R  Y  V    512

1563 GGGAAGCAGGCGCGCAAGTTCCAGACGTGGTCCGTGGCCGGCTACCTGGTGGCCAAGATG  1622
513   G  K  Q  A  R  K  F  Q  T  W  S  V  A  G  Y  L  V  A  K  M    532

1623 CTCCTGGACGACCCCTCCCACCTGCGGATCGTGGCGCTGGAGGACGACAGCCACTCCCGG  1682
533   L  L  D  D  P  S  H  L  R  I  V  A  L  E  D  D  S  H  S  R    552

1683 GCTCCCTTCCTTAAGCGCTCCAACTCGTGCCCATGACCCCAGGCTGCGCCGCGCTGCATA  1742
553   A  P  F  L  K  R  S  N  S  C  P  *

1743 CCACGCCCTTTTTGCCCACACCGAGACTACGAGTACAGCCACAAAGTTTCGTTATGAATT  1802
1803 CTGATTCGCGACAACAGGAGCATAGTGGAGGAGAGGATACTTAGTTACTTACCCACGCAT  1862
1863 TGCTGACCACGCGGTGCAGTGCATTCTTCTTACATAAGTATAACGAACCAGTAGTGATGT  1922
1923 CTTCTACCGTGCTGCGAAGAGGAGGATGACAAGATACCGAGTATTGTATGTATGTACTAG  1982
1983 GTAGGTACTATCCGTTCGTCAGATCGGATTCTACTAAACTGGTGAAGATTTTGGTGGGTC  2042
2043 AGGCTAGCGTTGCGTCCGGATGCGGAATTCTGAATCATCTGGTAGATGCGGAACTCTAAA  2102
2103 CCATCTGGTGGAGCTTTATTATGTTGAAGGAACCGAAGAAAAAAACTGTATTTTCTCCTT  2162
2163 TTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAT  2222
2223 TCGCGA                                                        2228
```

Figure 4.2 Full-length SNI sequences (DNA and amino acids). Initiation codon (ATG) highlighted.

The sequenced sugarcane cDNA was 2228 bp in length, with an open reading frame (ORF) of 564 amino acids.

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```
L.temulentum  1 ------------------------------------------------------------
D. carota     1 MNTTCIAVSNMRPCCRMLLSCKNSSIFGYSFRKCDHRMGTNLSKKQFKVYGLRGYVSCRG
S.officinarum 1 -------------------------------------------------------MVQCT

L.temulentum  1 -----MRKRGTNSFYRTLGGPPKFPELRPVECQCQRIDDLAGVIKAGNGTWAN-------
D. carota    61 GKGLGYRCGIDPNRKGFFGSGSDWGQPRVLTSGCRRVDSGGRSVLVNVASDYRNHSTSVE
S.officinarum 6 QPPPQLKLPESKITELTDDENHDSPPKPEKRTRMHHIERHRSCVVTLSDIELN-------

L.temulentum 49 ----------------------------DMVNKASQVLGDVAVPGQALGGNASLSGNPEKV
D. carota   121 GHVNDKSFERIYVRGGLNVKPLVIERVEKGEKVREEEGRVGVNGSNVNIGDSKGLNGGKV
S.officinarum59 ----------------------------GLQSRRL---LQTIEKSPGGGSQSSLHEETPT

L.temulentum 82 LPRRRNLSSVEDEAWDLLRESVVNYCGSPVGTIAANDPNDSNPANYDQVFIRDFIPSGIA
D. carota   181 LSPKREVSEVEKEAWELLRGAVVDYCGNPVGTVAASDPADSTPLNYDQVFIRDFVPSALA
S.officinarum88 DTNASHRHAIADAAWEALKGSIVYFRGQPIGTVATIDKSQGAALNYDQVFMRDFIPSALA

L.temulentum 142 FLLKGEYEIVRNFILHTLQLQSWEKTMDCHSPGQGLMPASFKVRTIPLDGDENATEEVLD
D. carota    241 FLLNGEGEIVKNFLLHTLQLQSWEKTVDCHSPGQGLMPASFKVKNVAIDGKIGESEDILD
S.officinarum148 FLMKGEHLIVKNFLVETARLQSREKMVDLFKLGQGVMPASFKVHHRNP----TQKTESLL

L.temulentum 202 PDFGEAAIGRVAPVDSGLWWIILLRAYGKCSGDLSVQERIDVQTGIKMILKLCLADGFDM
D. carota    301 PDFGESAIGRVAPVDSGLWWIILLRAYTKLTGDYGLQARVDVQTGIRLILNLCLTDGFDM
S.officinarum204 ADFGETAIGRVAPVDSGLWWIILLRAYTKWTGDNSLAESPNCQRAMHLILRLCLSEGCDT

L.temulentum 262 FPTLLVTDGSCMIDRRMGIHGHPLEIQALFYSALLSAREMLTPEDGSADLIRALNNRLVA
D. carota    361 FPTLLVTDGSCMIDRRMGIHGHPLEIQALFYSALRCSREMLIVNDSTKNLVAAVNNRLSA
S.officinarum264 SPALLCADGCSMIDRRMGIYGYPIEIQALFFMAMRCGLSLLK-QDSDADFVNHITKRIQA

L.temulentum 322 LSFHIREYYWVDMQKLNEIYRYKTEEYSYDAVNKFNIYPDQVSPWLVEWIPPKGGYFIGN
D. carota    421 LSFHIREYYWVDMKKINEIYRYKTEEYSTDAINKFNIYPDQIPSWLVDWMPETGGYLIGN
S.officinarum323 LSYHLHSYYWLDFQRLNDIYRYKTEEYSQTALNKFNVIPESIPDWIFDFMPSRGGYFIGN

L.temulentum 382 LQPAHMDFRFFSLGNLWSIVSSLATTQQSHAILDLIESKWSDLVAEMPLKICYPALENLE
D. carota    481 LQPAHMDFRFFTLGNLWSIVSSLGTPKQNESILNLIEDKWDDLVAHMPLKICYPALEYEE
S.officinarum383 VSPARMDFRWFCLGNFIAILSSLGPESRLKQYWILWKERWQELIGEMPLKICYPAMENQE

L.temulentum 442 WKIITGSDPKNTPWSYHNGGSWPTLLWQLTVASLKMNRPEIAAKAVEIAERRIATDKWPE
D. carota    541 WRVITGSDPKNTPWSYHNGGSWPTLLWQFTLACIKMKKPELARKAVALAEKKLSEDHWPE
S.officinarum443 WQIVTGCDPKNTRWSYHNGGSWPVLLWLLVAVSVKLGRPHLARRAVELMEQRLAKDDFPE

L.temulentum 502 YYDTKRARFIGKQSRLYQTWSIAGYLVAKQLLDKPDAARILWNDEDTEILN---AFS--T
D. carota    601 YYDTRRGRFIGKQSRLYQTWTIAGFLTSKLLLENPEMASKLFWEEDYELLESCVCAIGKS
S.officinarum503 YYDGKAGRYVGKQARKFQTWSVAGYLVAKMLLDDPSHLRIVALEDDSHSRAP--------

L.temulentum 557 NRKRGKKVLKKTYIV-
D. carota    661 GRKKCSRFAAKSQVV-
S.officinarum555 -------FLKRSNSCP
```

Figure 4.3 Comparison of NI sequences of *L. temulentum* (poison rye grass), *D. carota* (carrot) and *S. officinarum* (sugarcane) on amino acid level. Large regions of homology are indicated in blocks. Putative catalytic site (NDPN) is highlighted. Residues in red are identical in at least two of the three sequences.

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On an amino acid level the sugarcane sequence showed a 54.5 and 53.5% homology match to the *D. carota* and *L. temulentum* NI sequences, respectively (*figure 4.3*). The putative invertase catalytic site (NDPN) (highlighted in *figure 4.3*) is not present in the SNI sequence. The *D. carota* sequence has an additional approximately 56 amino acid residues (approximately 168bp) in the 5' region from the ATG of the cDNA not present in the current sequences from *S. officianarum* and *L. temulentum.* Physical characteristics of SNI were compared with *Lolium temulentum* and *Daucus carota* sequences as summarised in Table 4.1.

Table 4.1 Comparison of physical aspects of three NI sequences. The mature protein Mr is an approximation calculated from the amino acid usage.

Species	DNA ORF (bp)	Amino acid ORF (codons)	Approximate mature protein Mr (kDa)
Saccharum officianarum	1692	564	64.3
Lolium temulentum	1713	571	64.4
Daucus carota	1713	571	63

Three probes were prepared for Southern blot analysis of sugarcane, variety N19. The first was the entire 2228bp SNI cDNA. The second and third were prepared by restricting the 2228bp with Sma I resulting in a 1681bp and 547bp fragment. All probes were prepared using the Prime-it II Random primer labelling kit (Stratagene) with ^{32}P-dCTP. Each lane on all membranes had 10μg of fully digested gDNA (lanes 1 through 5 having digests of *Eco*R I , *Hinc* II, *Hind* III, *Sal* I respectively). In figure 4.4, blots A, B and C were probed with the 2228bp, 1681bp and 547bp fragments respectively.

A blot of sugarcane gDNA probed with 1163bp (prepared by restricting pENI 510 with *Hind* III and excising and purifying the desired band from an agarose gel) from the middle of the *L. temulentum* NI cDNA (*figure 4.5*) shows a similar banding pattern to figure 4.4A.

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A

1 2 3 4

21226 bp

2027 bp

831 bp

SNI cDNA (2228 bp)

B

1 2 3 4

21226 bp

2027 bp

831 bp

Sma I (1681)

C

1 2 3 4

21226 bp

2027 bp

831 bp

Figure 4.4 Southern blot analysis of N19 gDNA using fragments of a SNI cDNA as probes. Restriction endonucleases *Eco*R I (lane 1), *Hinc* II (lane 2), *Hind* III (lane 3), *Sac* I (lane 4). The full-length SNI cDNA (A), 1681 bp of the 5' region (B) and 547 bp from 3' region(C) were used as probes.

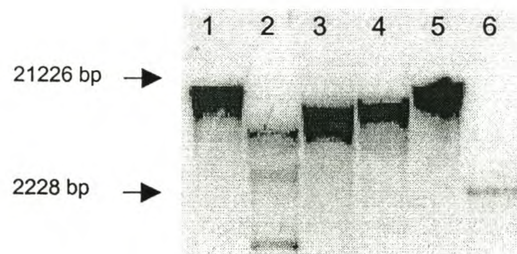

Figure 4.5 Southern blot analysis of N19 gDNA using 1163 bp of the *L. temulentum* SNI cDNA as a probe. Lanes 1 through 5 represent gDNA restricted with *Eco*R I, *Hinc* II, *Hind* III, *Sac* I and *Eco*R V respectively. Lane 6 contains the 2228 bp NI cDNA.

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4.3.2 *Construction and verification of transformation vectors harbouring the SNI cDNA*

Complete plasmid maps of pESNI 510 (*figure 4.6A*) and pASNI 510 (*figure 4.6B*) were verified by restriction mapping (*figures 4.7A and B, tables 4.2A and B*). Restriction fragments smaller than approximately 300 bp were too small to visualise.

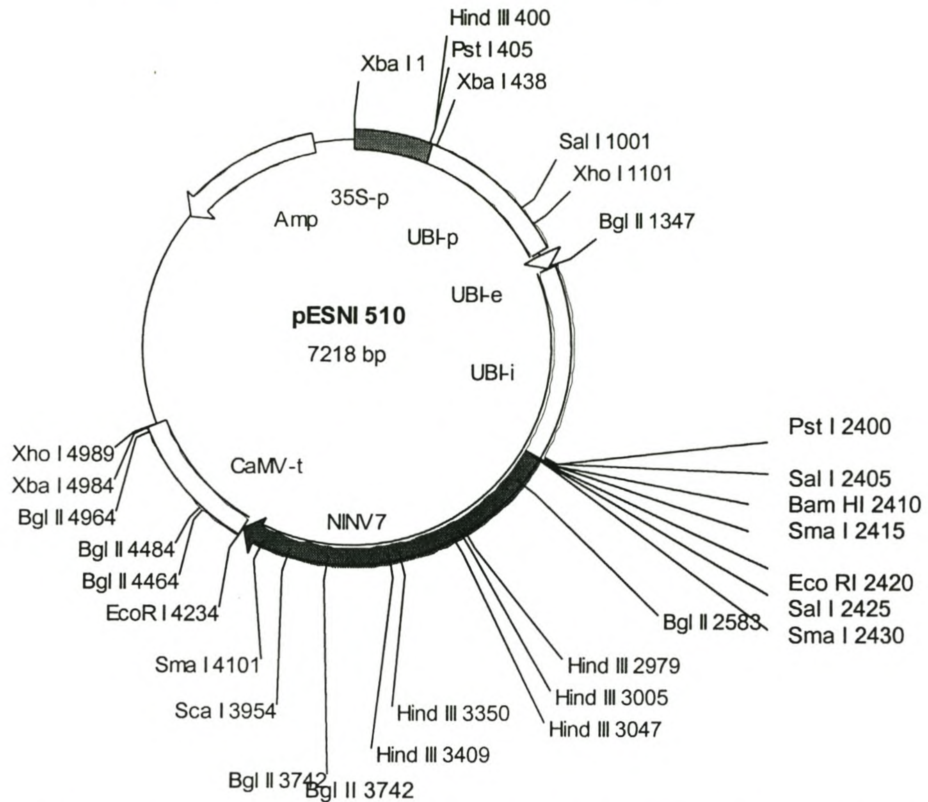

Figure 4.6A Plasmid map of pESNI 510 harbouring SNI cDNA in expression orientation.

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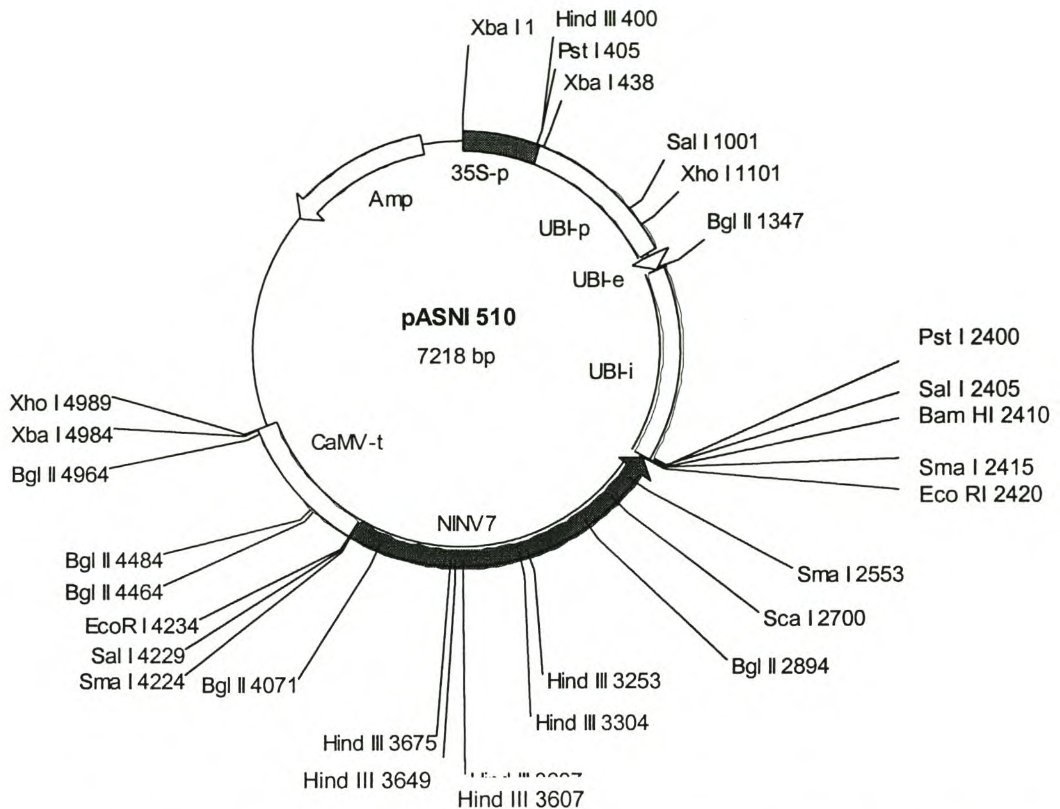

Figure 4.6B Plasmid map of pASNI 510 harbouring the SNI cDNA in the antisense orientation.

Table 4.2 Expected and achieved banding patterns of restriction endonuclease restriction mapping of pESNI 510 (A) and pASNI 510 (B)

A

Restriction endonuclease	Expected fragments (bp)	Achieved fragments (bp)
Bam HI	7218	7218
*Eco*R I	5404 + 1814	5404 + 1814
Hind III	4209 + 2579 + 303 + 59 + 42 + 26	4209 + 2579
Pst I	5223 +1995	5223 +1995
Sal I	5794 + 1404 +20	5794 + 1404
Sma I	5532 + 1671 + 15	5532 + 1671

B

Restriction endonuclease	Expected fragments (bp)	Achieved fragments (bp)
Bam HI	7218	7218
*Eco*R I	5404 + 1814	5404 + 1814
Hind III	3969 + 2853 + 303 + 51+42 + 26	3969 + 2853
Pst I	5223 + 1995	5223 + 1995
Sal I	3990 + 1824 + 1404	3990 + 1824 + 1404
Sma I	5409 + 1671 + 138	5409 + 1671

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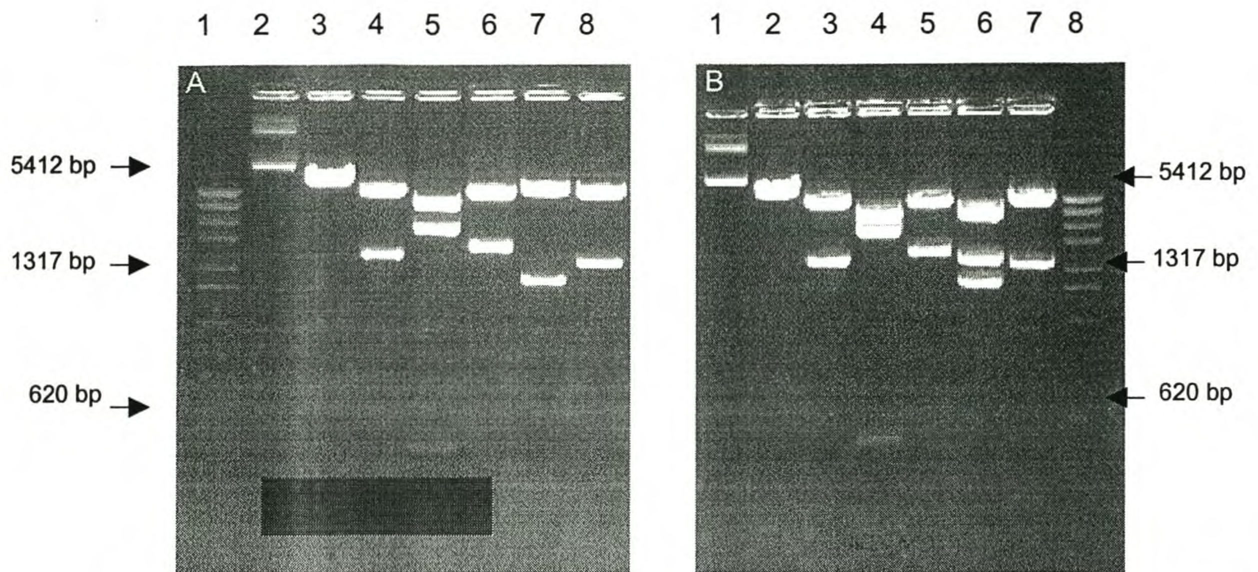

Figure 4.7 Verification of plasmid mapping by restriction endonuclease digestions and visualisation on a 1% agarose gel. pESNI 510 (A). pASNI 510 (A). Plasmids were restricted with *Bam* HI, *Eco*R I, *Hind* III, *Pst* I, *Sal* I and *Sma* I as represented in lanes 2 through 8. Molecular weight markers were loaded in lane 1.

4.3.3 Transformation with pENI 510 and pANI 510

Verification of pENI 510 and pANI 510 was achieved by restriction endonuclease mapping (*figures 4.8*, *table 4.3*).

Table 4.3 Expected and achieved banding patterns of restriction endonuclease restriction mapping of pENI 510 and pANI 510.

Restriction	Expected fragments (bp)		Achieved fragments (bp)	
endonuclease	pENI 510	pANI 510	pENI 510	pANI 510
Hind III	4547	3714	4547	3714
	1840	3173	1840	3173
	473	473	473	473
Pst I	4158	4586	4158	4158
	1995	1995	1995	1995
	1207	779	1207	1207

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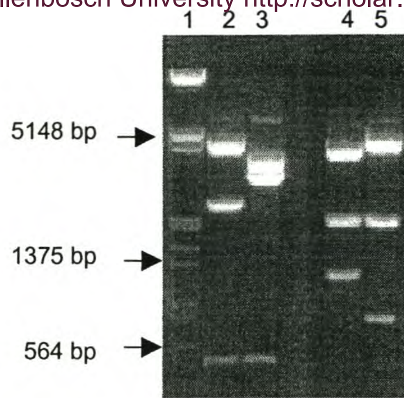

Figure 4.8 Verification of orientation of *L.temulentum* NI insert in pENI 510 and pANI 510 by restriction endonuclease digestions and visualisation on a 1% agarose gel. Lanes represent Molecular weight marker III (1), pENI 510 + *Hind* III (2), pANI 510 + *Hind* III (3), pENI 510 + *Pst* I (4), pANI 510 + *Pst* I (5).

Cobombardment with constructs carrying *L. temulentum* NI cDNA and selection gene *npt II* for sugarcane transformation resulted in few transgenics. None of the analysed plants successfully integrated the NI gene (*figure 4.9B*) however they all tested positive for *npt II* incorporation (verified by both PCR and DNA-DNA blot techniques) (*figures 4.9A*).

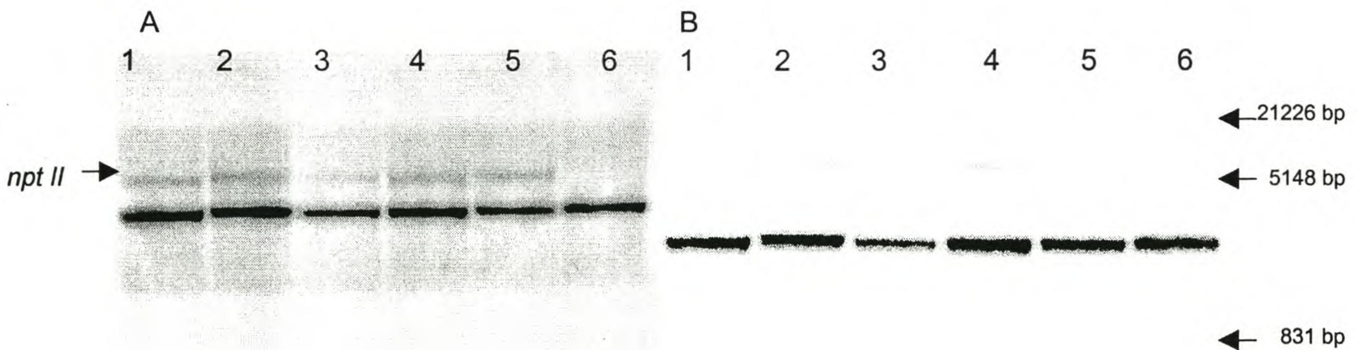

Figure 4.9 Southern blot analysis for transgene incorporation into the sugarcane genome; (A) *npt II* probe, (B) *L. temulentum* probe. Each lane represents a different transgene line: 1: A2, 2: B7, 3: C16, 4: D7, 5: Ona S04, 6: Nco 310 negative control.

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4.5 Discussion

Identification of the SNI cDNA was confirmed by the homology evident on both nucleotide and amino acid levels with confirmed NI sequences from both *Lolium temulentum* (poison rye grass) and *Daucus carota* (carrot). Areas of high homology between species are not located in any specific area of the gene, but rather distributed throughout the sequence (*figure 4.3*). Although these areas have not previously been implicated in the activity of the enzyme they may be important, hence their conservation between species.

The sequence generated for the SNI cDNA codes for an ORF of approximately 564 amino acid residues. Previous studies focussing on the metabolic characteristics of SNI have determined molecular weights between 58 kDa (Vorster and Botha, 1998) and 66 kDa (Del Rosario and Santisopasri, 1977). The predicted molecular weight from this cDNA ORF is approximately 64.3 kDa (*table 4.1*). This falls within the reported range of previously predicted molecular weights. Variation of molecular weights could be as a result of inaccuracy in chromatographic techniques, or the possibility that this cDNA codes for a different isozyme than measured in the metabolic studies. This discrepancy could be solved by expressing the cDNA in a bacterial or yeast system and determining physical and metabolic characteristics of the resultant protein.

Southern blot analysis (*figure 4.4*) indicates that SNI has a low genetic complexity; a postulation from the relatively few bands present when probing restricted gDNA with the expressed SNI sequence. These results imply that SNI has a low copy number in the sugarcane genome, as opposed to being a member of a multi-gene family. SNI has been implicated as an important enzyme in the sucrose accumulation cycle (Vorster and Botha, 1999). Separated polypeptides display a single protein signal at approximately 58 kDa in western blot analysis (Vorster and Botha, 1999), further suggesting that SNI is the product of a single gene. With a view to both understand and improve sucrose yield in sugarcane, these two factors; low genetic complexity and metabolic importance, have made SNI an ideal target for genetic manipulation.

With this in mind, we predicted that a NI sequence from a plant related to sugarcane could be used for overexpression and/or antisense manipulation of the enzyme's activity. However, co-bombardment with the *L. temulentum* full-length cDNA (trait

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gene) in an antisense orientation (*Appendix A, figure 4.10*) and a construct harbouring the *nptII* gene (selection gene) (*Appendix A, figure 4.11*) resulted in only a few transgenic plants. All of these plants tested negative for the trait gene (Southern Blot analysis, *figure 4.9B*) and positive for the selection gene (Southern blot analysis, *figure 4.9A*). After transformation, callus was subcultured on a medium containing sucrose as a carbon source. It is possible that a potential decrease in NI activity, caused by transformation, may have inhibited the plant's ability to produce sufficient hexoses from the source for germination and growth. This could have affected the efficiency of transformant regeneration. Perhaps further attempts at transformation using NI fragments should include equimolar quantities of hexoses in the subculturing media. Future work will be aimed at genetic manipulation using constructs containing the SNI sequence that have been successfully constructed in this project.

The SNI constructs (pESNI 510 and pASNI 510, *figures 4.6A and B*) were made with a view to manipulate SNI activity in all tissues, hence the use of a tandem promoter construct containing the 35S and Ubiquitin promoter sequences. Both plasmids' construction were verified (*figure 4.6 and table 4.2*), and will be included in a transformation program.

The verification of the full length SNI cDNA allows for further investigations of this sucrose metabolising enzyme. Not only can transcript levels in wild-type plants be examined to determine regulation of expression, but genetic manipulations can now be attempted. These modifications have the potential to influence sucrose accumulation. Thus, transgenic lines will help further elucidate the processes of sucrose accumulation and possibly generate commercial lines with higher sucrose content.

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4.5 Appendix A.

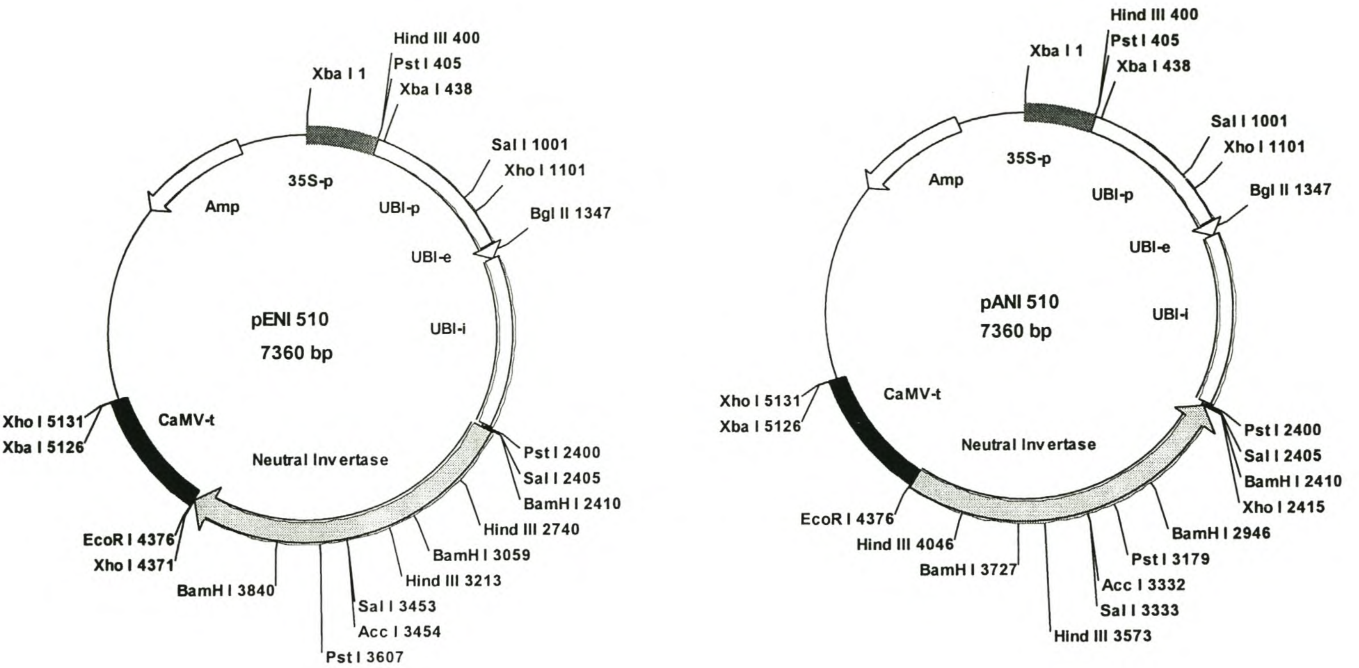

Figure 4.10. Plasmid maps of pENI 501 and pANI 510.

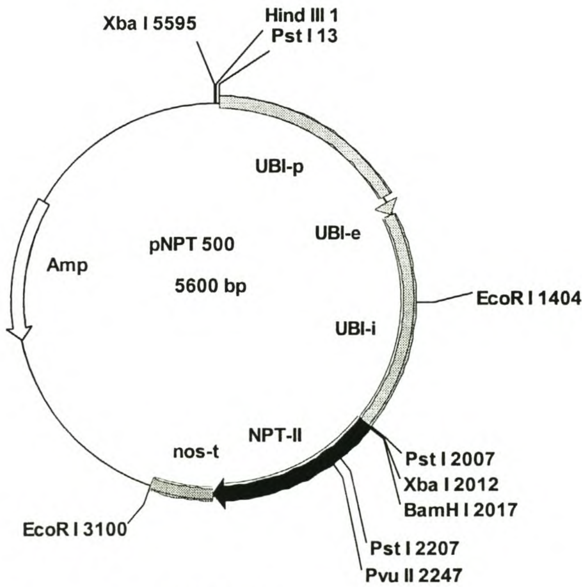

Figure 4.11. Plasmid map of pNPT 500.

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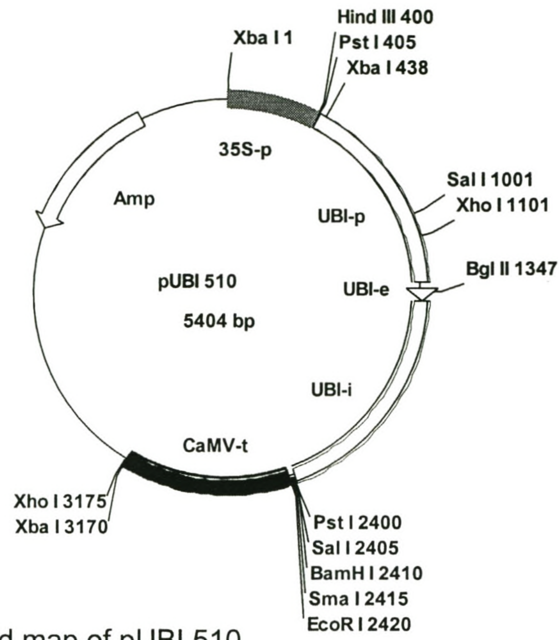

Figure 4.12. Plasmid map of pUBI 510.

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CHAPTER 5

RNA and protein expression of neutral invertase throughout the sugarcane plant

Abstract

Sugarcane neutral invertase (SNI) hydrolyses sucrose in the cytosol. Previous inconsistent reports of the distribution of enzyme activity in sugarcane have necessitated further investigation. Here we investigated SNI gene copy number as well as RNA expression patterns in the different tissues of the sugarcane plant. The aim of the work was to describe SNI expression before and after protein translation. SNI protein levels and sugar contents were also determined in the same tissues. Southern blot analysis showed the enzyme has a low copy number. SNI transcript expression was observed in all tissues of the sugarcane plant: roots, internodes, leaf roll and leaves. In culm tissues where sucrose content was low and hexose contents were high, SNI transcript and protein levels were higher than in tissues devoted to sucrose storage. This suggests that SNI is involved in growth metabolism.

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5.1 Introduction

Although there is considerable literature from as early as 1963 reporting activities and expression patterns of sugarcane neutral invertase (SNI, β-D-fructofuranoisdase, EC 3.2.1.26) (Hatch et al., 1963; Del Rosario and Santisopasri, 1977; Batta and Singh, 1986; Lingle and Smith, 1991; Zhu et al., 1997; Ebrahim et al., 1998), this has focussed on maximum extractable activities measured using spectrophotometric assays. Further studies, using polyclonal antibodies on protein blots, confirmed measured extractable invertase activities (Vorster and Botha, 1998; Vorster and Botha, 1999; Rose and Botha 2000).

The SNI protein has been well described in terms of its physical characteristics. These include molecular weight, ranging between 58 and 66 kDa (Vorster and Botha, 1999 and Del Rosario and Santisopasri, 1977 respectively), subunit aggregation as well as biochemical properties (pH optima ranging between 7.0 and 7.5) (Vorster and Botha, 1998; Del Rosario, 1977). Inhibitors include the reaction products glucose and fructose and various heavy metals (Vorster and Botha, 1998). SNI displays Michaelis Menten kinetics (Vorster and Botha, 1998).

Along with these descriptions of the enzyme's characteristics, many studies have reported its distribution within the sugarcane culm. Early work (Hatch et al., 1963; Batta and Singh, 1986) showed an increase in SNI activity as tissues matured. In more recent studies, an almost opposite distribution pattern has been observed with SNI activity decreasing with tissue maturity (Lingle, 1997; Zhu et al., 1997; Ebrahim et al., 1998; Vorster and Botha, 1999; Rose and Botha, 2000). The recent data negates the former with good reason. Early experiments were carried out with extracts insufficiently protected against protease activity and subjected to long desalting processes.

Up until now the cDNA for SNI had not been cloned (see *chapter 4* for full sequence and descriptions) and therefore any study at gene and transcript expression level has not been possible. Although SNI distribution patterns seem to have been established by concurring reports of extractable activities in the last few years, valuable information could be gathered by studying the SNI transcript expression in various tissues of the sugarcane culm.

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The aim of this study was, therefore, to characterise SNI expression at the gene, transcript and protein levels, and to compare these patterns with extractable contents of sucrose, glucose and fructose.

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5.2 **Materials and methods**

5.2.1 General

All restriction enzymes were obtained from Promega unless otherwise stated.

5.2.2 Plant material

Sugarcane, variety N19, was used for all extractions. DNA was extracted from leaf roll tissue. RNA, proteins and sugars were all extracted from the same tissue samples. Tissues were sampled from roots, leaves and internodes. Internodes were sequentially numbered from internode one (the internode attached to the leaf with the first exposed dewlap) through 17 as maturity increased. Young, middle and old leaves were defined as actively growing, actively photosynthesising and senescing, respectively.

5.2.3 Genomic DNA extraction

Genomic DNA was extracted as previously described (*chapter 4, section 4.2.5).*

5.2.4 Southern blot analysis

Southern blot analysis was carried out as previously described (*chapter 4, section 4.2.7).*

5.2.5 RNA extraction

RNA was extracted according to to a modified method of Bugos et al. (1995). Tissue was ground to a fine powder in liquid nitrogen and added (1:2 (w/v)) to 25:24:1 phenol:chloroform:isoamyl alcohol and vortexed. Thereafter homogenisation buffer (0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl and 1% (w/v) SDS) was added in same volume as the 25:24:1 phenol:chloroform:isoamyl alcohol. Sodium acetate (pH 5.2) was added to a final volume of 0.1 M, the emulsion was mixed and incubated on ice for 15 min followed by a 15 min centrifugation at 12 000 xg, 4°C. The aqueous phase was transferred to a new tube and one volume isopropanol added, followed by incubation at -70°C for at least 30 min. Precipitated RNA was pelleted by centrifugation at 10 000 xg for 10 min at 4°C. The pellet was washed with 70 % (v/v) ethanol, centrifuged for 5 min at 10 000 xg, 4°C and air dried. RNA was resuspended in 750 μL DEPC (Diethyl pyrocarbonate) treated water and insolubles removed by

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centrifugation at 10 000 xg for 5 min at 4°C. The supernatant was transferred to a microcentrifuge tube, and RNA precipitated using LiCl (final concentration of 2M) and incubating overnight at 4°C. RNA was recovered by centrifugation at 12 000 xg for 15 min at 4°C. The pellet was washed with 70 % (v/v) ethanol, centrifuged at 10 000 xg for 5 min and air dried. The pellet was resuspended in DEPC treated water and all remaining insolubles removed by centrifugation at 10 000 xg for 5 min at 4°C. RNA was quantified spectrophotometrically.

5.2.6 Northern blot analysis

12 μg RNA per sample were loaded on a 2 % (w/v) agarose gel (made up with 1X TBE (Tris-Borate/EDTA electrophoresis buffer). RNA was separated at 150 V for 10 min and then at 120 V until the dye front had run 8 cm. The gel was trimmed and stained in ethidium bromide solution (approximately 0.4 $\mu g.mL^{-1}$) for 15 min. RNA was viewed under UV light. The gel was then equilibrated in 10X SSC for 20 min, and the membrane wet in water and equilibrated in 10X SSC for 10 min. RNA was transferred onto the membrane by upward capillary blotting overnight at room temperature using 10X SSC. After transfer the membrane was removed, rinsed in 5X SSC, excess liquid evaporated and UV cross-linked on both sides for 1.5 min at 1200 $mJ.cm^{-2}$.

Probe preparation, prehybridisation and hybridisation of membranes, washing steps, exposure and developing of films was carried out as in the Southern blot analysis method (*chapter 4, section 4.2.7).*

5.2.7 Protein extraction and western blot analysis

Soluble proteins were extracted from the same tissues as RNA. Soluble proteins from liquid nitrogen ground tissue were extracted (approximately 1:4 (w/v)) in a buffer containing 100 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7), 1 mM EDTA, 10 mM DTT, 0.5 mM Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrogenchloride), 10 % (v/v) glycerol and 2 % (w/v) insoluble polyvinylpolypyrrolidine (PVPP). Samples were vortexed and insoluble cell components removed by centrifugation at 13 000 rpm for 10 min at 4°C. Protein concentrations were determined spectrophotometrically, according to the method of Bradford (1976) adjusted for microtiter plate application and using immunoglobulin G (IgG) as a standard.

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Proteins were separated on SDS-PAGE gel as previously described (Rose and Botha, 2000). Twenty μg total protein was loaded per sample. Western blot analysis was done as previously described (Rose and Botha, 2000; *chapter 3*) using a polyclonal antibody raised against *Beta vulgaris* NI.

5.2.8 Sugar extraction and determination

Soluble sugars were extracted in a buffer containing 70 % (v/v) ethanol and 50 mM Tris (pH 7). Tissue was incubated at 70°C overnight in buffer (1:10 (w/v)). Insoluble cell components were removed by centrifugation at 13 000 rpm for 10 min at 4°C. Sucrose and hexose concentrations in the supernatant were determined spectrophotometrically (Bergmeyer and Bernt, 1974). Buffers used for the assay were: (A) 150 mM Tris-HCl (pH 8) and 5 mM $MgCl_2$; (B) 100 mM Citrate (pH 5) and 5 mM $MgCl_2$. Sucrose was hydrolysed by adding 5 μL sample, 40 μL Buffer B and 5μL (10 U) invertase into the well of a microtiter plate and incubating for 15 min at room temperature. Hexose assays were run concurrently, replacing the invertase with ddH_2O. Glucose (in both the hexose and sucrose assays) was determined by adding 200 μL Buffer A containing 32 mM NADP (reduced nicotinamide-adenine phosphate dinucleotide) and 1.4 mM ATP (adenosine 5'-triphosphate) making the assay volume 250 μL. The reaction was started with HK and G6PDH (Hexokinase and Glucose-6-Phosphate dehydrogenase, EC numbers EC 2.7.1.1 and EC 1.1.1.49 respectively), allowing the reaction to run to completion and measuring the change in absorbance at 340 nm caused by the reduction of NADP on a plate reader (PowerwaveX, Biotek Instruments Inc.). Fructose was determined by adding PGI (Phosphoglucoisomerase, EC 5.3.1.9) to the reaction, allowing the reaction to run to completion and measuring the further change in absorbance caused by reduction of NADP.

Percentage Purity was calculated as the amount of sucrose as a percentage of the total sugar pool.

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5.3 Results

Cellular constituents (RNA, proteins and sugars) were extracted from leaf roll, leaves, internodes and roots of commercial sugarcane variety N19. DNA was extracted from the leaf roll.

5.3.1 Southern blot analysis

Southern blot analysis using a SNI cDNA as a probe exhibited a simple banding pattern (*figure 5.1*). This is visualised where gDNA from sugarcane was digested with *Eco*R I (*figure 5.1, lane 2)* and consequent hybridisation signals displayed only two major bands. This SNI fragment used for the probe had only one *Eco*R I restriction site.

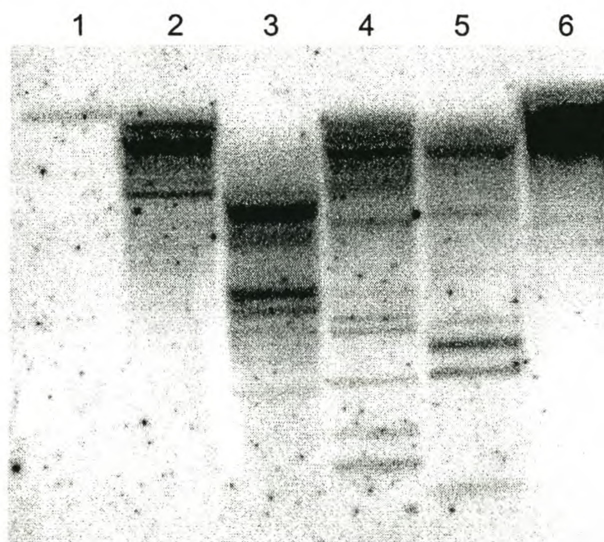

Figure 5.1 Southern analysis of SNI. A full-length SNI cDNA was used to probe completely digested gDNA (1: undigested, 2: *Eco*R I, 3: *Hinc*II, 4: *Hind*III, 5: *Sac*I, 6: *Eco*RV).

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5.3.2 *Northern and western blot analyses*

RNA extracted from 10 tissues was visualised on a 2% agarose gel (*figure 5.2*). With the exception of internode 3 sample, loading was evidently uniform. This was possibly due to the difficulties in accurately determining RNA concentrations in crude preparations.

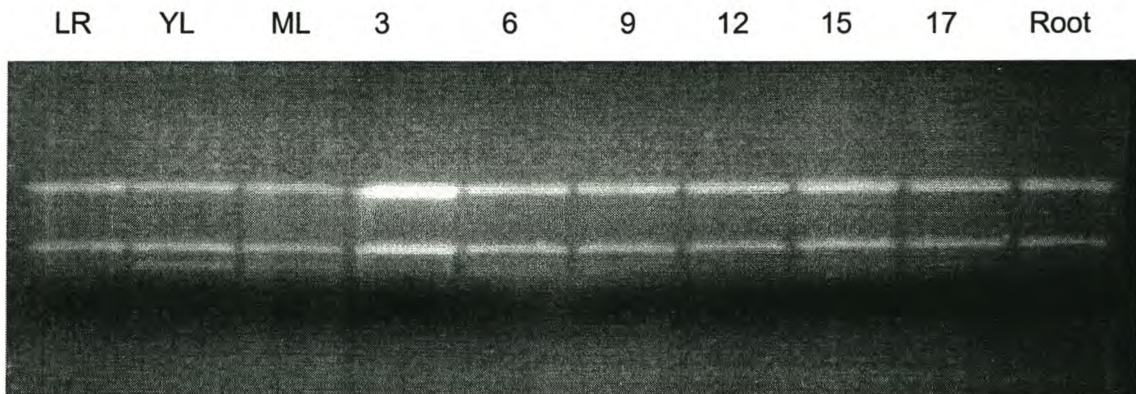

Figure 5.2 RNA extracted for Northern blot from Leaf roll (LR), young leaf (YL), middle leaf (ML), internodes 3, 6, 9, 12, 15, 17 and roots.

The SNI cDNA was used as a probe in a northern blot analysis (*figure 5.3A*). Hybridisation signals were detected in each tissue type (leaf roll, young leaf, medium leaf, internodes 3, 6, 9, 12, 15, 17, and root). It would appear that in the internodal tissues there was a slightly higher transcript expression in the younger culm tissues (3 and 6) than the older internodal tissues (15 and 17).

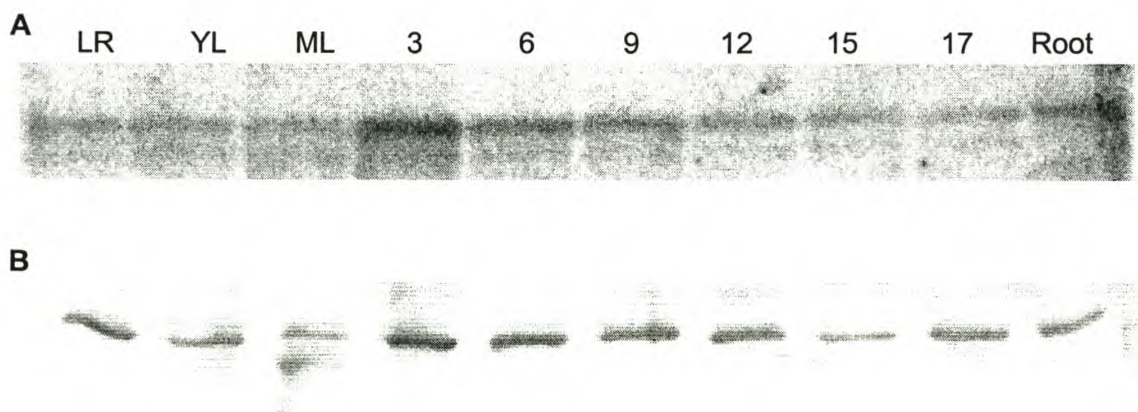

Figure 5.3 Neutral invertase expression at RNA (A) and protein (B) level. The analyses were achieved by Northern and Western blot techniques respectively. Each lane represents a different tissue; LR: leaf roll; YL: young leaf; ML: medium leaf; 3 through 17 represent internodal tissue with the root sample in the last lane.

Reflecting the RNA profile SNI protein was detected in every tissue sampled. SNI protein levels appeared to be higher in young growing tissues such as leaf roll and the

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immature internodes of the culm (*figure 5.3B*). The SNI protein profile of culm tissues exhibited a similar pattern of expression as enzyme activity profiles described by Vorster and Botha (1999); increasing from internode 3 and peaking between internodes 4 and 6 and dropping again to a low level at internode 10.

5.3.3 Sucrose and hexose levels

Soluble sugar profiles were similar to previously reported patterns (Ebrahim et al., 1998; Vorster and Botha, 1999; Botha and Black, 2000): sucrose contents increasing from young to old internodes, and hexoses peaking at internode 6 and rapidly declining in the older internodes (*figure 5.4*). Both glucose and fructose levels were notably higher in leaf roll than in any other tissue resulting in a purity of 45.2%, almost 30 % lower than that in any of the other tissues sampled (*table 5.1*). By internode 9 %purity had reached 98%, over 20% higher than in internode 3.

Table 5.1 Percentage Purity (sucrose:total sugars) of extracts from sugarcane tissues. Values are the mean of at least three samples.

Tissue	% purity	Tissue	% purity
Internode 3	74.2	Leaf roll	45.2
Internode 6	88.7	Young leaf	84.3
Internode 9	98.2	Medium leaf	94.1
Internode 12	98.7	Old leaf	96.5
Internode 15	98.6	Root	98.2
Internode 17	99.5		

Sugar levels in internodal tissues showed significant correlations with protein contents (determined as relative density values from western blot analysis). From data of sugars and protein between internodes 3 and 15, it would appear that the higher the sucrose content the lower the SNI content (*figure 5.5A*), although the relationship was not significant. Positive correlations between either fructose or glucose and SNI protein content (*figure 5.5B and C*) were evident.

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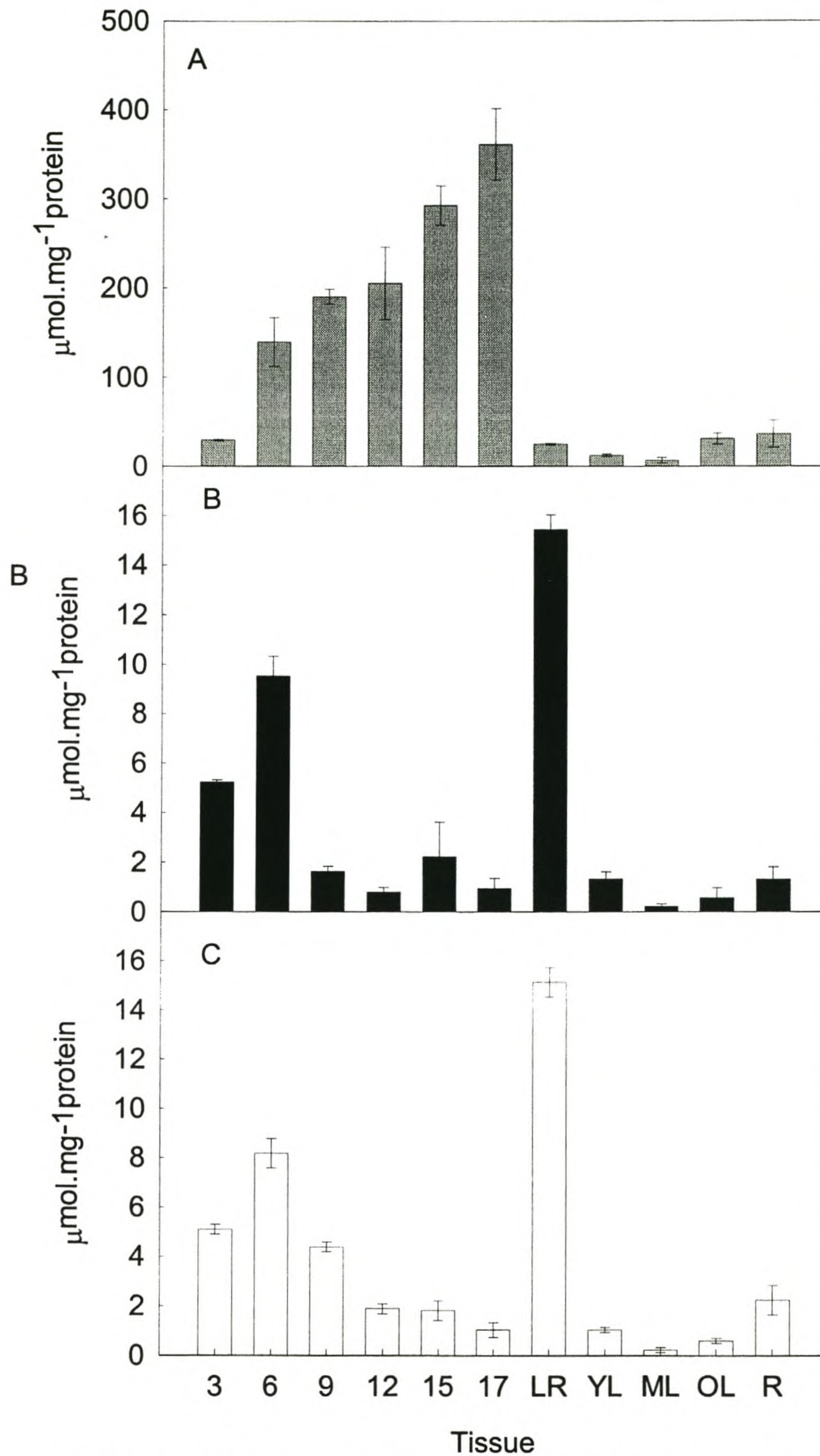

Figure 5.4 Soluble sugars were extracted from the same tissue samples as RNA and protein (internodes 3, 6, 9, 12, 15 and 17; leaf roll (LR), young leaf (YL), medium leaf (ML), old leaf (OL) and root (R)) and levels determined using an enzyme coupled spectrophotometric assay. Panels A, B and C show contents of sucrose, glucose and fructose, respectively. Data points are the mean of at least three samples ± se.

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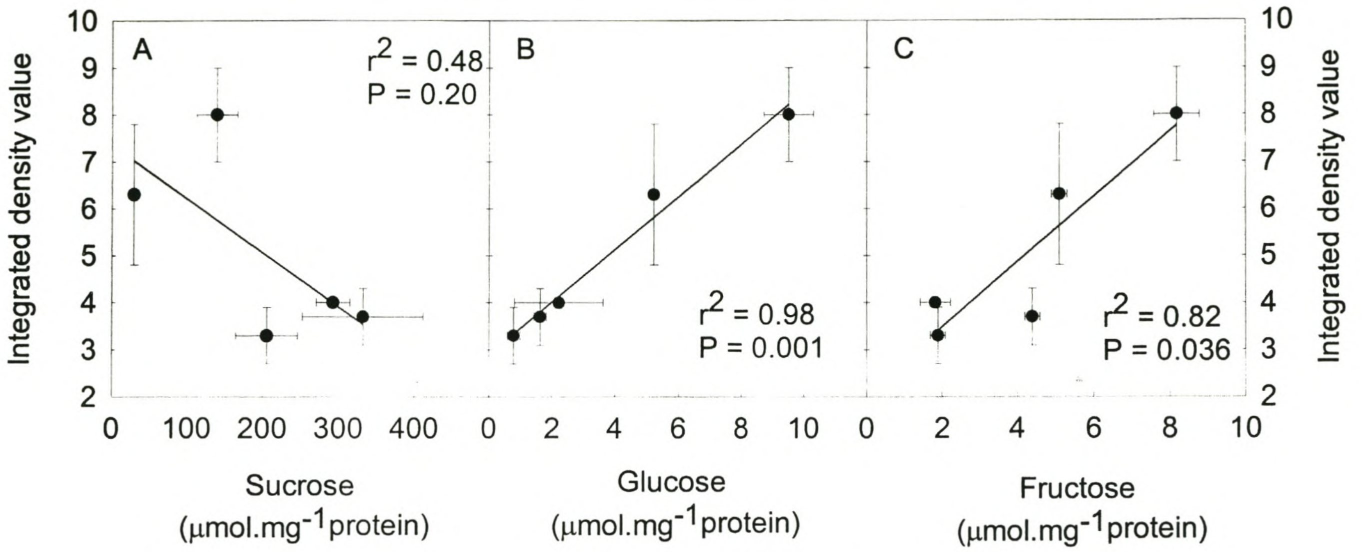

Figure 5.5 Neutral Invertase protein content (integrated density value calculated from western blot analysis) plotted against sucrose (A), glucose (B) and fructose (C) content from the same tissues. Data points are the mean of at least three samples ± se.

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5.4 Discussion

Because of its constitutive presence in the cytosol, NI has access to sucrose being translocated from one compartment to another within the cell. In sugarcane, the activity (measured on both a fresh weight and protein mass basis) of this enzyme has been widely studied both in isolation and in context with other sucrose metabolising enzymes (Hatch et al., 1963; Del Rosario and Santisopasri, 1977; Batta and Singh, 1986; Lingle and Smith, 1991; Zhu et al., 1997; Ebrahim et al., 1998; Vorster and Botha, 1998; Vorster and Botha, 1999; Rose and Botha 2000). Observations drawn from available literature are that (i) SNI activity increases proportionally to its protein content, (ii) SNI levels are variable within the same tissue between seasons, (iii) SNI activity appears to be highest in immature tissues, and (iv) SNI activity is not solely responsible for the levels of accumulation of sucrose in storage tissues, but forms an integral part of the cycle of sucrose accumulation.

From the Southern analysis it is evident that SNI has a fairly simple genetic pattern (*figure 5.1*). The fact that there were two major bands evident from gDNA restricted with an endonuclease that cuts once in the probe (a full length SNI cDNA fragment), could suggest that there are few, possibly only one, isoform of the enzyme at gene level. In support of this, carrot NI is potentially a single copy gene (Sturm et al., 1999). From a biochemical point of view this makes the potential study and manipulation of this enzyme considerably easier than if it was the product of a multigene family.

Until recently, the gene sequence for SNI was not available (see *chapter 4, section 4.3.1*). Obtaining the full-length cDNA allowed us to examine the expression of SNI at transcript level. Northern analysis confirmed that transcripts were present in all tissue types in the sugarcane plant: leaves, culm and root tissues. The constitutive expression of SNI transcript (*figure 5.3A*) seems to be at low levels throughout the plant. There appears to be a higher transcript expression in internodes 3 and 6 than other tissues (*figure 5.3A*). These are tissues where active growth is occurring (Lingle and Smith, 1991) and also where high SNI activities have been measured (Ebrahim et al., 1998; Vorster and Botha, 1999).

Protein blots (*figure 5.3B*) confirm results of previous studies of SNI distribution, where activity peaked between internodes 4 and 6 and decreased again in the older internodes (Zhu et al., 1997; Ebrahim et al., 1998; Vorster and Botha, 1999).

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Sucrose, glucose and fructose contents were determined in all tissues (*figure 5.4A, B and C)*. Leaf tissues showed the lowest sucrose levels (μmol.mg^{-1} protein), while the disaccharide's content increased from young to mature tissues in the culm (*figure 5.4A*). A trend is apparent in the culm: that in young tissues where sucrose is low, SNI transcript and protein levels are higher than in mature tissues (*figure 5.3A and B, figure 5.5A),* suggesting that the enzyme could make a contribution to the supply of hexoses in young tissues.

The plant material used in this investigation (sugarcane, variety N19) was harvested early in the spring. This variety is characterised by its rapid growth, and therefore requires hexoses for growth and biosynthesis. SNI is an enzyme potentially responsible for the supply of these metabolites. In support of this, the high hexose contents in leaf roll (a tissue dedicated to growth) resulted in a purity (sucrose:total sugars) of under 50% (*table 5.1*). In this tissue SNI protein levels were also higher than other leaf tissues (*figure 5.3B*).

Between internodes 3 and 15 a positive correlation between SNI protein content and both hexoses was observed (*figure 5.5B and C*). We have not identified a correlation like this previously (Rose and Botha, 2000), and along with this the lack of significant relationship between the enzyme content and sucrose here does not concur with what we have previously reported (Rose and Botha, 2000, *figure 3.3*). This possibly means that the relationship between SNI and its substrate and products are not causal across seasons, but rather dependent on the metabolic environment determined by environmental and cyclic factors. It is also possible that because having attempted to correlate a cytoplasmic enzyme with total cell sugar levels the true relationship has been masked.

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5.5 Conclusions

SNI is expressed in all tissues of the sugarcane plant. A similar trend in distribution is observed in transcript and protein levels where a slightly higher activity is observed in young growing tissues. SNI is an important enzyme in the cycle of sucrose accumulation displaying trends between its protein level and both its substrates and product, confirming it as a good target for genetic manipulation.

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CHAPTER 6

Sucrose hydrolysis and carbon partitioning in low and high sucrose-storing sugarcane genotypes

(Manuscript in preparation)

Abstract

It was hypothesised that the high sucrose-storing capacity of a sugarcane genotype would be partly dependant on the rate of sucrose breakdown. Sucrose content was 10-fold higher in maturing internodes of variety N19 than US6656-15. Fluxes involved in sucrose synthesis and hydrolysis were investigated using equimolar hexoses and [^{14}C]-fructose as a carbon source for tissue discs of young and maturing internodal tissue of sugarcane, varieties N19 and US6656-15. In internode 7 of both varieties over 30% of the label in the water soluble fraction was recovered in sucrose after a 4 h incubation period. The amount of label incorporated into glucose via invertase hydrolysis was 3 times higher in mature internodal tissues of US6656-15 than in N19. Taking metabolic compartmentation into account, hydrolysis of sucrose via invertase made a significant contribution to the net turnover of sucrose. However, there appear to be other factors influencing the net accumulation of sucrose in storage tissues of sugarcane.

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6.1 Introduction

During the stage of sucrose accumulation in sugarcane, carbon is channelled away from insoluble cell components, respiratory and phosphorylated metabolites, amino and organic acids into sucrose (Whittaker and Botha, 1997). Sucrose accumulation results from the difference between synthesis and degradation. Enzymes responsible for both synthesis and degradation have been identified in tissues of sucrose accumulation in cell suspension cultures (Wendler et al., 1990) and internodes (Zhu et al., 1997; Ebrahim et al., 1998). Sucrose is accumulated against a concentration gradient (i.e. accumulating more sucrose in more mature tissues as opposed to spreading the concentration evenly between internodes), aided by intracellular compartmentation. Three compartments involved in the process of sucrose accumulation were identified in early investigations, and termed the outer space (apoplast and cell walls), metabolic compartment (cytosol) and storage compartment (vacuole) (Sacher et al., 1963). A cycle involving synthesis and degradation of sucrose has been described in both young and old tissues (Whittaker and Botha, 1997; Vorster and Botha, 1999; Bindon and Botha, 2000).

In sugarcane internodal tissues, many steps in the cycle of sucrose accumulation have been implicated as key points of regulation. These focal points have included the enzymes responsible for synthesis: SPS and SuSy (Botha and Black, 2000) and degradation: AI and SuSy, and to a lesser degree NI (Zhu et al., 1997; Ebrahim et al., 1998; Vorster and Botha, 1999; Botha and Black, 2000; Rose and Botha, 2000). The majority of these studies have been based on data gained in *in vitro* experiments (Lingle, 1997; Zhu et al., 1997; Ebrahim et al., 1999; Ebrahim et al., 1998; Botha and Black, 2000; Rose and Botha, 2000). Maximum extractable activities of the above mentioned enzymes have been correlated with absolute sugar contents in an effort to identify which enzymes appear to play crucial roles in accumulation. SPS is regarded as the enzyme responsible for the bulk of synthetic reactions, as its activity exceeds that of SuSy up to three-fold in older internodes (Botha and Black, 2000). Significant positive correlations have been reported between sucrose and SPS (Botha and Black, 2000), and between sucrose and the difference between SPS and AI (Zhu et al., 1997; Ebrahim et al., 1998). No significant correlation between SuSy activity and sucrose content has been observed (Botha and Black, 2000). In light of this, SPS and AI have been implicated as important catalysts for the consequent accumulation of sucrose. The contribution of SNI to net turnover of sucrose has not been studied as extensively, and until recently no correlation between its activity

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(even in combination with other enzymes) and sucrose content has been reported. A significant negative relationship between SNI and sucrose content has been observed across young and old internodes, with a tight correlation in the growing section (bottom) of internodes throughout the culm (Rose and Botha, 2000). Because of its cytosolic positioning, SNI has the potential to degrade all synthesised sucrose if its activity exceeds the ability of the cell to synthesise and partition the sucrose into the storage compartment. This makes SNI an important enzyme to remember when examining sucrose accumulation.

Taking these studies into account, further work examining aspects of synthesis and degradation in an intact tissue system was required. Using radio-labelled carbon sources the relative fluxes into and out of sugar pools can be calculated. In a commercial, high-sucrose storing variety, flux into sucrose increased almost 300 % between immature and actively accumulating tissues (Bindon and Botha, 2000). Return of label from sucrose into fructose after feeding labelled glucose gives an indication of net sucrose degradation. This degradative flux was detected only in immature tissues after a 3 h labelling period. The net turnover in all tissues was high enough to facilitate accumulation of sucrose.

However, despite all these studies, factors directly affecting net accumulation are still unclear. Using young and maturing internodes from both an ancestor non-sucrose storing variety (US6656-15) and a commercial variety (N19) (also comparing seasonal variation in this variety) we attempted to determine whether sucrose accumulation is significantly influenced by (i) sucrose synthesis rates, (ii) sucrose hydrolysis, (iii) partitioning of source carbon into water solubles other than sugars, or (iv) compartmentation of sucrose into the vacuole.

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6.2 Materials and methods

Mature plants of sugarcane (variety N19) were harvested in the early morning in winter (August) and summer (January) from Welgevallen experimental farm in Stellenbosch, South Africa. Sugarcane, variety US6656-15, was harvested in early morning in summer from tunnel grown plants in Stellenbosch, South Africa. Internode one of the culm was defined as the internode attached to the leaf with the first exposed dewlap. Internode 4 was selected to represent immature tissue. Internode 7 represented tissue already actively accumulating sucrose. Tissues samples of each section were taken from three individual culms. The internodes were divided in half to render top and bottom sections of the internode. Longitudinal sections, with a diameter of 6 mm, were excised using a cork borer. One mm think slices of the sections were cut using a hand microtome and the discs then immersed in wash buffer (25 mM K-Mes (pH 5.7), 250 mM mannitol).

6.2.1 [^{14}C]-radiolabelling experiments

Tissue discs were washed for at least 15 min. Excess liquid was removed and 20 discs were placed in a 250 mL Erlenmeyer flask containing 1.5 mL incubation buffer (25 mM K-Mes (2-[N-morpholino]ethanesulfonic acid (potassium salt) (pH 5.7), 250 mM mannitol, 5 mM glucose, 5 mM fructose, and [U-^{14}C]-fructose as noted in the results section). Tissue was vacuum infiltrated for approximately 5 seconds and the flasks sealed with rubber stoppers. Tissue was incubated for 4 h on a rotary shaker at room temperature. Ten discs were removed after 2 h for sugar and label incorporation determinations. $^{14}CO_2$ released during incubation was collected at various time points in vials containing 500 μL 12% (w/v) KOH. For CO_2 determination, 4 mL scintillation cocktail (Ultima Flo™ M, Packard) was added to the KOH vials and counted for 10 min in a Beckman LS 1801 scintillation counter to determine released radioactivity. On completion of incubation, tissues were rinsed with ice-cold ddH_2O and washed for 10 min in wash buffer (25 mM K-Mes (pH 5.7), 250 mM mannitol, 1 mM $CaCl_2$). Excess buffer was removed and tissues flash frozen until extraction.

6.2.2 Sugar extractions

Disks were chopped and extracted in buffer containing 50 mM Tris (pH 7) and 70 % (v/v) ethanol in a ratio 10:1 (discs:mL buffer), at 70°C overnight. Extracts were then

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centrifuged at 13 000 rpm for 10 min and the supernatant removed and transferred to a new tube. The remaining pellet was washed with 400 μL extraction buffer, centrifuged as before, supernatant removed and added to the original one mL. This rendered all solubles from the extract in a volume of 1.4 mL. 20 μL of this was counted in 4 mL scintillation cocktail (Ultima Flo™ M) and counted for 10 min in a Beckman LS 1801 scintillation counter to determine radioactivity in total soluble fraction. 1 mL of the total solubles were passed through Alumina A and TC-18 cartridges in tandem to remove all acidic and aromatic compounds, rendering an extract containing only the neutral fraction, i.e. the sugars. The neutral fraction was reduced completely in a vacuum centrifuge. The dried sugars were resuspended in 200 μL 10 % (v/v) isopropanol and prepared for HPLC (high performance liquid chromatography) separation by passing through 4μm filters (Millipore).

6.2.3 HPLC analysis of [^{14}C]-sugars

Sugars were separated by HPLC (high performance liquid chromatography, Shimadzu SCL-10AVP system). Samples were fractionated using a Supelco™ LC-NH_2 column. The run time was between 12 and 16 minutes, with a flow rate of 1.5 $mL.min^{-1}$ using 80% (v/v) HPLC-grade acetonitrile as the mobile phase. Amounts of ^{14}C in separated sugars were determined by liquid scintillation chromatography (Radiomatic A-500 inline radio-chromatography detector).

6.2.4 Enzymatic sugar determinations

Sucrose, glucose and fructose were determined from the same samples passed through the HPLC using an enzyme-coupled spectrophotmetric assay (Bergemeyer and Bernt, 1974) adapted to microtiter plate format as previously described (*chapter 5, section 5.2.8)*

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6.3 Results

6.3.1 Seasonal variations

6.3.1.1 Sugars

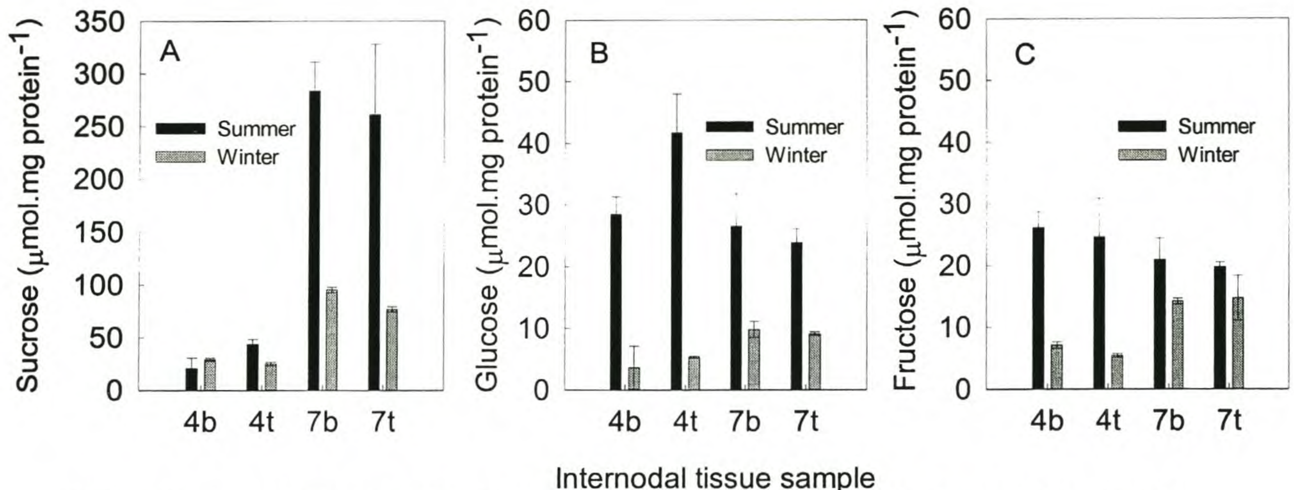

Figure 6.1 Sugar content (sucrose (A), glucose (B)and fructose (C)) of N19 internodal tissues in summer (late December) and winter (mid August). The bottom and top of internodes 4 (4b and 4t) and internode 7 (7b and 7t) were sampled. Data are the mean of at least 3 reps ± se.

Sucrose levels in internode 7 of N19 were three times higher in the summer than winter (*figure 6.1A*). In all tissues sampled hexose contents were higher in summer than winter (*figure 6.1B and C*). Young growing tissues exhibited up to 8-fold more hexoses in the summer as compared with winter contents.

6.3.1.2 Flux into sugars

In order to determine flux into different sugar pools, tissue discs were incubated in a medium containing [^{14}C]-fructose for 4 h. Thereafter sugars were extracted, fractionated, and concentration and label incorporation determined.

In previous studies examining the differences of sugar content and enzyme activities at temperatures above and below optimum, marked differences were noted. Both hexose and sucrose contents were higher in all internodes in canes grown at 27°C as opposed to 15°C. Maximum extractable activities of SPS, SuSy, SAI and NI were lower in all internodes of tissues grown at lower temperatures (Ebrahim et al., 1998). In light of what appears to be a general decrease in metabolism in tissues grown at

temperatures below optimum, we made the specific activity of the external medium higher in the winter than the summer. This was in order that return of label into all pools of interest could be detected after a 4 h incubation. In the summer, experimental conditions were such that external specific activity of fructose was 37 Bq.nmol^{-1}, and 55.5 Bq.nmol^{-1} in the winter. We have taken this into account in our calculations by expressing data as percentages of a standard pool in each season.

In the winter all tissues incorporated similar amounts of label into sucrose (*table 6.1*). However, in the summer incorporation of label differed almost 2 fold, increasing from internode 4 to internode 7 (*table 6.2*).

Table 6.1 Incorporation of label into components of the sugar pool in tissues sampled in the winter. Labelling medium contained [^{14}C]-fructose (55.5 Bq.nmol^{-1}). Values are the mean of 2 reps ± se.

Tissue section	kBq.mg^{-1}protein		Percentage
	Sucrose	Glucose	Glucose/Sucrose
4b	7.0 ± 0.5	0.29 ± 0.03	4.20 ± 0.09
4t	9.1 ± 0.9	0.24 ± 0.02	2.63 ± 0.04
7b	6.7 ± 0.2	0.26 ± 0.01	3.92 ± 0.24
7t	6.5 ± 0.7	0.24 ± 0.01	3.75 ± 0.64

Table 6.2 Incorporation of label into components of the sugar pool in tissues sampled in the summer. Labelling medium contained [^{14}C]-fructose (37 Bq.nmol^{-1}). Values are the mean of 3 reps ± se.

Tissue section	kBq.mg^{-1}protein		Percentage
	Sucrose	Glucose	Glucose/Sucrose
4b	7.8 ± 0.5	2.38 ± 0.67	29.61 ± 0.07
4t	8.9 ± 2.3	0.31 ± 0.02	3.94 ± 0.01
7b	13.9 ± 1.2	0.32 ± 0.05	2.36 ± 0.00
7t	16.9 ± 1.6	0.43 ± 0.06	2.59 ± 0.01

In tissues of internode 4 the percentage label in glucose as a function of label in sucrose was higher in the summer than winter (*tables 6.1* and *6.2)*. This is especially evident in the bottom region of the internode where in the summer the ratio of label in glucose to sucrose is over 7 times higher than any other tissue in the summer or winter.

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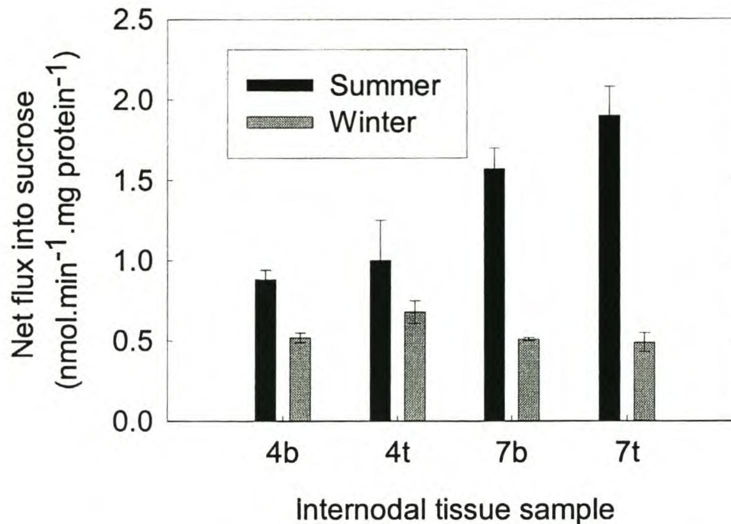

Figure 6.2 Net flux into sucrose (nmol.min^{-1}.mg^{-1}protein) calculated from the change in amount of label in the sucrose pool over 4 h as a function of the external medium specific activity (55.5 and 37 Bq.nmol^{-1} in the winter and summer respectively). Data points are the mean of 2 or 3 reps (winter and summer respectively) ± se.

Assuming the internal hexose pool was in equilibrium with the external labelling medium, net flux rates into sucrose were determined from the increase in label appearing in the sucrose pool as a function of the cytosolic hexose specific activity (*figure 6.2*). In all tissues, net flux into sucrose was higher in the summer than the winter. In internode 4 tissues the difference was 1.5 fold. In more mature tissues, the increase in sucrose turnover from winter to summer was over 300 %. This data is an underestimation of the true rate values, as the internal specific activity used for calculations had not yet reached isotopic equilibrium with the external medium. In light of this, trends can be noted, but conclusions from absolute values are risky. However, the apparent increase in net turnover accounts for the increase in sucrose content as seen in figure 6.1.

Sucrose hydrolysis increased in all tissues from the winter to the summer (*table 6.3*). This data was calculated using the determined internal specific activity of sucrose.

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Table 6.3 Net flux into glucose calculated using sucrose internal specific activity after 4 h incubation with [^{14}C]-fructose (55.5 and 37 Bq.nmol^{-1} in winter and summer respectively. Values are the average 2 or 3 reps (winter and summer respectively) ± se.

Tissue section	Net flux into glucose (nmol.min^{-1}.mg^{-1}protein)	
	winter	summer
4b	5.6 ± 1.0	14.0 ± 3.9
4t	3.0 ± 0.4	7.0 ± 1.4
7b	14.1 ± 0.8	24.9 ± 4.4
7t	11.2 ± 1.7	29.5 ± 6.2

6.3.2 Variation between varieties

Plant material was harvested in early summer. A low sucrose storing variety, US6656-15, and a high sucrose storing variety, N19, were selected. Internodal tissues were sectioned into discs and incubated in a medium containing [^{14}C]-fructose (37 Bq.nmol^{-1}) for 4 h. The consequent distribution of label in the total soluble pool, with special attention to the sugars, was determined. Protein content between the two varieties did not differ, although a decrease in protein content from internode 4 (0.036 mg.disk^{-1}) to internode 7 (0.026 mg.disk^{-1}) was observed.

6.3.2.1 Sugars

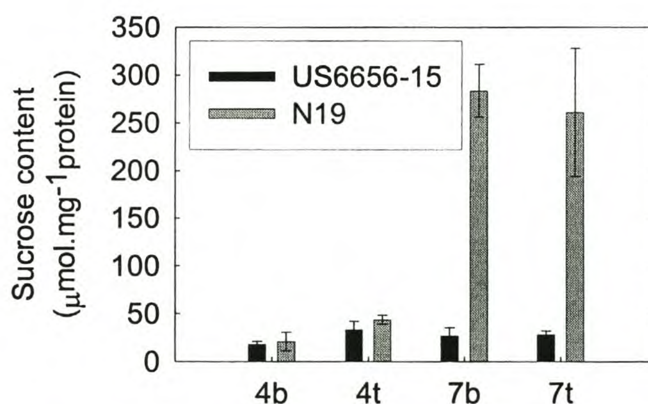

Figure 6.3 Sucrose content (µmol.mg^{-1}protein) in low sucrose storing (US6656-15) and high sucrose storing (N19) sugarcane varieties. Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively). Data points are the mean of 3 reps ± se.

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In immature tissues (internode 4) sucrose content was similar between varieties. Sucrose content of maturing culm tissue differed dramatically between varieties. In internode 7, US6656-15 had a sucrose content ($\mu mol.mg^{-1}$protein) at least nine-fold lower than the same tissue of N19 (*figure 6.3*). In young internodes (internode 4) glucose content was higher in N19 than US6656-15 (*table 6.4*).

Table 6.4 Hexose content ($\mu mol.mg^{-1}$protein) in low sucrose storing (US6656-15) and high sucrose storing (N19) sugarcane varieties. Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively). Values are the mean of 3 reps ± se.

Tissue section	$\mu mol.mg^{-1}$protein			
	Glucose		Fructose	
	US6656-15	N19	US6656-15	N19
4b	10.3 ± 1.0	28.5 ± 2.8	9.8 ± 2.8	26.1 ± 2.6
4t	23.5 ± 4.4	41.7 ± 6.3	28.2 ± 3.6	24.6 ± 6.3
7b	25.5 ± 3.6	26.5 ± 5.3	12.7 ± 5.1	20.9 ± 3.5
7t	28.8 ± 4.3	23.9 ± 2.2	24.1 ± 2.6	19.7 ± 0.8

6.3.2.2 ^{14}C-label distribution

Table 6.5 Incorporation of label into the total soluble pool after 4h labelling incubation in [^{14}C]-fructose. Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of varieties US6656-15 and N19. Values are the mean of 3 reps ± se.

Tissue section	Total solubles ($kBq.mg^{-1}$protein)	
	US6656-15	N19
4b	29.3 ± 2.7	29.3 ± 1.8
4t	25.0 ± 1.5	18.9 ± 4.7
7b	33.9 ± 6.2	30.6 ± 1.1
7t	38.6 ± 1.6	35.1 ± 1.3

After a 4h labelling period incorporation of ^{14}C into the total soluble pool was similar between varieties (*table 6.5*). The cytosolic pool of US6656-15 reached isotopic equilibrium faster than that of N19. This is seen by the apparent tendency towards steady release of CO_2 in US6656-15 giving a near linear response(r^2 = 0.964) (*figure 6.4*) as opposed to N19 (r^2 = 0.936).

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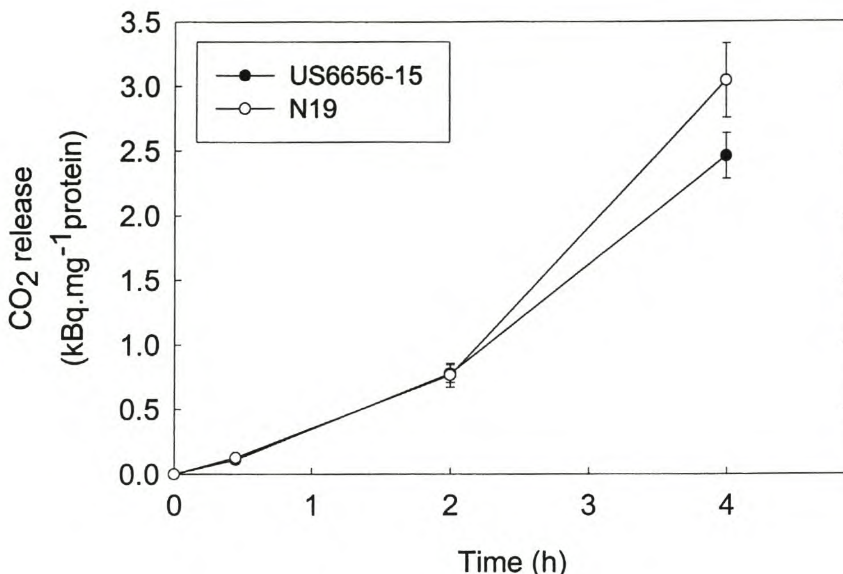

Figure 6.4 Release of $^{14}CO2$ over a 4 h labelling period using [^{14}C]-fructose as a source of label. Sugarcane internodal tissue discs from varieties US6656-15 (A) and N19 (B) were used. Data are the mean of 12 reps ± se.

6.3.2.3 *Labelled sugars*

Over the 4 h labelling period, percentage incorporation of label into sucrose (as a function of the total solubles) did not differ significantly between varieties. In internode 7 tissues of both varieties, over 30% of label in the total soluble pool was recovered in sucrose. Within the total soluble pool, the percentage incorporation of label into sucrose was higher in the bottom of maturing than immature internodal tissues. In the top of the internode the difference was not evident (*table 6.6*).

Profiles of the amount of label incorporated into sucrose over 4 h differ between varieties. It is apparent in internode 7 tissues of N19 that the capacity to label the sucrose pool has not reached saturation (*figure 6.5B*). However, in three of the tissues of US6656-15 sampled the incorporation of label into sucrose appears to be reaching its maximum (*figure 6.5A*).

Table 6.6. Label distribution in total solubles pool after a 4 h incubation with [^{14}C]-fructose. Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of varieties US6656-15 and N19. Values are the mean of 3 reps ± se.

Variety	Internodal tissue	% label as a proportion of total solubles			
		Fructose	Glucose	Sucrose	Total solubles (excl. sugars)
US 6656-15	4b	9.1 ± 1.6	3.6 ± 0.4	24.3 ± 4.4	63.0 ± 6.4
	4t	11.6 ± 0.9	3.4 ± 0.4	34.8 ± 1.2	50.2 ± 2.4
	7b	4.7 ± 0.9	2.0 ± 0.8	31.4 ± 8.8	62.0 ± 9.1
	7t	7.2 ± 0.3	2.8 ± 0.6	39.0 ± 1.9	51.0 ± 2.4
N19	4b	12.6 ± 1.6	5.7 ± 1.3	19.1 ± 1.2	62.7 ± 3.5
	4t	8.9 ± 1.8	1.3 ± 0.3	33.4 ± 0.3	56.4 ± 2.1
	7b	7.0 ± 0.9	0.8 ± 0.1	32.4 ± 1.6	59.9 ± 2.0
	7t	7.5 ± 0.9	0.9 ± 0.1	34.2 ± 2.0	57.3 ± 1.0

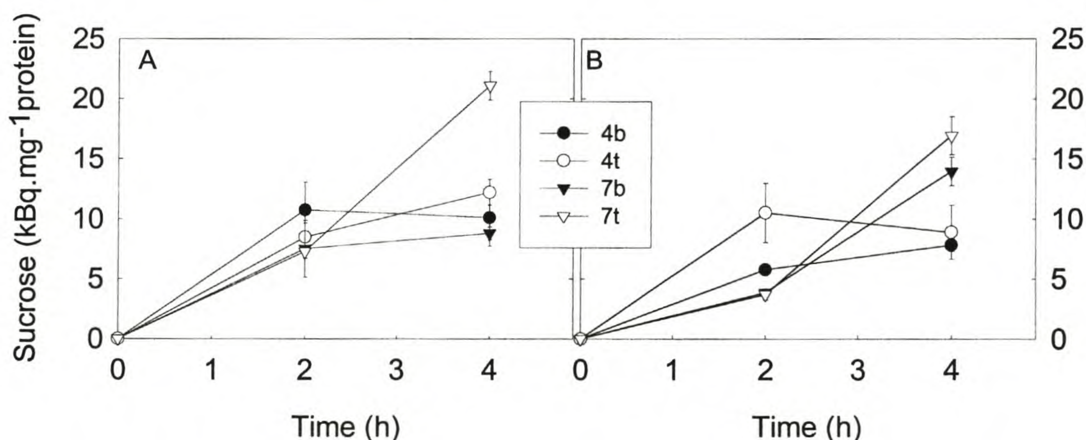

Figure 6.5 Incorporation of label into the sucrose pool (kBq.mg^{-1}protein) over a 4 h incubation in [^{14}C]-fructose (37 Bq.nmol^{-1}). Sugarcane varieties US6656-15 (A) and N19 (B) were selected. Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively). Data points are the mean of 3 reps ± se.

This is further emphasised by the 9 fold difference in internode 7 internal specific activities of sucrose (Bq.µmol^{-1}) between US6656-15 and N19 (*table 6.7*). The

difference is caused by the sucrose content and not the amount of label incorporated into the sucrose pool (*figure 6.3* and *figure 6.5*).

Table 6.7 Internal sucrose specific activity (Bq.μmol^{-1}) after a 4 h incubation in a medium containing [^{14}C]-fructose (37 Bq.nmol^{-1}). Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of varieties US6656-15 an N19. Values are the mean of 3 reps ± se.

Tissue section	Sucrose	
	US 6656-15	N19
4b	550.9 ± 76.3	508.7 ± 159.4
4t	395.2 ± 79.9	211.5 ± 34.5
7b	270.1 ± 45.9	54.6 ± 3.6
7t	762.4 ± 102.4	62.4 ± 6.7

6.2.3.4 Sucrose turnover

Net turnover of sucrose was determined as the difference in appearance of label into the sucrose pool over a specific time frame. Over the 4 h labelling period US6656-15 and N19 rates of sucrose turnover (Bq.min^{-1}.mg^{-1}protein) did not reflect the observed differences in sucrose contents. In fact, US6656-15 displayed slightly higher rates of net flux into sucrose in the top of internode 7 (*figure 6.6*) where its content was ten times lower than that of N19 (*figure 6.3*).

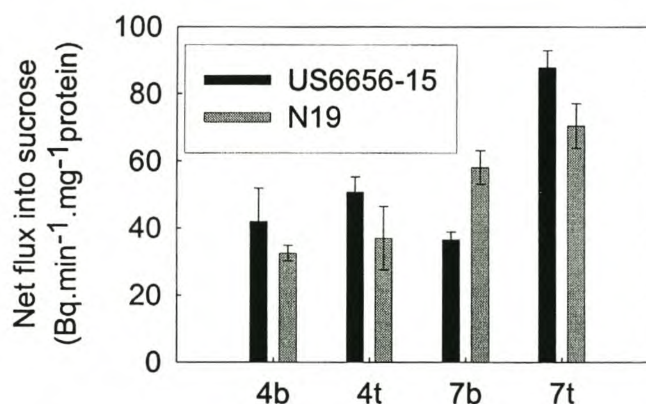

Figure 6.6 Net flux into sucrose (Bq.min^{-1}.mg^{-1}protein) after a 4 h incubation with [^{14}C]-fructose (37 Bq.nmol^{-1}). Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of varieties US6656-15 an N19. Data points are the mean of 3 reps ± se.

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Label recovered in the glucose pool after labelling with [^{14}C]-fructose is a result of invertase hydrolysis, as the only pathway for the production of free glucose from fructose is via hexose phosphorylation and subsequent randomisation of label in the moieties of sucrose and its hydrolisation by one of the invertases. This can therefore give us an indication of the relative contribution of invertase to sucrose cycling between different tissues- whether internode or variety.

Table 6.8 Incorporation of label into glucose after 4 h labelling internodal tissues in [^{14}C]-fructose (37 Bq.nmol^{-1}). Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of variety US6656-15. Values are the mean of 3 reps ± se.

Tissue section	Glucose (kBq.mg^{-1}protein)
4b	1.5 ± 0.3
4t	1.2 ± 0.2
7b	1.0 ± 0.4
7t	1.5 ± 0.3

After 4 hours of labelling, three of the US6656-15 tissues incorporated over 3 times more label into glucose than those of N19 (*tables 6.2 and 6.8*). The exception were tissues sectioned from the bottom of internode 4; where N19 partitioned over one and a half times more label into glucose than US6656-15.

Assuming the cytosolic sucrose pool was in isotopic equilibrium with that of the external medium (37 Bq.nmol^{-1}), the rate of sucrose hydrolysis (nmol.min^{-1}.mg^{-1}protein) in N19 was only higher than that of US6656-15 in the bottom of internode 4 (*figure 6.7A*).

Assuming the cytosolic and vacuolar pools were in isotopic equilibrium and that sucrose concentration was the same between the two compartments, flux into glucose was calculated using the measured internal specific activity (*figure 6.7B*). This produced a very different looking set of data, with N19 hydrolysis rates higher in three of the four tissues sampled.

Assuming that in US6656-15 the sucrose is exclusively in the cytosol sucrose hydrolysis rates were calculated from measured specific activities. Assuming that 90% of the sucrose in N19 tissues was in the vacuole, and only 10% of the labelled sucrose had been transported into the vacuole, sucrose hydrolysis rates were

calculated from a sucrose specific activity nine times lower than the measured amount. It is evident, therefore, that sucrose hydrolysis rates were higher in US6656-15 than N19 (*figure 6.7C*) in all tissues sampled.

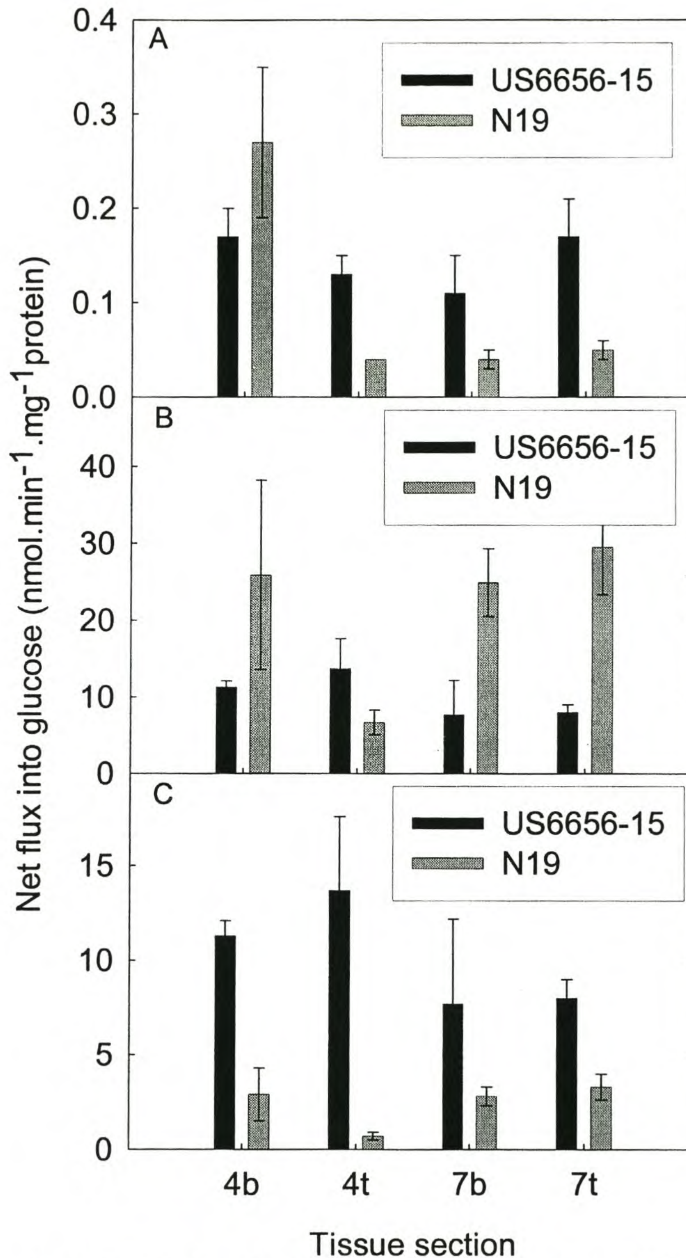

Figure 6.7 Net flux into glucose as an indication of invertase hydrolysis. Calculated from the appearance of ^{14}C-glucose after a 4 h incubation in [^{14}C]-fructose (37 Bq.nmol^{-1}) and external specific activity (A), internal sucrose specific activity (B) and adjusted internal sucrose specific activity (C). Tissue samples were sectioned from the bottom and top of internode 4 (4b and 4t, respectively), the bottom and top of internode 7 (7b and 7t, respectively) of varieties US6656-15 an N19. Data points are the mean of 3 reps ± se.

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6.4 Discussion

6.4.1 Increase in net flux into sucrose increases sucrose content between seasons

In order to further elucidate the mechanisms of sucrose accumulation in sugarcane internodal tissues, two varieties were selected (US6656-15 and N19). Seasonal variations were also determined in one variety (N19) to compare parameters involved in the process of sucrose storage in sugarcane. US6656-15 is a low-sucrose storing variety. In contrast, N19 is a high-sucrose storing variety whose best features (besides its early season, high sucrose storing ability) include rapid germination, canopy formation and growth. Young developing (internode 4) and actively accumulating (internode 7) tissues were sampled. We further divided the internodes into sections distinguished by morphological and anatomical features (Rose and Botha, 2000). The bottom section is viewed as the cell division and growth area, whereas the top does not continue growing and active sucrose accumulation is thought to be the main function.

The two varieties differ very evidently in their sucrose contents (μmol.mg protein^{-1}). N19 shows sucrose content profiles of a commercial sugarcane variety; increasing up to 10-fold from internode 4 to internode 7 (*figure 6.3*). However, this was not the case with tissues of US6656-15, where no sucrose gradient between internodes was evident.

Between seasons, N19 also shows a 3-fold increase in sucrose content in internode 7 tissues (*figure 6.1A*). Higher sucrose contents are evident in the summer, as tissues are both actively growing and storing sucrose in the warmer months (Lingle, 1997).

Incubation of N19 tissue discs in medium containing [^{14}C]-fructose resulted in incorporation of label into both the sucrose and glucose pools after 4 h. Even though the specific activity of the external medium was 1.5 times lower in the experiments done in the summer, incorporation of label into the above mentioned sugar pools was higher in all tissues sampled in the summer than the winter (*tables 6.1* and *6.2*). The amount of label incorporated into the sucrose pool was similar in all tissues in the winter (*table 6.1*), possibly a result of lower accumulating activity in this season. In the summer a clear distinction between internodes 4 and 7 is illustrated by a higher incorporation of label into the sucrose pool of internode 7 (*table 6.2*). This could be

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indicative of an increased sucrose storage function in the summer months, a characteristic of this variety. In support of this, net flux into sucrose ($nmol.min^{-1}.mg^{-1}$protein) was significantly higher in internode 7 than internode 4 in tissues sampled in the summer (*figure 6.2*). Net turnover of sucrose was higher in all tissues in the summer. What is important to note about net flux into sucrose, is that there was a difference between seasons, and that in the summer there was a distinction between young and maturing internodes. The calculated net flux into sucrose is determined by assuming that the cytosolic fructose pool is at isotopic equilibrium with the external medium. However, determined internal specific activities for fructose are not close to the external value. Although this value is diluted by the vacuolar pool of hexoses, the cytosolic pool will still have a lower specific activity than that of the external medium. Therefore calculations using these values underestimate the actual flux rate.

Hexose contents were higher in all tissues in the summer as opposed to the winter (*figure 6.1A* and *B*). Incorporation of label into the glucose pool as a function of label in the sucrose pool was higher in summer months in young tissues (*tables 6.1* and *6.2*). Especially notable is the large percentage of label in the glucose pool of tissues from the bottom of internode 4 in the summer. This tissue is conventionally regarded as dedicated to growth. Partitioning of label into the glucose pool could therefore indicate an increased supply of substrate for growth and respiration. Calculating the net flux into glucose from sucrose (assuming the cytosolic sucrose pool was at equilibrium with the external fructose pool) showed an overall increase in hydrolysis rates between seasons (*table 6.2*). This supports the hypothesis that metabolism was upregulated in the summer, in this case to possibly supply substrates for growth.

The increase in net flux into sucrose ($nmol.min^{-1}.mg^{-1}$protein) between seasons was always high enough to compensate for the observed difference in sucrose content.

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6.4.2 *Partitioning of sucrose to the vacuole could cause differences in accumulation between varieties*

Tissues from varieties N19 (commercial, high-sucrose storer) and US6656-15 (ancestor, low sucrose-storer) were assayed in the summer.

Both varieties incorporated similar amounts of label into the total soluble pool after a 4 h incubation in [^{14}C]-fructose (*table 6.5*). This can, therefore, not be the point, between varieties, at which assimilates are differentially channelled in order to result in the significant differences in accumulated sucrose content (see *figure 6.3*).

Percentage of ^{14}carbon in the total soluble pool directed into sucrose did not differ between varieties (*table 6.6*). This could therefore not account for the 10-fold difference in sucrose content evident in maturing tissues between varieties. N19 partitions a lower percentage carbon into glucose in older internodes than US6656-15. This could potentially affect the net accumulation of sucrose.

In maturing internodes, sucrose content in US6656-15 was only 10% of that determined in N19 (*figure 6.3*). However, over a 4 h incubation in medium containing 37 Bq.nmol^{-1} [^{14}C]-fructose incorporation of label into sucrose (KBq.mg protein^{-1}) showed different patterns (*figure 6.5*). In three of the four tissues of US6656-15, incorporation of label into the sucrose pool appears to be saturated after only 2 h of the labelling (*figure 6.5A*). This suggests that the entire sucrose pool had been labelled. This can only occur if there is either a very rapid exchange of assimilates between the cytosol (where sucrose is synthesised) and the vacuole (where sucrose could be stored), or if the cell does not have the ability to partition sucrose to the vacuole effectively. The high-sucrose storing variety, N19, showed a exponentially increasing incorporation of label into the sucrose pool (*figure 6.5B*) in internode 7. It is possible that this variety has the ability to effectively partition sucrose away from the cytosolic compartment, thus accumulating significant quantities of the disaccharide in maturing internodes.

Reaching isotopic equilibrium in the cytosol appears to be faster in US6656-15 as a near-linear response of $^{14}CO_2$ release is evident over the 4 h labelling period (*figure 6.4*). If all sucrose in the cell is in the cytosolic compartment, all pools in the cycle of sucrose turnover will be rapidly labelled. It follows that all pools downstream, through

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to CO_2 release, will reach equilibrium quickly. $^{14}CO_2$ release from N19 was still increasing exponentially at the termination of incubation. This suggests that the intermediate pools in the cytosol had not yet reached isotopic equilibrium. This could be as a result of effective partitioning of synthesised [^{14}C]-sugars (including sucrose and the hexoses) to the vacuole, effectively removing them from the futile cycle in the cytosol, and thus causing a delay in reaching isotopic equilibrium.

In internode 7 tissues sucrose specific activities in the cell are between 5 and 12 times higher in US6656-15 than N19(*table 6.7*). The capacity for the sucrose specific activity to continue increasing is higher in N19 because of its high sucrose content.

Between varieties the net flux into sucrose (Bq.min^{-1}.mg^{-1}protein) did not consistently reflect the increase in sucrose content. In young internodes there is no significant difference in calculated net turnover of sucrose between varieties (*figure 6.6*). In the same tissues there is no significant difference in sucrose content. However, in the older internodes the 10-fold difference in content is not reflected by the small differences in net flux into sucrose.

From the data of incorporation into sucrose and CO_2 (*figures 6.4* and *6.5*), we suggest that the cytosolic pool of US6656-15 reached isotopic equilibrium faster than that of N19. Over a short labelling period of 4 h it is possible that the vacuolar pool of sucrose in N19 has been only partially labelled, and that the bulk of label in the fractionated sucrose was in fact in the cytosol. From the distribution of label within the total soluble pool (*table6.6*) it is evident that after 4 h similar amounts of label are present in sucrose between varieties. However, the incorporation of label into the sucrose pool of internode 7 tissues from N19 is still exponentially increasing after 4 hours. This can only mean that the potential to label sucrose is not nearly being met. After 4 h the cytosolic sucrose pool may be tending to equilibrium (i.e. the entire sucrose cycle is in isotopic equilibrium with other pools in the cytosol), and only after this can [^{14}C]-sucrose be accumulated in the vacuole at a constant rate. So if the labelling period was in fact longer we would possibly notice a continued increase in incorporation of label into sucrose (kBq.mg^{-1}protein), and an apparently higher rate of net sucrose turnover. In keeping with this, the tendency for US6656-15 tissues to bring both cytosolic and total sucrose pools to isotopic equilibrium could indicate that the vacuole is not playing a significant role in sucrose metabolism, and that observed flux rates into sucrose are in fact a reflection of the truth.

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It is therefore tempting to propose that partitioning of sucrose to the vacuole (*figure 6.8)* is a main factor contributing to the accumulation of sucrose in high sucrose storing varieties.

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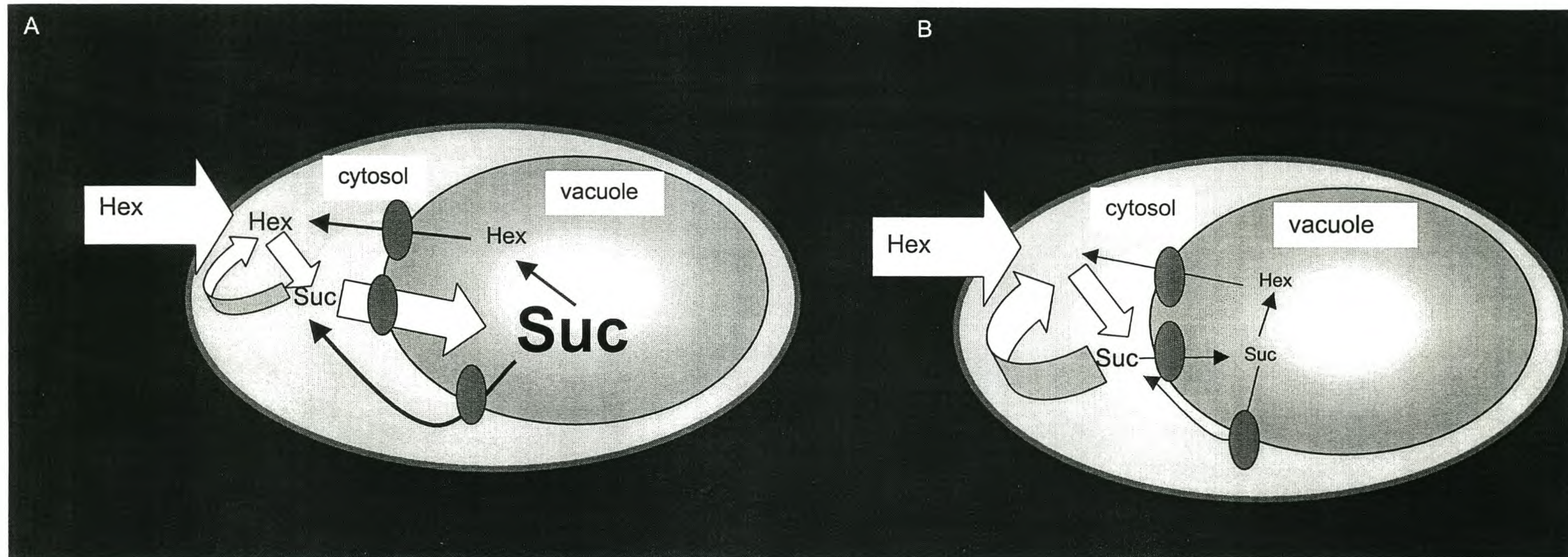

Figure 6.8 Proposed difference in the mechanism of sucrose accumulation in internode 7 tissues of N19 (A) and US6656-15 (B). Suc = sucrose, Hex = hexose. Circles on the tonoplast could represent either transport proteins or protein channels.

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6.4.3 Sucrose hydrolysis affects net accumulation

In young tissues of US6656-15, glucose contents are at least half that of N19 (figure 3A). Incorporation of label into glucose from [^{14}C]-fructose was only higher in the bottom of internode 4 of N19 compared with US6656-15 (*table 6.8)*. In order to compare sucrose hydrolysis rates between varieties we compared three methods of calculations based on three assumptions. Assuming the hydrolysed sucrose pool was in isotopic equilibrium with the external medium (i.e. 37 Bq.nmol^{-1}), sucrose hydrolysis rates were higher in all tissues of US6656-15, except the bottom region of internode 4, where the higher N19 hydrolysis rate could be contributing to growth metabolism (*figure 6.7A*). However, we know that the internal pools are not in equilibrium with the external pool because the measured sucrose specific activities were at least 50 times lower than the external (*table 6.7*).

Hydrolysis rates were then estimated using the determined internal specific activity of sucrose. It was observed that N19 rates were higher than those of US6656-15 in the internode 7 tissues (*figure 6.7B*). This cannot be a reflection of reality because the amount of label appearing in the glucose pool of US6656-15 was higher in these tissues after 4 h than in N19. Not only this, but the internal specific activity is not a true representation of N19 cytosolic sucrose specific activity. This is because approximately 90% of the sucrose in the cell is in the vacuole (assuming that the vacuole occupies 90% of the cell volume, and the concentration of sucrose is equal between vacuole and cytosol), and an estimation of only 10 % of the [^{14}C]-sucrose is in the vacuole (*figure 6.8*). Therefore N19 hydrolysis rates have been over estimated, but US6656-15 hydrolysis rates could be representative of reactions in the cell.

Sucrose specific activities from N19 were adjusted in order to compensate for the above mentioned vacuolar dilution factor. It was assumed that, because the difference in specific activity between varieties was up to 9-fold and that it appears that US6656-15 sucrose is primarily in the cytosol, the specific activity of sucrose in the cytosol of N19 was 9 times higher than the measured value. From this, hydrolysis rates were recalculated. In all tissues US6656-15 hydrolysis rates were far higher than that of N19 (*figure 6.7C*).

Although the percentage label incorporated into glucose appears small it is always over 1% (*table 6.6*). Keeping in mind that this data was collected over a short

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labelling period and that an increased incubation would possibly give a higher rate, hydrolysis is affecting the sucrose content of the cell. It is also clear that a low-sucrose storing variety has a higher sucrose hydrolysing rate.

6.5 Conclusions

Between seasons an increase in sucrose content would appear to be the result of a general up-regulation of metabolism. Differences between low- and high-sucrose storing varieties would appear to be the ability to partition sucrose to the vacuole of maturing internodal tissues.

When studying these processes using radio-labelled substrates to trace pathways of metabolism, one must be careful in calculating absolute flux rates when intra-cellular compartmentation could cause erroneous calculations. In the cycle of sucrose accumulation, it is evident that hydrolysis is an important factor that can easily be underestimated. The sucrose hydrolysing enzymes in the cytosol (where sucrose is synthesised) are therefore good targets for further investigation. As NI activity decreases with internode age (Vorster and Botha, 1999; Rose and Botha, 2000) and is always higher than AI activities it is an ideal target for genetic modification to manipulate sucrose content, possibly decreasing sucrose breakdown and consequently increasing net sucrose turnover.

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CHAPTER 7

General discussion

Understanding the steps involved in sucrose accumulation has the potential to effectively target specific points of control in order to improve sucrose yield in sugarcane. In an effort to gain such understanding we have focussed on the enzyme neutral invertase (NI), responsible for the cytosolic hydrolysis of sucrose. Reports dating back to the early 1960's have described sugarcane NI (SNI) activity and its biochemical properties; such as molecular weight, subunit aggregation, K_m and V_{max} values as well as *in vivo* and *in vitro* inhibitors (*chapter 2, section 2.3.1.2*). Because of differences in experimental techniques, there has been little consistency between reported data. Along with this, it would appear that both technology and available information had limited the potential for further research. In this project we have addressed both the problems of inconsistency and limitations by exploiting various biochemical and molecular techniques to attempt to describe this enzyme's expression at gene, transcript and protein levels on both a total tissue and cellular basis.

Detailed analysis of maximum extractable SNI activities showed that in young growing tissues of the sugarcane culm, where sucrose is low and hexoses are highest, the enzyme's activity was highest (*chapter 3, sections 3.3.1 and 3.3.2;* Rose and Botha, 2000). The difference between expression patterns viewed here and in the older studies (Hatch et al., 1963; Batta and Singh, 1986), could be explained by differences in experimental approaches. In recent years, more attention has been paid to the use of protease inhibitors while extracting enzymes for assay, and considerably reducing the time of desalting procedures. Our data confirms recent reports of SNI activity distribution (Lingle and Smith, 1991; Ebrahim et al., 1998; Vorster and Botha, 1999). Acquiring a polyclonal antibody (raised against *Beta vulgaris* NI) in a previous project (Vorster and Botha, 1998) that recognised SNI, allowed us to confirm the observed activity distribution, determined by enzyme coupled assays, using western blot analysis. We observed that the maximum extractable SNI activity was directly proportional to the amount of SNI protein in the assayed extract. It could therefore be postulated that SNI is regulated *in vivo* before protein translation (i.e. the mature protein is conformationally altered to change activity, e.g. by phosphorylation), or by fine control mechanisms such as feed back inhibition by its products glucose or fructose (Morell and Copeland, 1984;

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Lee and Sturm, 1996; Ross et al., 1996; Vorster and Botha, 1999). In mature internodal tissues of sugarcane, where sucrose contents are high and hexose contents are low, the proposed competitive inhibitory effect of fructose (Lee and Sturm , 1996) could become negligible because of the relatively high levels of sucrose. However, inhibition by glucose may still play a role because of its proposed non-competitive nature.

The importance of SNI in the cycle of sucrose synthesis and degradation has not, until now, been extensively investigated. Acid invertases (AI) (both soluble and cell wall bound isoforms) have been the subject of many studies in sugarcane metabolism. Acid invertase has often been singled out as a sucrose degrading enzyme that makes a major contribution to the futile cycling of sucrose (Zhu et al., 1997; Ebrahim et al., 1998; Ma et al, 2000; Zhu et al, 2000). However, in a recent study where a yeast invertase clone was targeted to the vacuole, cytosol and cell wall of sugarcane suspension cultures, it was observed that an increase in cytosolic invertase activity caused a dramatic decrease in cellular sucrose levels and severe phenotypic problems (Ma et al, 2000). This could imply that the amount of SNI has an important role in ultimate amount of accumulated sucrose. By comparing endogenous SNI activities and sucrose levels in the same tissues from the sugarcane culm, we observed that these two factors were negatively correlated in a linear relationship (*chapter 3, section 3.3.2*). However, the significance of this relationship was not consistent between seasons (*chapter 5, section 5.3.3*). From this, and the fact that in previous work it was shown that SNI activity varies both between individual plants and between seasons (Lingle and Smith, 1991), we could deduce that SNI and total sucrose contents vary in a complex system of interlinked components (not excluding the likelihood that SNI and the cytosolic sucrose content are intimately linked). Sugarcane neutral invertase potentially plays an important role in the amount of total sucrose accumulated because of its location in the cell and central positioning in the pathways of the sucrose cycle.

We also examined the role of hydrolysis (caused by all cellular invertases) in sucrose accumulation by exploiting techniques of radiolabelled flux analyses. By feeding [^{14}C]-fructose to culm tissues of two varieties: US6656-15 and N19 (low- and high- sucrose storing varieties, respectively), we could propose the effect of hydrolysis on the sucrose pool. What is important to note here is that sucrose content in internode 7 of N19 was ten-fold that in the equivalent tissue of US6656-15. Over the 4 h labelling period it

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appeared that both varieties synthesised sucrose at similar rates, and this could therefore not explain the difference in net accumulation. However, two imperative points were observed. Firstly, over a short labelling period it would seem difficult to bring a cell, whose sucrose content is as high as that of N19, to isotopic equilibrium (*chapter 6, section 6.3.2.3*). Thus, rates determined for both synthesis and degradation may be underestimated. What we did observe was that even with this underestimation, hydrolysis rates were always above one percent of the synthesis rate (*chapter 6, section 6.3.2.4*). Although this sounds an insignificant figure, when considering the amounts of sucrose produced by the industry, a one percent difference in sucrose yield could make a notable contribution. Although we were not able to separate the contributions of acid and neutral invertases, we believe that, in light of its cellular location and amount of activity in comparison with AI (Vorster and Botha, 1999) SNI is an important role player in the breakdown of sucrose.

Secondly, we observed that respiratory and sucrose pools in US6656-15 would appear to reach equilibrium faster than those in N19. As a result of differential partitioning of sucrose between the cytosol and vacuole, it would appear that N19 has the ability to remove sucrose from the "metabolic" compartment to the "storage" compartment. In support of this, differences in sucrose content between the winter and summer N19 internodal tissues were always in the same magnitude as the differences in sucrose net turnover rate (*chapter 6, section 6.3.1*). From these facts we have proposed that between varieties differences between cytosolic sucrose content and the ability to partition sucrose to the vacuole is another important factor in its accumulation.

Future work aimed at improving sucrose yields in sugarcane could therefore focus on either decreasing hydrolysis or increasing the ability to partition sucrose to the vacuole.

Because of sugarcane's high ploidy, the potential for enzymes to belong to a multigene family is high. To date no work examining SNI expression at DNA and RNA level has been reported. Here we report the first complete sequence for an expressed SNI gene. From Southern blot analysis we observed that SNI has a low, possibly single, gene copy number. This could be further confirmed from the single protein product observed in western blot analysis. These facts make the potential for genetic manipulation far

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simpler than if the enzyme had multiple copies that interact or are expressed in different tissues.

RNA expression patterns mimicked those observed at a protein level (*chapter 5, section 5.3.2*). This further confirms the hypothesis that SNI could be regulated at the level of transcription. Because of the trends observed between SNI, sucrose and hexose levels it is tempting to hypothesise that these sugars have the ability to act as signal molecules in leading to the differential expression of the SNI protein. It could also be possible that SNI plays an integral role in the cytosolic equilibrium of sugar levels, not only affecting the supply of substrates to growth and respiration, but also affecting the transcript expression of a multitude of enzymes encoded for in the nuclear genome.

From all of the work in this project it is evident that SNI plays a crucial role in the cycle of sucrose accumulation in sugarcane internodal tissue. Future work will be aimed at the down-regulation of the enzyme using vectors constructed in this project. This work has provided both a platform for future research and the answers to critical questions: (i) SNI is found in all tissues of sugarcane on both transcript and therefore protein level, with higher expression in immature tissues; (ii) the SNI expressed gene sequence shows homology to other plant NI but not AI, suggesting its independent development; (iii) SNI has a low gene copy number; (iii) total hydrolysis (including the SNI component) contributes significantly to the net turnover of sucrose; and (iv) the ability to partition sucrose away from the cytosol to the vacuole may be a determining factor in the efficiency of commercial sugarcane varieties to accumulate sucrose.

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CURRICULUM VITAE

Susan Rose

PERSONAL INFORMATION	
Surname	Rose
First Names	Susan
Identity no.	7708240148081
Date of Birth	24th August 1977
Sex	Female
Marital Status	Single
Nationality	South African
Home Language	English
Other Languages	Afrikaans
Qualifications	Matric/Grade 12 B.Sc. (Botany and Biochemistry) M.Sc. Plant Biotechnology (in progress)

ACADEMIC HISTORY	
Secondary School	St.Mary's DSG (Kloof)
Matriculation subjects	English, Afrikaans, Science, Mathematics, Accountancy, Art
Tertiary Institution	University of Stellenbosch, Stellenbosch South Africa (1996-2001)
Undergraduate subjects	Biochemistry, Botany, Chemistry Microbiology
Postgraduate subjects	Integrated Plant metabolism, Plant molecular biology

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Research experience to date:

1998: February - August

Project Title: ***Agrobacterium*** **mediated transformation of sugarcane**

This project fulfilled the requirement of a six-month course in my final year of undergraduate Botany studies.
The work was carried out in the Institute for Plant Biotechnology, University of Stellenbosch
Supervisors: Mr. J.H. Groenewald and Prof. F.C. Botha.
Techniques:
1. Tissue culture preparation of plant tissue for transformation.
2. Verification of vectors used for transformation.
3. Preparation of *Agrobacterium* strains for transformation.
4. Transformation of both tobacco and sugarcane tissues.
5. Identification of putative transgenics at a transient expression level using histochemical dye assays.

1998: January – December

Research assistant :

This was a part time position held at the Institute for Plant Biotechnology. Procedures included routine initiation and maintenance of plant cultures (both callus and whole plant material). On a more molecular level transgenic plants generated by other researchers were screened to verify the identity of putative transgenic lines using PCR.

1999-2001

Project Title: **Sucrose accumulation and the expression of neutral invertase in sugarcane**

This project was designed to fulfil the requirements for an MSc. (Plant Biotechnology) and is still in progress.
All work is being carried out at the Institute for Plant Biotechnology under the
Supervisor: Prof. F.C. Botha.
Aim: To describe the endogenous distribution of neutral invertase (NI) in different tissues of the sugarcane culm and its relation to soluble sugar levels.
Techniques:
1. Enzyme linked spectrophotometry enzyme activity assays.
2. Western blot analysis.
3. ^{14}C radio-labeling flux analysis.
4. Enzymatic and HPLC sugar determinations.
5. Plant transformation vector construction.
6. Plant transformation via direct particle bombardment.
7. PCR
8. Southern blot analysis.
9. Northern blot analysis.

Publications: S. Rose and F.C. Botha. Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. *Plant Phys. and Biochem.* (2000) 38: 11, pp 819-824