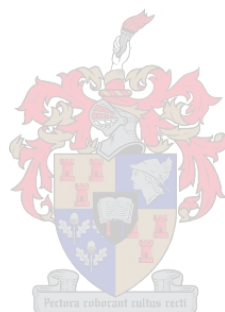


**CARBON TURNOVER AND SUCROSE METABOLISM IN THE  
CULM OF TRANSGENIC SUGARCANE PRODUCING  
1-KESTOSE**

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Thesis presented in fulfillment of the requirements for the degree of  
Master of Science at the University of Stellenbosch

Supervisors: Professor F.C. Botha and Professor B.I Hockett  
December 2007

*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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December 2007

## ABSTRACT

Carbon partitioning was investigated in sugarcane (*Saccharum* spp. hybrids) that was genetically modified with sucrose: sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) from *Cynara scolymus*. This enzyme catalyses the transfer of a fructosyl moiety from one sucrose molecule to another to produce the trisaccharide 1-kestose. Molecular characterisation of four sugarcane lines, regenerated after transformation, confirmed that two lines (2153 and 2121) were transgenic, with at least one intact copy of 1-SST present in line 2153, and a minimum of five copies (or portions thereof) present in line 2121. The novel gene was successfully transcribed and translated in both lines, as confirmed by cDNA gel blot hybridisation and HPLC analysis respectively.

Kestose production was stable under field resembling conditions and levels of this trisaccharide progressively increased with increasing internodal maturity from  $7.94 \pm 2.96 \text{ nmol.g}^{-1}$  fresh mass (fm) in internode 6 to  $112.01 \pm 17.42 \text{ nmol.g}^{-1}$  fm in internode 16 of 2153, and by  $1.05 \pm 0.93 \text{ nmol.g}^{-1}$  fm from the youngest to the oldest internode in line 2121. Sugarcane line 2153 contained 100 times more 1-kestose than 2121 in the oldest sampled internode hence the lines were referred to as high- and low-1-kestose producers. The production of 1-kestose did not reduce sucrose levels in the transgenics, instead they contained significantly higher levels of sucrose than the control line NCo310 ( $p < 0.01$ ,  $N=72$ ). The production of this alternative sugar in addition to elevated sucrose levels significantly increased the total sugar content in the transgenic lines ( $p < 0.01$ ,  $N=72$ ). Moreover, the high-1-kestose producer had statistically more total sugar than the low-1-kestose producer ( $p < 0.01$ ,  $N=72$ ).

Soluble acid invertase (SAI) and neutral invertase (NI,  $\beta$ -fructofuranosidase EC 3.2.1.26) from non-transgenic sugarcane internodal tissues were separated and partially purified. Kinetic analysis of the purified invertases revealed two isoforms of SAI eluting at approximately 100 mM KCl in a linear gradient while NI eluted at approximately 500 mM KCl. The final specific activities of SAI and NI were  $88.57 \text{ pkat.mg}^{-1}$  protein and  $92.31 \text{ pkat.mg}^{-1}$  protein, respectively. This implied a 16- fold purification of SAI, and 4- fold purification of NI. The pH optimum for NI was 7.0 and that for soluble acid invertase less than 5.0. Due to the broad pH activities of the invertases, activities significantly overlapped between pH 4.5 and 7.0. The affinity of these invertases for 1-kestose hydrolysis was tested. The invertases displayed hyperbolic saturation kinetics for sucrose, and had low affinities for 1-kestose with  $K_m$  values ranging from 50 - 247 mM. Furthermore, the presence of 200 mM 1-kestose had

an inhibitory effect on SAI-mediated sucrose hydrolysis reducing activity to 51 % and 54 % for isoform 1 and 2 respectively.

To determine whether carbon allocation had been altered by the expression and activity of 1-SST, <sup>14</sup>C whole-plant radiolabelling experiments were conducted. Radiolabelled CO<sub>2</sub> was fed to the leaf subtending internode 5 and the allocation of carbon to different parts of the culm was assessed. There was no significant difference in the distribution of total radiolabel down the culm of the three sugarcane lines ( $p > 0.05$ ,  $N = 72$ ). However, the percentage of total radiolabel in the water-soluble fraction per internode in the high-1-kestose producer was significantly higher than the other two lines ( $p < 0.01$ ,  $N = 72$ ). As a result, the percentage radiolabel in the water-insoluble fraction in this transgenic was concomitantly lower than in the other lines. Carbon was therefore redirected from the water-insoluble fraction to the water-soluble fraction to account for the additive production of 1-kestose. The expression of 1-SST in sugarcane therefore established an additional carbohydrate sink by the flow of carbon from the sucrose pool into 1-kestose. This did not lead to a depletion of the sucrose pool, but rather stimulated carbon channelling into this pathway, thereby increasing the non-structural carbohydrate content of the plant in one of the transgenics.

The work described in this study is the first to report on carbon partitioning in 1-kestose-producing sugarcane grown under field resembling conditions. It contributes significantly to an improved understanding of carbon partitioning in the culm, and demonstrates that an alternative sugar can be produced in sugarcane under field resembling conditions.

## OPSOMMING

Koolstofverdeling is in genetiese gemanipuleerde suikerriet (hibriede van *Saccharum* spp.) bestudeer. Die genetiese verandering is te weeg gebring deur die *Cynara scolymus* geen wat vir die ensiem sukrose: sukrose 1-fruktosieltransferase (1-SST; EC 2.4.1.99) na suikerriet oor te dra. Aanvanklike molekulêre karakterisering het aangedui dat twee van die vermeende transgeniese lyne (2153 and 2121) die transgeen bevat. Lyn 2153 het een en lyn 2121 ten minste vyf intakte kopieë van die transgeen bevat. Die analyses het duidelik aangetoon dat die transgeen suksesvol in beide hierdie transgeniese lyne uitgedruk word.

Stabiele kestose produksie het onder normale omgewingstoestande plaasgevind. Die vlakke van kestose onder hierdie toestande het tussen  $7.94 \pm 2.96 \text{ nmol.g}^{-1}$  vars massa (vm) in internode 6 tot  $112.01 \pm 17.42 \text{ nmol.g}^{-1}$  vm in internode 16 gewissel. Duidelike verskille in die hoeveelheid kestose in die transgeniese suikerriet was ook tussen die twee transgeniese lyne waarneembaar. Lyn 2153 het 'n honderdmaal meer kestose as lyn 2121 bevat en daarom word daar na hierdie twee lyne as hoë- en lae vlak kestose produseerders verwys. Die akkumulering van kestose het nie tot 'n verlaging in die sukrose vlakke gelei nie. Inteendeel, dit het in die algemeen tot 'n toename in sukrose inhoud gelei ( $p < 0.01$ ,  $N=72$ ). As gevolg van die produksie van betekenisvolle vlakke van kestose en die toename in sukrose het die transgeniese lyne hoë vlakke totale suiker as die kontrole plante bevat ( $p < 0.01$ ,  $N=72$ ). Die hoë kestose produseerder het ook meer totale suiker as die lae kestose produseerder bevat ( $p < 0.01$ ,  $N=72$ ).

Oplosbare suurinvertase (SAI) en neutrale invertase (NI,  $\beta$ -fructofuranosidase EC 3.2.1.26) is uit suikerriet internodale weefsel geïsoleer en gedeeltelik gesuiwer voordat die ensieme kineties gekarakteriseer is. Daar is twee isovorme van SAI in suikerriet teenwoordig en die vorme kan van mekaar geskei word met behulp van ionuitruilingschromatografie. Die een vorm het by 100 mM en die ander eers by 500mM KCl ge-elueer. Die finale spesifieke aktiwiteit van die isovorme was  $88.57 \text{ pkat.mg}^{-1}$  proteïen en  $92.31 \text{ pkat.mg}^{-1}$  proteïen vir die SAI en neutrale invertase vorme. Die pH optimum vir NI was 7.0 en vir SAI 5.0. As gevolg van die breë pH optimum is daar tussen pH 4.5 en 7.0 groot oorvleueling in die isovorme se aktiwiteit. Albei die isovorme toon hiperboliese substraat kinetika vir sukrose en beide het 'n baie lae affiniteit ( $K_m$  50 - 247 mM) vir 1-kestose. Verder is dit duidelik dat 1-kestose ook SAI aktiwiteit onderdruk. In die teenwoordigheid van 200mM kestose word die aktiwiteite van die twee SAI isovorme met 51 % en 54 % gerem.

Om te bepaal of die uitdrukking van 1-SST koolstofverdeling in suikerriet verander is  $^{14}\text{C}$  heel-plant merking uitgevoer. Vir die doel is  $^{14}\text{CO}_2$  aan die blaar wat aan internode 5 gekoppel is gevoer. Geen betekenisvolle verskille in die verspreiding van totale radioaktiwiteit in die stingels van die transgeniese en kontrole plante waargeneem nie ( $p > 0.05$ ,  $N = 72$ ). Die persentasie radioaktiwiteit in die wateroplosbare fraksie was egter betekenisvol hoër in die hoë kestose produseerder as die ander twee lyne gewees ( $p < 0.01$ ,  $N = 72$ ). Dit het daar toe gelei dat minder radioaktiwiteit in die transgeniese lyne na die wateroplosbare fraksie verprei is. Dit beteken dat die genetiese modifiseer tot 'n herverdeling van koolstof na suiker gelei het en dit het ten koste van die water onoplosbare fraksie gebeur. Die uitsrukking van 1-SST in suikerriet lei tot koolstof vloei vanaf sukrose na kestose en as gevolg daarvan word 'n addisionele koolstof swelgpunt geskep. In stede van die nuwe suiker produksie ten koste van sukrose is koolstof vanaf die onoplosbare fraksie na die suikerpoel gekanaliseer.

Die werk wat in die tesis beskryf word is die eerste wat aandui dat 'n nuwe suiker onder veldtoestande in suikerriet geproduseer kan word sonder dat die sukrose vlakke nadelig beïnvloed word. Die herkanalisering van koolstof na die suikerpoel ten koste van die water onoplosbare fraksie, dra grootliks by om ons kennis oor die regulering van koolstofmetabolisme in suikerriet te verbreed.

FOR MY MOM,  
WHO IN TIMES OF HER OWN PERSONAL STRUGGLE, NEVER FAILED TO  
PROVIDE ME WITH ENDLESS LOVE, CARE AND ENCOURAGEMENT.

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And finally

"To Him who is able to do immeasurably more than all we ask or imagine, according to his power that is at work within us, to Him, be glory in the church and in Christ Jesus throughout all generations, for ever and ever, Amen."

Ephesians 3: 20-21



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## LIST OF ABBREVIATIONS

$A_{260}$	absorbance at a wavelength of 260 nanometres
$A_{280}$	absorbance at a wavelength of 280 nanometres
<i>asd</i>	aspartic semialdehyde dehydrogenase gene
bp	nucleic acid base pair
Bq	Becquerel
BSA	bovine serum albumin
cDNA	copy deoxyribonucleic acid
cm	centimeter
d	day
dATP	deoxy-adenosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxy-guanine triphosphate
DLU	density light units
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
DTT	1,4-dithiothreitol
dTTP	deoxy-thymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
FBPase	fructose-1,6-biphosphatase (EC 3.1.3.11)
fm	fresh mass
fru	fructose
g	gram
<i>g</i>	gravitational force
glc	glucose
h	hour
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high pressure liquid chromatography
J	Joule
KCl	potassium chloride
$K_m$	concentration of substrate that produces half maximal activity
L	litre
M	molar
mg	milligram
MgCl <sub>2</sub>	magnesium chloride
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mol	moles
NaCl	sodium chloride
NaH[ <sup>14</sup> C]O <sub>3</sub>	radiolabelled sodium bicarbonate
NaOAc	sodium acetate
NaOH	sodium hydroxide

ng	nanogram
NI	neutral invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26)
nm	nanometre
nmol	nanomoles
$^{\circ}\text{C}$	degrees Celsius
pg	picogram
PCR	polymerase chain reaction
PFP	pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90)
pKat	picoKatal
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SAI	soluble acid invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26)
SDS	sodium dodecyl sulphate
SE	standard error
SPS	sucrose phosphate synthase (UDP-glucose: D-fructose-6-P 2- $\alpha$ -D-glucosyl-transferase, EC 2.4.1.14)
SSC	sodium chloride-sodium citrate buffer
SSPE	saline-sodium phosphate-EDTA buffer
suc	sucrose
SuSy	sucrose synthase (UDP-glucose: D-fructose 2- $\alpha$ -D-glucosyl-transferase, EC 2.4.1.13)
TBE	tris-borate/EDTA electrophoresis buffer
TE	tris/EDTA buffer
TENS	tris-HCl/EDTA/NaCl/SDS buffer
TLC	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	units
UDP-glucose	uridine 5'-diphosphate glucose
UV	ultraviolet
v	volume
V	Volt
$V_{\text{max}}$	maximum rate under substrate saturating conditions
1-FFT	fructan: fructan 1-fructosyltransferase (EC 2.4.1.100)
1-SST	sucrose: sucrose 1- fructosyltransferase (EC 2.4.1.99)
$\mu\text{A}$	microampere
$\mu\text{g}$	microgram
$\mu\text{l}$	microlitre
$^{14}\text{C}$	radiolabelled carbon
$^{14}\text{CO}_2$	radiolabelled carbon dioxide
$^{14}\text{C}$ - glc	radiolabelled glucose
$^{14}\text{C}$ - suc	radiolabelled sucrose
$\lambda$	wavelength



## CHAPTER 1

### GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) has the capacity to store large quantities of sucrose in the culm tissues (Moore, 1995; Komor, 2000). It has been reported that under ideal conditions, commercial hybrid cultivars store up to half of their dry mass in sucrose (Bull and Glasziou, 1963; Wendler *et al.*, 1990; Whittaker and Botha, 1997; Botha and Black, 2000). It has been suggested that this is the net result of continual hydrolysis and synthesis of sucrose (Sacher *et al.*, 1963; Hawker and Hatch, 1965; Wendler *et al.*, 1990; Zhu *et al.*, 1997). Simultaneous hydrolysis and synthesis of sucrose has been reported in immature (Sacher *et al.*, 1963) and mature tissue (Hawker and Hatch, 1965) as well as sugarcane cell suspension cultures (Wendler *et al.*, 1990). Carbon in the sucrose pool is recycled and returned to the hexose pool indicative of significant cycling of sucrose (Bindon and Botha, 2002), even in mature sugarcane internodes that are accumulating sucrose. Such a cycle could play an important role in sucrose accumulation. Numerous reviews have focussed on sucrose accumulation in the sugarcane culm, but the biochemical basis for the regulation of this process is still poorly understood (Moore, 1995; Moore, 2005; Rae *et al.*, 2005).

Sacher and colleagues (1963) described the spatial separation of the cytosol and vacuole; two compartments within the cell responsible for sucrose metabolism and storage respectively. Both compartments contain enzymes capable of sucrose hydrolysis namely sucrose synthase (SuSy, EC 2.4.1.13) and neutral invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26) in the cytosol, and soluble acid invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26) in the vacuole.

The invertases form an integral part in this cycling and have been suggested as potential key regulators of sucrose accumulation in sugarcane parenchyma cells (Hatch and Glasziou, 1963; Gayler and Glasziou, 1972). However, later research discounted their importance (Veith and Komor, 1993). Sucrose phosphate synthase (SPS, EC 2.4.1.14) (Hatch, 1964), sucrose synthase (SuSy, EC 2.4.1.13) (Goldner *et al.*, 1991) and the balance between the activities of several enzymes (Wendler *et al.*, 1990; Zhu *et al.*, 1997; Komor, 1994) have also been implicated as potential regulatory points for sugarcane sucrose accumulation.

It is unknown whether sucrose turnover occurs primarily in the cytosol, vacuole, or in both cellular compartments. If turnover is solely cytosolic then the point of regulation is

neutral invertase. Turnover in both compartments would underpin both neutral and acid invertase as targets for regulation of cellular sucrose. Alternatively, soluble acid invertase may be the primary point of regulation if turnover is found to be purely vacuolar. The vacuole composes 90 % of the total cellular volume (Komor, 1994; Komor, 2000) hence hydrolysis of sucrose in the vacuole is likely to play an important role in sucrose accumulation. Previous studies have failed to elucidate the contribution of vacuolar sucrose hydrolysis to sucrose accumulation. Therefore a significant gap in current understanding of sucrose metabolism lies in the inability to discriminate the reactions involved in sucrose hydrolysis in the vacuole, from those in the cytosol. Hence the compartment and resident enzymes responsible for regulation of cellular sucrose levels have not yet been pinpointed.

This information is a prerequisite for a better understanding of sucrose accumulation in sugarcane for two reasons. Firstly, sucrose in the vacuole is the result of sucrose movement from the cytosol and across the tonoplast through active transport, diffusion or both. The presence of a group translocator for active transport of sucrose across the tonoplast has been previously postulated (Thom and Maretzki, 1985) but later dismissed (Maretzki and Thom, 1988). Regardless of the transport mechanism, the process will be dependent on the sucrose concentration in the cytosol. Carbon cycling between the sucrose and the hexose pool is energetically expensive (Hatzfeld and Stitt, 1990; Hill and ap Rees, 1994; Dieuaide-Noubhani *et al.*, 1997; Rontein *et al.*, 2002; Alonso *et al.*, 2005) and will probably have a negative impact on the uptake of compounds and accumulation of storage products in sugarcane. Secondly, since sucrose synthetic enzymes are functional in the cytosol, re-synthesis of sucrose is not possible in the vacuole. Hence, sucrose hydrolysis in the vacuole is likely to negatively impact cellular sucrose accumulation. It is probable that the sucrose content in the vacuole may regulate the total sucrose load in the cell. Differentiation of sucrose metabolism in the two compartments will unravel the complex interactions they share and elucidate the impact that each compartment plays in the regulation of cellular sucrose metabolism.

Physical separation of the cytosol and vacuole, by vacuolar isolation has been attempted (Thom *et al.*, 1982; Thom and Komor, 1984) but this technique is laborious, problematic and often inaccurate, as data do not reflect the functioning of an intact vacuole *in vivo*. Genetic modification provides a tool for approaching this problem. The expression of an endogenous gene can be modified (Groenewald and Botha, 2007; Rossouw *et al.*, 2007), or a gene from an exogenous source can be introduced

(Uchimiya *et al.*, 1989; Hellwege *et al.*, 1997). In this study, the latter approach was followed in attempt to differentiate between cytosolic and vacuolar metabolism. The gene coding for sucrose: sucrose 1- fructosyltransferase (1-SST; EC 2.4.1.99) from *Cynara scolymus* was introduced into sugarcane. This enzyme catalyses the transfer of a fructosyl moiety from one sucrose molecule to another, resulting in the trisaccharide 1-kestose.

1-Kestose was selected as the alternative sugar of choice because it is a trisaccharide, synthesised from sucrose that is novel to sugarcane. Furthermore, the 1-SST cDNA contained a signal sequence targeting it for vacuolar expression (Hellwege *et al.*, 2000). This would create an additional sink for carbon in the vacuole and allow for the cytosolic and vacuolar reactions to be distinguished. On the basis that 1-kestose is a fructan (Pollock, 1986; Cairns and Pollock, 1988) and that the hydrolysis of fructans by invertases has been widely reported (Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003), it was assumed that 1-kestose would be susceptible to hydrolysis by cellular invertases. Plant invertases are  $\beta$ -fructofuranosidases that recognise and hydrolyse the bonds adjacent to fructose moieties. On the grounds of structural similarity of sucrose and 1-kestose, invertase-mediated hydrolysis of 1-kestose would be likely to have a similar affinity as that for sucrose. Monitoring of this turnover of 1-kestose would infer sucrose hydrolysis in the vacuole and give an indication of the contribution this makes to cellular sucrose hydrolysis. This would further elucidate the relationship between vacuolar and cytosolic sucrose hydrolysis.

This is the first study to genetically introduce 1-SST into sugarcane for the ultimate production of 1-kestose in intact potted sugarcane grown under field resembling conditions. It was hypothesised that the synthesis of 1-kestose from sucrose would reduce sucrose levels. Hence, 1-kestose would be produced at the expense of sucrose.

The aim of this study was to distinguish between vacuolar and cytosolic sucrose hydrolysis in sugarcane, by the genetic introduction of 1-SST. This gene was targeted for vacuolar expression and catalysed the production of 1-kestose from sucrose. Four potted sugarcane lines grown under field resembling conditions were characterised on a molecular level to establish 1-kestose production and to investigate the impact of the genetic event on sucrose levels (Chapter 3). To assess the ability of sugarcane invertases to hydrolyse this novel sugar, neutral and soluble acid invertase were

extracted and partially purified from a non-transgenic line. Their kinetic properties when using sucrose and 1-kestose as substrates were characterised and compared (Chapter 4). Furthermore, whole plant radiolabelling was used to investigate carbon partitioning down the culm of transgenic sugarcane lines to assess the effect of the genetic event on carbon allocation, and to determine 1-kestose turnover (Chapter 5).

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## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

This review focuses on sucrose metabolism in sugarcane (*Saccharum* spp. hybrids) with specific reference to the lack of understanding surrounding the relative contribution and regulation that cytosolic and vacuolar sucrose turnover play in cellular metabolism. An alternative approach to address this knowledge gap is discussed.

#### 2.2 The importance of sucrose

Sucrose is a nonreducing disaccharide composed of an  $\alpha$ -D-glucopyranose and a  $\beta$ -D-fructofuranose moiety joined by a  $\beta(1\rightarrow2)$  hydrolytic linkage (Buchanan *et al.*, 2000). Sucrose is the principle product of photosynthesis and a major form in which carbon is translocated in sugarcane plants from the source (leaves) to the sink (meristems and culm). This was confirmed in a study where sucrose radiolabelled in the fructose moiety was fed to leaves and asymmetry of the translocated sugar was retained (Hatch and Glasziou, 1964). Furthermore, sucrose is the primary storage sugar in some plants (Kruger, 1997; Chibbar and Båga, 2003). This is particularly true of sugarcane which is capable of storing large quantities of sucrose in the culm tissues (Moore, 1995; Komor, 2000). It has been reported that commercial hybrid cultivars can store up to half their dry mass in sucrose (Bull and Glasziou, 1963; Wendler *et al.*, 1990; Whittaker and Botha, 1997; Botha and Black, 2000). On the basis of the fundamental role of sucrose in plants, and the commercial importance of sugarcane (Moore, 1995), knowledge pertaining to the regulation, synthesis, breakdown and subsequent partitioning of sucrose is considered essential.

#### 2.3 Current understanding of sucrose metabolism

Radiolabelling studies and enzymatic measurements on sugarcane tissue discs indicated that sucrose is probably present in at least three distinct cellular compartments (Sacher *et al.*, 1963). It is assumed that these are the 'outer space' (apoplast and cell walls), 'metabolic compartment' (cytosol) and 'storage compartment' (vacuole).

Degradation of sucrose occurs in all three compartments. It is facilitated by the action of sucrose synthase (SuSy, EC 2.4.1.13) and the invertases ( $\beta$ -fructofuranosidase, EC 3.2.1.26). The invertases are characterised according to their subcellular localisations, pH optima and biochemical properties namely cell wall (insoluble, acid), vacuolar



(soluble, acid), and cytosolic (soluble, neutral) invertase (for review see Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). Regardless of their resident compartment, invertases catalyse the irreversible hydrolysis of sucrose to glucose and fructose. All plant invertases recognise and hydrolyse the bond adjacent to fructose moieties.

Soluble acid invertase (SAI) has been extracted from numerous sources including sugar beet (Giaquinta, 1979), rice (Isla *et al.*, 1995), barley (Obeland *et al.*, 1993; Nagaraj *et al.*, 2005) and Japanese pear fruit (Hashizume *et al.*, 2003). The  $K_m$  values for sugarcane SAI range from 2 mM to 13 mM (Gayler and Glasziou, 1972; Del Rosario and Santisopasri, 1977) which are similar to the range reported for SAI from other sources (1 mM – 14 mM). Activity of SAI also declines with increasing internodal maturity (Zhu *et al.*, 1997) such that levels are highest in rapidly growing sugarcane tissues.

Sugarcane neutral invertase (NI)  $K_m$  determinations of 25 mM (Hatch *et al.*, 1963) and 9.8 mM (Vorster and Botha, 1998) are similar to those reported for NI extracted from other sources (10 mM – 20 mM, Lee and Sturm, 1996; Ross *et al.*, 1996). Early studies on NI suggested that levels of this cytosolic enzyme increased with internodal maturation (Hatch *et al.*, 1963). However, more recent work shows the opposite relationship, with NI declining with increasing internodal maturity (Lingle, 1997; Vorster and Botha, 1999; Rose and Botha, 2000). These discrepancies could be due to sampling techniques or inactivation of the enzyme during extraction.

Several studies have highlighted the important contribution of invertases to sucrose metabolism in sugarcane parenchyma cells (Hatch and Glasziou, 1963; Gayler and Glasziou, 1972). Since a gradient of maturation and sucrose accumulation exists down the culm (Moore, 1995; Whittaker and Botha, 1997; Zhu *et al.*, 1997; Botha and Black, 2000; Rose and Botha, 2000; Moore, 2005; Rae *et al.*, 2005), while SAI and NI activities are lowest in the mature internodes, this implicates the importance of other points of regulation in sucrose accumulation. The sole contribution of one type of enzyme is likely to be small in light of the complexity of the sucrose accumulating process.

Synthesis of sucrose occurs exclusively in the cytosol and is catalysed by sucrose synthase (SuSy, EC 2.4.1.13) and sucrose phosphate synthase (SPS, EC 2.4.1.14), although the latter enzyme is reported to be more dominant in this role. This is on the basis that SPS activity is three-fold that of SuSy in mature internodes and due to the

existence of a positive correlation between SPS activity and sucrose content (Wendler *et al.*, 1990; Botha and Black, 2000). Sucrose phosphate synthase (Hatch, 1964), SuSy (Goldner *et al.*, 1991) or a combination of several enzymes (Wendler *et al.*, 1990; Komor, 1994) have also been implicated as regulatory points for sucrose accumulation.

The importance of compartmentation in metabolism has been emphasised by a study that targeted yeast invertase to either the apoplast, cytosol or vacuole in tobacco (Sonnewald *et al.*, 1991). The yeast-derived invertase, in addition to the endogenous plant invertase, interfered with metabolism suggesting sensitivity of the compartments to metabolic imbalance.

## 2.4 Sucrose cycling

Sugarcane culm tissues accumulate high quantities of sucrose (for review see Moore, 1995). Although the biochemical basis for the regulation of sucrose accumulation is poorly understood (for review see Moore, 1995; Moore, 2005; Rae *et al.*, 2005), it has been suggested that sucrose accumulation is the net result of hydrolysis and synthesis of sucrose (Wendler *et al.*, 1990; Zhu *et al.*, 1997).

Sucrose accumulation is characterised by a continuous cycle of sucrose synthesis and degradation that was first reported in immature sugarcane tissue (Sacher *et al.*, 1963). Thereafter, the simultaneous synthesis and degradation during accumulation or utilisation of sucrose has been reported for a wide range of tissues including mature sugarcane tissue (Hawker and Hatch, 1965), sugarcane cell suspension cultures (Wendler *et al.*, 1990), as well as for pea (*Pisum sativum*) roots (Hargreaves and ap Rees, 1988), *Chenopodium rubrum* cells (Hatzfeld and Stitt, 1990), potato (*Solanum tuberosum*) tubers (Hatzfeld and Stitt, 1990; Geigenberger and Stitt, 1991), ripening banana (*Musa cavendishii*) (Hill and ap Rees, 1994), maize (*Zea mays*) root tips (Hatzfeld and Stitt, 1990; Dieuaide-Noubhani *et al.*, 1997), and tomato (*Lycopersicon esculentum*) fruit (N'tchobo *et al.*, 1999; Nguyen-Quoc and Foyer, 2001).

This apparently 'futile' cycle is energetically expensive and reported to use between 3 % and 70 % of the ATP produced by the cell (Hatzfeld and Stitt, 1990; Hill and ap Rees, 1994; Dieuaide-Noubhani *et al.*, 1997; Rontein *et al.*, 2002; Alonso *et al.*, 2005). This range is dependent on the tissue. Furthermore, supply of ATP was not affected by carbon starvation nor did the synthesis and degradation of sucrose stop under sugar starvation (Dieuaide-Noubhani *et al.*, 1997). This suggests that even under carbon starvation, sucrose turnover plays an essential role (Dieuaide-Noubhani *et al.*, 1997). It

has been suggested that the cycling of sucrose and hexoses between the cytosolic and vacuolar compartments allows the cells to respond in a sensitive manner to minor changes in the net flux (for review see Moore, 1995).

As the principle product of photosynthesis, major translocated form of carbon and the primary storage carbohydrate in sugarcane, sucrose plays a pivotal role in plant metabolism (Sonnewald *et al.*, 1991). And, on the basis of the close relationship between SuSy, and sink strength (Xu *et al.*, 1989), uridine 5'-diphosphate glucose (UDP-glucose) is central to carbon partitioning in sink tissues. There is also an intimate association between UDP-glucose and cytosolic pyrophosphate (PPi) in plant tissues. The value and importance of UDP-glucose in sucrose metabolism was demonstrated by altering PPi metabolism in tobacco plants. An aberrant phenotype which included the accumulation of photoassimilate in source leaves, loss of chlorophyll and stunted plant growth was observed on introduction of a PPi-hydrolysing enzyme that prevented reactions leading to glycolysis and thereby led to an increase in UDP-glucose and sucrose synthesis. The original phenotype could be restored by by-passing the UDP-glucose dependent flow of carbon into respiration (Lerchl *et al.*, 1995; Geigenberger *et al.*, 1996). Respiration, cell wall/fibre synthesis (water-insolubles) and sucrose synthesis (water-solubles) are the three major processes in which UDP-glucose plays an integral part and these pathways compete for available UDP-glucose. Carbon partitioning studies in tissue slices (Botha *et al.*, 1996; Whittaker and Botha, 1997; Bindon and Botha, 2001, 2002) showed that maturation of internodal tissues corresponds to the redirection of carbon to sucrose at the expense of water-insoluble matter, respiration, amino acids, organic acids and phosphorylated intermediates (Botha *et al.*, 1996; Whittaker and Botha, 1997; Bindon and Botha, 2001, 2002). A 66 % increase in radiolabel present in the sucrose pool was evident in internode 9 after feeding, whilst carbon allocation to respiration, and fibre decreased by 9 % and 14 % respectively (Bindon and Botha, 2002). Sugarcane cell suspension cultures under nitrogen limiting conditions also allocated more carbon to sucrose than structural matter and respiration (Veith and Komor, 1993).

Thirty percent of carbon is recycled between the sucrose and hexose pools (Whittaker and Botha, 1997; Bindon and Botha, 2002). Although sucrose cycling has been widely reported in numerous tissue types, it remains unknown whether this turnover refers to that in the cytosol, vacuole, or both compartments. There is a pressing need to resolve whether sucrose turnover refers to cytosolic or vacuolar or both compartments, as this

would pinpoint the enzymatic point of regulation and indicate the contribution of vacuolar sucrose hydrolysis to cellular sucrose turnover.

Since the vacuole occupies approximately 90 % of total cellular volume (Komor, 1994; Komor, 2000), it is likely that the majority of sucrose is stored in this compartment which probably plays an important role in sucrose accumulation (Rae *et al.*, 2005). It is generally accepted that soluble acid invertase resides in the vacuole (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). Hence, this enzyme may in turn play a significant role in sucrose accumulation.

Sucrose in the vacuole is a consequence of movement from the cytosol and across the tonoplast through active transport, diffusion or both. The active transport of sucrose across the tonoplast has been previously postulated (Thom and Marezki, 1985) but the presence of a group translocator was subsequently dismissed as the result was an artefact of vacuolar preparations contaminated with plasmamembranes (Marezki and Thom, 1988). More recently, a sucrose H<sup>+</sup>/ antiport mechanism has been postulated for vacuolar sucrose transport (Getz, 1991; Getz, 2000). Irrespective of the mechanism of sucrose transport across the tonoplast and into the vacuole, the process will be dependent on the cytosolic sucrose concentration. Not only is vacuolar hydrolysis of sucrose dependent on the cytosolic concentration of sucrose, but the lack of sucrose synthesising enzymes within the vacuole implies that hydrolysis of sucrose in this compartment is likely to have a negative impact on cellular sucrose storage.

The importance of this compartment in the regulation of cellular sucrose hydrolysis is yet to be elucidated. There is, therefore, a significant gap in the current understanding of sucrose metabolism because of this inability to successfully distinguish between the reactions involved in sucrose hydrolysis within the vacuole, from those within the cytosol *in vivo*.

## **2.5 Approaches to distinguish cytosolic and vacuolar sucrose reactions**

Vacuolar isolation is one means to physically separate the reactions involved in the vacuole from those in the cytosol. Sugarcane vacuoles were isolated from cell suspension cultures, and their ability to transport 3-O-methyl glucose against a concentration gradient was tested (Thom *et al.*, 1982; Thom and Komor, 1984). This technique was found to be laborious and purification of the vacuolar preparations was problematic (Moore, 1995). Hence, data from isolated vacuoles did not reflect the functioning of intact vacuoles *in vivo*.

Another approach for understanding the reactions involved in the cytosolic and vacuolar compartments is study of their resident enzymes. Endogenous enzymes involved in sucrose metabolism in sugarcane have been isolated and their molecular and kinetic properties characterised (Botha *et al.*, 1996; for review see Tymowska-Lalanne and Kreis, 1998; Vorster and Botha, 1998; Botha and Black, 2000; Rose and Botha, 2000; Bosch *et al.*, 2004). The measurement and monitoring of these enzymes have provided invaluable insight into their functioning *in vitro*. However, it is likely that this approach has been exhausted.

## **2.6 Genetic manipulation as an alternative approach**

Despite the major advancements that have been made over the past decade by the aforementioned approaches, an alternative approach is required for investigating sucrose metabolism that will yield nascent information. Genetic modification facilitates the specific manipulation of metabolism by the up- or down-regulation of endogenous reactions, or by the introduction of a gene from an exogenous source that catalyses a novel metabolic reaction (for review see Turk and Smeekens, 1999). This approach has been successfully conducted for the up- and down- regulation of endogenous expression of genes involved in sucrose metabolism, and for the genetic introduction of exogenous genes for sucrose and fructan metabolism (Table 2.1).

Genetic manipulation by the introduction of an exogenous gene that catalyses a novel reaction in the plant by using an endogenous sugar as a substrate, allows for the conversion of an endogenous carbohydrate to another product. The impact of these genetic modifications on the plant can infer the functional role of the enzyme involved in the perturbed step of metabolism. There is only one report of alternative sugar production in sugarcane (Birch and Wu, 2005; Wu and Birch, 2007). The gene encoding bacterial sucrose isomerase which catalyses the conversion of sucrose to isomaltulose (another disaccharide) was introduced into glasshouse-grown cane. This modification yielded high sucrose concentrations and in turn increased the soluble carbohydrate content (Birch and Wu, 2005; Wu and Birch, 2007). However, the partitioning of carbon between sugar and fibre was not altered in these lines (Birch and Wu, 2005; Wu and Birch, 2007). Rather, these transgenic lines showed increased photosynthesis and sink strength, and the increased photosynthate was stored as sugar.

**Table 2.1** Some of the genetic modifications that have been made to plant metabolism by the up- or down-regulation of endogenous genes, or by the introduction of genes from an exogenous source.

<b>Metabolism</b>	<b>Gene target<sup>1</sup></b>	<b>Origin</b>	<b>Introduced into</b>	<b>Result</b>	<b>Reference</b>
<b>Sucrose</b>	Chimeric cytosolic and vacuolar invertase	Yeast <sup>2</sup>	Tobacco ( <i>Nicotiana tabacum</i> )	- stunted growth - reduced root formation - starch and sucrose accumulated in leaves	Sonnewald <i>et al.</i> , 1991
	PPase	Bacterial ( <i>Escherichia coli</i> )	Tobacco ( <i>Nicotiana tabacum</i> )	- sugar accumulated in leaves - stunted growth - chlorophyll loss	Lerchl <i>et al.</i> , 1995 Geigenberger <i>et al.</i> , 1996
	Chimeric cytosolic invertase	Yeast <sup>2</sup>	Tobacco ( <i>Nicotiana tabacum</i> )	- bypass PPI-dependent sucrose synthesis - wildtype phenotype restored	Lerchl <i>et al.</i> , 1995
	SPS (up-regulate)	N.A. <sup>3</sup>	Tomato ( <i>Lycopersicon esculentum</i> )	- sucrose concentration doubled	Chibbar and Båga, 2003
	PFP (down-regulate)	N.A. <sup>3</sup>	Potato ( <i>Solanum tuberosum</i> )	- reduced starch content - slightly higher sucrose concentration	Chibbar and Båga, 2003
	FBPase (up-regulate)	Spinach ( <i>Spinacia oleracea</i> )	Tobacco ( <i>Nicotiana tabacum</i> )	- FBPase activity increased 4- and 6-fold	Chibbar and Båga, 2003
	SI	Bacterial ( <i>Erwinia rhapontici</i> )	Sugarcane ( <i>Saccharum</i> spp. hybrids)	- isomaltulose production - doubled total sugar content - increased photosynthesis, sucrose transport and sink strength	Birch and Wu, 2005 Wu and Birch, 2007
	PFP (down-regulate)	N.A. <sup>3</sup>	Sugarcane ( <i>Saccharum</i> spp. hybrids)	- PFP activity reduced up to 70 % and sucrose concentrations increased in young internodes - no activity detected in old tissues	Groenewald and Botha, 2007
	NI (down-regulate)	N.A. <sup>3</sup>	Sugarcane ( <i>Saccharum</i> spp. hybrids)	- reduced hexose concentration - increased sucrose concentration	Rossouw <i>et al.</i> , 2007
	<b>Fructan</b>	1-SST	Globe artichoke ( <i>Cynara scolymus</i> )	Potato ( <i>Solanum tuberosum</i> )	- 1-kestose and nystose produced
1-SST		Jerusalem artichoke ( <i>Helianthus tuberosus</i> )	Sugar beet ( <i>Beta vulgaris</i> )	- short oligofructans produced	Sévenier <i>et al.</i> , 1998
1-SST and 1-FFT		Globe artichoke ( <i>Cynara scolymus</i> )	Potato ( <i>Solanum tuberosum</i> )	- high molecular weight inulin produced	Hellwege <i>et al.</i> , 2000

<sup>1</sup> PPase (inorganic pyrophosphatase, EC 3.6.1.1); SPS (sucrose phosphate synthase, EC 2.4.1.14); PFP (pyrophosphate: fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.90); FBPase (fructose-1,6-biphosphatase, EC 3.1.3.11); SI (sucrose isomerase, EC 5.4.99.11); NI (neutral invertase, EC 3.2.1.26); 1-SST (sucrose: sucrose 1-fructosyltransferase, EC 2.4.1.99); 1-FFT (fructan: fructan 1-fructosyltransferase, EC 2.4.1.100). <sup>2</sup> Yeast species not specified. <sup>3</sup> Not applicable since the endogenous gene was manipulated.

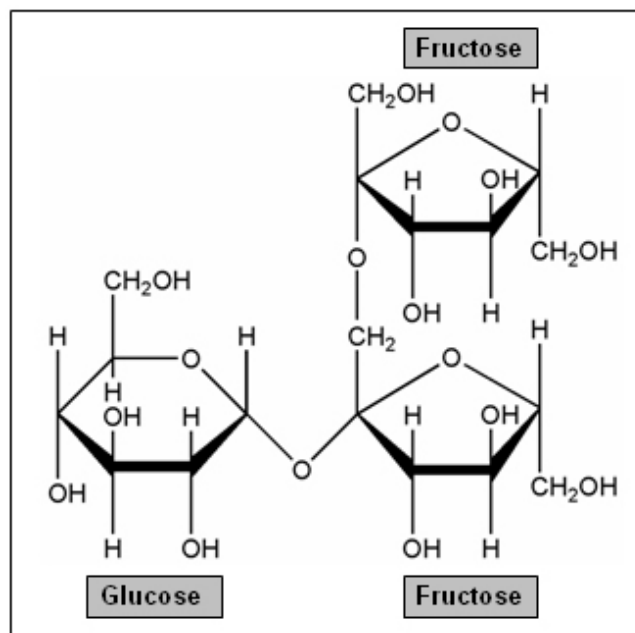
The manipulation of carbohydrate metabolism by the specific introduction of a gene from an exogenous source for the conversion of sucrose to an alternative product is a novel approach towards clarifying the relationship between cytosolic and vacuolar sucrose metabolism. Carbon partitioning and successful degradation of the alternative product by endogenous enzymes may be two potential hurdles of this approach.

### **2.7 Fructan biosynthesis: synthesis of 1-kestose from sucrose**

Sucrose is the major storage compound in sugarcane. Sucrose is also the precursor for fructan biosynthesis. Between 12-15 % of higher plants use fructans (oligo- and poly-fructosyl sucrose) as their major carbohydrate (Pollock and Cairns, 1991) which are stored in the vacuole (Simpson and Bonnett, 1993). Fructan is synthesised from sucrose by the action of two or more different fructosyltransferases. The classic model of fructan synthesis by Edelman and Jefford (1968), describes the formation of 1-kestose (G1-2F1-2F, Figure 2.1) from sucrose by sucrose: sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99). This enzyme catalyses the transfer of a fructosyl moiety from one sucrose molecule to another, to produce the trisaccharide 1-kestose, plus glucose as a by-product. Another enzyme, fructan: fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100), adds fructose residues onto 1-kestose in a sequential manner to form longer fructan chains. The combined action of these two fructosyltransferases results in the formation of a mixture of fructan molecules with varying chain lengths (Edelman and Jefford, 1968). Five classes of structurally different fructans exist in higher plants: inulin, levan, mixed levan, inulin neoseries and levan neoseries. These are distinguished in terms of their linkages and the plant species in which they are found (Vijn and Smeekens, 1999). 1-Kestose is the shortest fructan of the  $\beta$ -1,2-linked inulin.

These fructosyltransferase enzymes from various plant sources have been purified to homogeneity and their incubation with sucrose resulted in the formation of inulin with a maximum degree of polymerisation of twenty fructosyl units (Henry and Darbyshire, 1980, Koops and Jonker, 1996, Van den Ende and Van Laere, 1996)

In addition to the isolation and characterisation of these enzymes, cDNA encoding 1-SST has been isolated and cloned from globe artichoke (Hellwege *et al.*, 1997), Jerusalem artichoke (Van der Meer *et al.*, 1998), onion (Vijn *et al.*, 1998), tall fescue (Lüscher *et al.*, 2000), dandelion (Van den Ende *et al.*, 2000), and barley (Nagaraj *et al.*, 2004).



**Figure 2.1** Molecular structure of 1-kestose. This trisaccharide, which is the shortest fructan of the inulin type, is composed of sucrose (one glucose and one fructose moiety) plus a fructose moiety with a  $\beta(2-1)$  linkage between the fructosyl unit and sucrose.

Transformation of sugar beet and potato with cDNA encoding 1-SST resulted in the synthesis of 1-kestose, nystose (G1-2F1-2F1-2F1) and low molecular weight fructans (Hellwege *et al.*, 1997; Sévenier *et al.*, 1998). High molecular weight inulin was produced when 1-SST and 1-FFT cDNA from globe artichoke was expressed in potato (Hellwege *et al.*, 2000) (Table 2.1).

Some invertases hydrolyse fructans (Cairns and Ashton, 1991; Pollock and Cairns, 1991; Cairns, 1993; Bonnett and Simpson, 1993; Simpson and Bonnett, 1993). 1-kestose is the shortest fructan of  $\beta$ -1,2-linked inulin type, hence these reports of fructan hydrolysis are likely to include of 1-kestose (Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003). However, there have been no previous reports documenting the affinity of sugarcane invertases for 1-kestose, probably because fructans are not naturally synthesised in sugarcane nor has sugarcane been previously transformed with a fructosyltransferase enzyme.

To date, there is no reported evidence of 1-kestose production in sugarcane grown under field resembling conditions. The production of 1-kestose from sucrose by the genetic introduction of 1-SST may facilitate the discrimination of the cytosolic and vacuolar pools. Based upon numerous reports of fructan hydrolysis by invertases



(Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003), monitoring of vacuolar 1-kestose turnover may infer sucrose turnover and further elucidate sugarcane sucrose metabolism.

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## CHAPTER 3

### CHARACTERISATION OF TRANSGENIC LINES PRODUCING A NOVEL TRISACCHARIDE (1-KESTOSE)

#### 3.1 Abstract

Sucrose: sucrose 1- fructosyltransferase (1-SST; EC 2.4.1.99) catalyses the production of the trisaccharide 1-kestose from sucrose, by transferral of a fructosyl moiety from one sucrose molecule to another. Four sugarcane lines that were putative transgenics for 1-SST were analysed on a molecular level and their production of 1-kestose was assessed. Two of the sugarcane lines were confirmed to be transgenic with at least one intact copy of 1-SST present in line 2153 and a minimum of five copies (or portions thereof) present in line 2121. In both lines, the novel gene was successfully transcribed and translated as confirmed by cDNA gel blot hybridisation and HPLC analysis respectively. 1-Kestose levels progressively increased with increasing internodal maturity from  $7.94 \pm 2.96 \text{ nmol.g}^{-1} \text{ fm}$  in internode 6 to  $112.01 \pm 17.42 \text{ nmol.g}^{-1} \text{ fm}$  in internode 16 of 2153, and by  $1.05 \pm 0.93 \text{ nmol.g}^{-1} \text{ fm}$  from the youngest to the oldest internode in sugarcane line 2121. Line 2153 contained 100 times more 1-kestose than 2121 in the oldest sampled internode. The production of 1-kestose did not decrease sucrose levels in the transgenics, instead they contained significantly higher levels of sucrose ( $p < 0.01$ ,  $N=72$ ) than the control line NCo310. Moreover, both transgenics produced significantly more total sugar than NCo310 ( $p < 0.01$ ,  $N=72$ ), with 2153 having statistically more total sugar than 2121 ( $p < 0.01$ ,  $N=72$ ). 1-Kestose is therefore produced in addition to sucrose, rather than at the expense of it. This study is the first to report the production of 1-kestose in transgenic sugarcane grown under field resembling conditions.

#### 3.2 Introduction

The purpose of this study was to establish the production of 1-kestose from sucrose in sugarcane by the genetic introduction of 1-SST from *Cynara scolymus*. The 1-SST transgene product was targeted to the vacuole such that cytosolic and vacuolar reactions involving sucrose hydrolysis could be discriminated. This study focuses on the characterisation of four sugarcane lines grown under field resembling conditions and investigates the impact of the introduction of 1-SST on sucrose levels.

Carbon partitioning studies in sugarcane (*Saccharum* spp. hybrids) have furthered our knowledge of the regulation of sucrose metabolism. However, our understanding is limited with respect to compartmentation of sucrose in the vacuole and the cytosol. It is widely accepted that a cycle of simultaneous synthesis and breakdown of sucrose occurs in young tissues (Sacher *et al.*, 1963) as well as in mature sugarcane tissues that are accumulating sucrose (Hawker and Hatch, 1965). Enzymes catalysing the synthesis (sucrose synthase, EC 2.4.1.13 and sucrose phosphate synthase, EC 2.4.1.14) and breakdown (sucrose synthase, EC 2.4.1.13 and invertases,  $\beta$ -fructofuranosidase, EC 3.2.1.26) of sucrose are implicated in this energetically expensive cycling.

Soluble acid invertase (SAI,  $\beta$ -fructofuranosidase, EC 3.2.1.26) is located in the vacuole; a storage compartment comprising 90 % of the cellular volume (Komor, 1994; Komor, 2000). A strong negative relationship is evident between sucrose storage and SAI, such that SAI levels are highest in the young internodes and sucrose hydrolysis supplies hexoses for active growth (Zhu *et al.*, 1997). Since the sucrose synthetic enzymes are confined to the cytosol, vacuolar sucrose synthesis is impossible. Hence, SAI is primarily responsible for sucrose hydrolysis in the vacuole. However, the extent to which intracellular sucrose concentration is determined by vacuolar SAI is yet to be established (Komor, 2000).

The study of vacuolar metabolism is notoriously difficult. Previous attempts to achieve physical discrimination of the cytosol from the vacuole in sugarcane (Thom *et al.*, 1982; Thom and Komor, 1984) were unsuccessful because the lengthy technique yields data that do not reflect the functioning of an intact vacuole *in vivo*. Enzymatic conversion of vacuolar sucrose to an alternative product, that is also susceptible to hydrolysis, is another approach to distinguish between the hydrolysis reactions in the vacuole from those in the cytosol. This would involve genetic modification. This approach has been used to up- or down-regulate endogenous genes or to introduce a gene from an exogenous source, to yield an alternative product (Uchimiya *et al.*, 1989).

Sucrose: sucrose 1- fructosyltransferase (1-SST, EC 2.4.1.99) is an enzyme native to fructan-accumulating plants, that catalyses the production of the trisaccharide 1-kestose from sucrose. 1-Kestose is the primary intermediate in fructan synthesis between sucrose and fructan in fructan-synthesising plants (Pollock, 1986; Cairns and Pollock, 1988). Other plants that have been genetically modified to include this enzyme

are potato (*Solanum tuberosum*, Hellwege *et al.*, 1997; Hellwege *et al.*, 2000) and sugar beet (*Beta vulgaris*, Sévenier *et al.*, 1998). Both studies report the successful production of 1-kestose, nystose and short oligofructans. Another enzyme fructan: fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) was also introduced into potato, which subsequently added fructose moieties onto 1-kestose to produce high molecular weight inulins (Hellwege *et al.*, 2000).

The current study reports the characterisation of four sugarcane lines regenerated following transformation with 1-SST and grown under field resembling conditions. The most appropriate transgenic sugarcane lines were selected for further analysis, and the effect of the novel transgene on sucrose levels was investigated.

### 3.3 Materials and Methods

#### 3.3.1 Transgenic sugarcane lines

Sugarcane (*Saccharum* spp. hybrid) lines 2121, 2153, 2167 and 2168 were obtained from Bayer BioScience GmbH (Potsdam, Germany). These lines of the commercial cultivar NCo310, had been regenerated following transformation (Klein *et al.*, 1987; Christou, 1993; Helenius *et al.*, 2000) with plasmid pML1 (Section 3.3.3) containing the gene encoding sucrose: sucrose 1-fructosyl transferase (1-SST) from *Cynara scolymus* (Hellwege *et al.*, 1997; Hellwege *et al.*, 2000). Transformation of the sugarcane lines with pML1 was carried out at the Institute for Plant Biotechnology, University of Stellenbosch (Nell, 2006) and the lines were micropropagated through tissue culture (George, 1993). An untransformed field-grown NCo310 line was used as a control and was not propagated through tissue culture at any stage.

#### 3.3.2 Sugarcane growth and maintenance

Culms were stripped of their leaves and cut into nodal sections called setts; each bearing a single intact axillary bud. These setts from the transgenic lines were planted in speedling trays along with those from non-transgenic NCo310 which served as the control line. Vegetative seedlings were maintained in a glasshouse and exposed to natural daylight and temperatures between 26 °C and 28 °C. Overhead sprinklers watered the plants for 3 minutes (min) twice daily, while 1 g.L<sup>-1</sup> Hygrotech seedling nutrient mix fertilizer (Hygrotech, Pretoria, SA) was used weekly. After 30 days (d), the seedlings were transferred to large pots (37 cm diameter x 35 cm height) in an outside fenced terrace where they were kept under contained conditions. The seedlings were watered twice daily for 15 min each using dripper irrigation (4 L.h<sup>-1</sup>), and fertilised every

3 weeks with 20 g per pot of 5.1.5 (46) fertilizer (N:20.9 %, P:4.2 %, K: 20.9 %) (Kynoch Fertilizer Ltd., Randburg, SA).

### 3.3.3 Transformation vector pML1

Plasmid pML1, owned and constructed by Bayer BioScience GmbH (Potsdam, Germany), included the cDNA of 1-SST from *Cynara scolymus* (Hellwege *et al.*, 1997; Hellwege *et al.*, 2000) under the control of a maize polyubiquitin promoter (*ubq*) with modified intron (Christensen *et al.*, 1992). Included within the cDNA was a signal sequence for the vacuolar expression of 1-SST (Hellwege *et al.*, 2000).

### 3.3.4 Bacterial stocks

An *E.coli* strain, G6MD2 (Schwartz, 1966), an aspartic semialdehyde dehydrogenase mutant (*asd*<sup>-</sup>) (*E.coli* Genetic Stock Centre, Yale University, CT, USA) was selected as a suitable host for pML1 which contains the *asd* gene. Glycerol stocks of *E.coli* strain G6MD2 exclusive and inclusive of pML1 were prepared by mixing bacterial cultures grown overnight at 30 °C in Luria Bertani (LB) medium (10 g.L<sup>-1</sup> NaCl, 5 g.L<sup>-1</sup> yeast extract, 10 g.L<sup>-1</sup> bactotryptone and 15 g.L<sup>-1</sup> agar, pH 7.0), with or without supplementation of 1 mM diaminopimelic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 10 mM glucose (Saarchem, Wadeville, South Africa) respectively, with sterile glycerol in a 5:7:1 ratio and flash-freezing in liquid nitrogen. Stab cultures of each were prepared according to Sambrook *et al.* (1989).

### 3.3.5 Growth of *E.coli* cells

Untransformed host bacterial cells were grown on solid LB medium supplemented with 1 mM diaminopimelic acid and 10 mM glucose for 16 h at 30 °C. A single colony was selected and grown in supplemented LB broth shaken at 300 rpm at 30 °C overnight. Plates were stored at 4 °C and used within 2 weeks.

### 3.3.6 Transformation of bacterial cells

The method of Cohen *et al.* (1972) was used to prepare competent *E.coli* cells and transform them with pML1, with the modification that the cells were grown at 30 °C and selective plates lacked the nutrient supplements (diaminopimelic acid and glucose) required by untransformed *E.coli* of G6MD2 strain. Plasmid was generated and isolated for the preparation of probe DNA for Southern and Northern analyses (see Section 3.7.4).

### 3.3.7 Southern blot analysis

#### *Genomic DNA isolation*

Total genomic DNA was isolated from leaf and leafroll tissue from young sugarcane plants by a modification of the method of Saghai-Marooft *et al.* (1984). Tissue (2 – 3 g fresh weight) was powdered in liquid nitrogen, distributed in 10 ml of warm extraction buffer (100 mM Tris-HCl pH 7.5 (Roche Diagnostics GmbH, Mannheim, Germany), 700 mM NaCl (Saarchem, Wadeville, South Africa), 50 mM EDTA pH 8.0 (ICN Biomedicals Incorporated, Aurora, Ohio, MA), 1 % (m/v) CTAB (BDH Limited, Poole, England), 140 mM 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)) and incubated for 90 min at 65 °C, with gentle periodic inversion. Chloroform: isoamylalcohol (Saarchem, Wadeville, South Africa) (24:1 v/v, 4 ml) was added, and the solution gently inverted for 10 min. Following centrifugation at 1652 g for 10 min at room temperature, the aqueous phase was removed and the step was repeated. Thereafter, the aqueous phase was incubated at room temperature for 30 min with pre-boiled RNase A (30 µl, 10 mg.ml<sup>-1</sup>, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). An equal amount of isopropanol (Saarchem, Wadeville, South Africa) was added and the DNA precipitated at 4 °C for 30 min. Centrifugation at 1452 g produced a pellet that was dried and resuspended in 1X TE (10 mM Tris pH 7.5 and 1 mM EDTA pH 8.0). Phenol: chloroform: isoamylalcohol (25:24:1 v/v, 2 ml) was added and the solution mixed and centrifuged for 5 min at 1452 g. The aqueous phase was retained and the DNA precipitated with 1/10 volume of 3 M NaOAc (Saarchem, Wadeville, South Africa) and 2 volumes of absolute ethanol at 4 °C for 30 min with periodic inversion. After centrifugation for 10 min at 1200 g, DNA pellets were air-dried, resuspended in 1X TE and quantified using a Hoefer<sup>®</sup> DyNA Quant<sup>®</sup> 200 (Amersham Pharmacia Biotec, San Francisco, CA, USA).

#### *DNA digestion and precipitation*

Genomic DNA (11 µg) was digested with 30 U of *Hind*III (Roche Diagnostics GmbH, Mannheim, Germany) overnight at 37 °C in a final reaction volume of 400 µl in restriction enzyme buffer (1 mM Tris-HCl pH 8.0, 0.5 mM MgCl<sub>2</sub>, 10 mM NaCl, 0.1 mM 2-mercaptoethanol) and 4 mM spermidine trihydrochloride. Digestion was boosted by the addition of 10 U of *Hind*III followed by incubation at 37 °C for 1 hour (h). Digestion was confirmed by electrophoresis of 1 µg DNA in 1 % (m/v) agarose gel (Laboratorios Conda, Madrid, Spain). Addition of 1/20 volume of 5 M NaCl and 2.5 volumes of absolute ethanol (Saarchem, Wadeville, South Africa) precipitated the DNA during

incubation at -85 °C for 1 h. Following centrifugation at 20800 *g* for 30 min, the supernatant was removed and the pellets were air-dried, resuspended in 1X TE, and incubated at 4 °C overnight. In addition, pML1 plasmid DNA (500 ng) was digested with 2 U *Hind*III for 2.5 h at 37 °C and 200 pg of this was added to digested carrier DNA (NCo310) for use as a positive control for Southern blotting.

#### *Blotting procedure*

Restricted genomic (10 µg) and plasmid (200 pg) DNAs were separated by 0.8 % (m/v) agarose gel electrophoresis using 0.5X TBE (90 mM Tris, 90 mM Boric acid, 10 mM EDTA pH 8.0) as a running buffer, and stained with ethidium bromide (1 µg.ml<sup>-1</sup>, final concentration) for 10 min with gentle shaking. Thereafter, gels were rinsed in deionised water and incubated for 20 min in 125 mM HCl to depurinate the DNA. After rinsing in deionised water, the DNA was denatured by placing the gel in a solution containing 1.5 M NaCl and 0.5 M NaOH (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 30 min with gentle shaking. An overnight upward capillary blot onto Hybond™ -N+ membrane (Amersham Biosciences UK Limited, Buckinghamshire, England) was performed according to the manufacturer's instructions using a denaturing solution (1.5 M NaCl and 0.5 M NaOH) as the transfer buffer. DNA was UV cross-linked to the membrane at 70 mJ.cm<sup>-2</sup>, the membrane briefly rinsed in 2X SSC and prehybridised overnight at 65 °C with 5X SSC (750 mM NaCl and 75 mM tri-sodium citrate), 5X Denhardt's solution (0.1 % (m/v) ficoll, 0.1 % (m/v) polyvinyl pyrrolidone, 0.1 % (m/v) bovine serum albumin), 0.5 % (m/v) SDS (Roche Diagnostics GmbH, Mannheim, Germany) and 200 µg.ml<sup>-1</sup> denatured, fragmented herringsperm DNA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

#### *Probe preparation*

Plasmid DNA miniprep, by alkaline lysis, was performed according to the method of Birnboim and Doly (1979) with the modification that 440 mM sucrose (Saarchem, Wadeville, South Africa) was used instead of 50 mM glucose. Plasmid quantification was estimated by comparison with a known quantity of markers separated in a 1 % (m/v) agarose gel. The plasmid identity was confirmed by restriction with either *Eco* RV, *Hind*III, or both *Eco* RV and *Hind*III, (6 U each, Roche Diagnostics GmbH, Mannheim, Germany), with a restriction enzyme buffer (1 mM Tris HCl, 0.5 mM MgCl<sub>2</sub>, 10 mM NaCl, 0.1 mM 2-mercaptoethanol, pH 8.0) at 37 °C for 2.5 h. The DNA probe was prepared by amplification of a 609 bp region of 1-SST in a PCR reaction of

50 µl total volume containing 5 ng pML1, 120 nM each of forward (SSTf, 5'CGAATGTGAAGCATGTGTT3') and reverse (SSTr, 5'GAAATACACAGGAAGTTG CTCAGA3') primers (Inqaba Biotechnical Industries, Hatfield, South Africa), and 3.5 mM MgCl<sub>2</sub>, 40 µM deoxynucleotide triphosphates (dNTPs), 1 U Taq DNA polymerase and Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1 % (v/v) Triton X-100). Reactions were conducted in a GeneAmp® PCR system 9700 (The Perkin-Elmer Corporation, Norwalk, CT, USA) under the following conditions: 1 cycle at 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The 609 bp amplicon of 1-SST was excised from the gel and purified through QIAquick spin columns (Qiagen GmbH, Hilden, Germany). The sequence of the probe DNA was compared to sequences in Genbank, using the BLAST algorithm (Altschul *et al.*, 1990) provided by the National Centre for Biotechnology Information (NCBI), to ensure that no homology was shared with other sugarcane sequences. Probe DNA was labelled using the Megaprime™ DNA labelling system (RPN 1606, Amersham Biosciences UK Limited, Buckinghamshire, England) where DNA (27 ng), random nonamer primers (2.5 µg) and deionised water were denatured by boiling for 5 min. After cooling, labelling buffer (dATP, dGTP and dTTP in Tris/HCl pH 7.5, 2-mercaptoethanol and MgCl<sub>2</sub>), Klenow DNA polymerase (2 U) and 925 kBq [ $\alpha$ -<sup>32</sup>P] dCTP (222 TBq.mmol<sup>-1</sup>, Amersham Biosciences UK Limited, Buckinghamshire, England) were added to a final volume of 50 µl, and incubated at 37 °C for 1 h. Unincorporated nucleotides were removed using a QIAquick nucleotide removal kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

#### *Hybridisation*

The labelled probe was denatured by boiling for 5 min, snap-cooled on ice, added to the prehybridisation solution (5X SSC, 5X Denhardt's solution, 0.5 % (m/v) SDS and 200 µg.ml<sup>-1</sup> denatured, fragmented herringsperm DNA) and the membranes hybridised at 65 °C overnight. The hybridisation solution was removed and the membrane was washed once with 2X SSC and 0.1 % (m/v) SDS, twice with preheated 1X SSC and 0.1 % (m/v) SDS and once with 0.1X SSC and 0.1 % (m/v) SDS, each at 65 °C for 10 min.

#### *Visualisation*

Following brief rinsing of membranes in deionised water, they were sealed in polyethylene film and exposed to Cyclone storage phosphorscreens (Packard



Instrument Company, Meriden, CT, USA) for 16 h. Data were captured and analysed using OptiQuant™ software version 3.1 for the Cyclone storage phosphorscreen imager (Model A431201, Packard Instrument Company, Meriden, CT, USA).

### 3.3.8. RNA isolation

Total RNA was isolated from internodal tissue (internode 4, 7, 10, 13) by a modified method of Bugos *et al.* (1995). Two to 4 g of tissue was powdered in liquid nitrogen, distributed in 10 ml TENS buffer (0.1 M Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 0.1 M NaCl, 0.1 % (m/v) SDS), together with 100 mM 2-mercaptoethanol and 10 ml phenol: chloroform: isoamylalcohol (25:24:1 v/v) and vortexed at high speed for 2 min. Following the addition of 700 µl of 3 M sodium acetate (pH 5.2), the solution was mixed, stored on ice for 15 min and centrifuged at 12000 g for 15 min at 4 °C. An equal volume of isopropanol was added to the aqueous phase and the solution was incubated at – 85 °C for 15 min and at – 20 °C for 2 h. The precipitated RNA was recovered by centrifugation at 10000 g for 10 min at 4 °C and the pellet washed with 5 ml 70 % (v/v) ethanol and centrifuged once again. Thereafter, the dried pellet was resuspended in 750 µl diethyl pyrocarbonate (DEPC) - treated water, centrifuged at 10000 g for 5 min at 4 °C and the supernatant transferred to a microcentrifuge tube. The RNA was precipitated by an overnight incubation at 4 °C with 2 M (final concentration) lithium chloride and DEPC-treated water in a final volume of 1 ml. Following centrifugation at 15300 g for 15 min at 4 °C, the pellet was washed with 1 ml of 70 % ethanol, centrifuged at 10600 g for 5 min and briefly air-dried. The pellet was resuspended in 100 µl of DEPC-treated water and quantified using a spectrophotometer (Beckman DU® 7500, Beckman Instruments Incorporated, Fullerton, CA, USA). RNA purity was also determined by obtaining the absorbance spectrum at wavelengths ( $\lambda$ ) of 260 nm and 280 nm. Thereafter, RNA was stored at - 85 °C.

### 3.3.9 Northern blot analysis

Total RNA (10 µg) was electrophoresed in 1.2 % (m/v) agarose formaldehyde gel, which was rinsed with DEPC-treated water for 15 min with agitation. Thereafter it was equilibrated with 20X SSC twice for 15 min each, prior to blotting onto a Hybond™ -N+ membrane (Amersham Biosciences UK Limited, Buckinghamshire, England) according to the manufacturer's instructions using 20X SSC as the transfer buffer. RNA was UV cross-linked to the membrane at 70 mJ.cm<sup>-2</sup> and the membrane prehybridised at 65 °C for 6 h with 5X SSPE (750 mM NaCl, 44 mM NaH<sub>2</sub>PO<sub>4</sub>, 6 mM EDTA pH 7.4), 5X

Denhardt's solution, 0.5 % (m/v) SDS and 200  $\mu\text{g}\cdot\text{ml}^{-1}$  denatured herringsperm DNA. The probe was prepared and the membrane hybridised as in section 3.3.7. except this membrane was washed twice with 2X SSPE, 0.1 % (m/v) SDS at room temperature for 10 min, once with 1X SSPE, 0.1 % (m/v) SDS at 65 °C for 15 min and once with 0.1X SSPE, 0.1 % (m/v) SDS at 65 °C for 10 min. Subsequent membrane exposure and signal visualisation was carried out according to 3.3.7.

#### *3.3.10 cDNA gel blot hybridisation*

Total RNA (3  $\mu\text{g}$ ) was denatured at 65 °C for 10 min along with oligo-dT primer (0.4  $\mu\text{g}$ ). After cooling to room temperature, 0.625X Expand™ reverse transcriptase buffer, 10 mM DTT, 0.5 mM dNTP and 20 U RNase inhibitor were added and incubated at 37 °C for 2 min. Thereafter, the RNA was reverse-transcribed by Expand™ reverse transcriptase (75 U, Roche Diagnostics GmbH, Mannheim, Germany) at 42 °C for 1 h in a total reaction volume of 20  $\mu\text{l}$ . The reaction was stopped by the addition of 45 mM EDTA. The reaction mix was then diluted 10X and 1  $\mu\text{l}$  amplified in a PCR reaction of 50  $\mu\text{l}$  total volume containing 120 nM each of forward (SSTf, 5'CGAATGTGAAGCATG TGTT3') and reverse (SSTr, 5'GAAATACACAGGAAGTTGCTCAGA3') primers, and 3.5 mM  $\text{MgCl}_2$ , 40  $\mu\text{M}$  dNTPs, 1 U Taq DNA polymerase and Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1 % (v/v) Triton X-100). Reactions were conducted in a GeneAmp® PCR system 9700 under the following conditions: 1 cycle at 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The resultant reverse transcribed (RT)-PCR products were separated in a 0.8 % (m/v) agarose gel run and visualised by staining with ethidium bromide (1  $\mu\text{g}\cdot\text{ml}^{-1}$ , final concentration). Overnight upward capillary blotting, probe preparation, blot hybridisation and visualisation was conducted according to section 3.3.7.

#### *3.3.11 Sugar extraction*

Soluble material was extracted from 100 mg of finely chopped core tissue from internodes 3, 6, 9, 12, 13 and 16, that were harvested in duplicate from 4 replicate stalks per sugarcane line. Internodes were numbered such that internode 3 was taken as the internode subtended by the leaf with the first exposed dewlap. HM extraction buffer containing 30 % (v/v) 100 mM HEPES (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) pH 7.8 together with 20 mM  $\text{MgCl}_2$  (Saarchem, Wadeville, South Africa) and 70 % (v/v) absolute ethanol was added to the tissue in a 1:10 (m/v) ratio and incubated

at 70 °C overnight. The extracts were centrifuged at 20800 *g* for 15 min and the supernatant retained. Additional extraction buffer was added to the tissue in a ratio of 1:10 (m/v), and this was incubated for 1 h at 70 °C. After centrifugation at 20800 *g* for 15 min the supernatant was again retained, and the remaining tissue washed twice with 500 µl of hot ultrapure deionised water (with a resistivity of 18.2 MΩ.cm<sup>-1</sup>) by vortexing. Following centrifugation for 5 min at 20800 *g*, the supernatant was removed and pooled with the supernatants retained from the previous steps, and stored at -20 °C.

### 3.3.12 Quantification of sugars by High Pressure Liquid Chromatography (HPLC)

Soluble extracts were filtered (4 mm nylon 0.45 µm syringe filters, National Scientific Company, Duluth, GA, USA) and diluted 50 times with ultrapure deionised water. Chromatographic separations were performed on a Waters Associates (Millford, MA) HPLC system equipped with a 1500 series pump, SAT/IN™ module, 100 µl injection loop and electrochemical detector (PAD, model 2465, set at a sensitivity of 50 µA) with gold electrode which was maintained at the following potentials and durations  $E_1 = +0.08$  V ( $t_1 = 0.4$  s);  $E_2 = +0.73$  V ( $t_2 = 0.4$  s);  $E_3 = -0.57$  V ( $t_3 = 0.2$  s). Upon injection of 5 µl of soluble extract using Waters 717 plus Autosampler, the sugars were separated within 20 min, through a CarboPac™ PA1 analytical column (4 x 250 mm, DIONEX Corporation, Sunnyvale, CA, USA) with adjoining CarboPac™ PA1 guard column (4 x 50 mm, DIONEX Corporation, Sunnyvale, CA, USA) using a mobile phase of vacuum-filtered (0.45 µm 47 mm, Millipore Corporation, Billerica, MA, USA), degassed 200 mM NaOH prepared with ultrapure deionised water at a flow rate of 1 ml.min<sup>-1</sup>. Eluted sugars were integrated and quantified using the Breeze software package (Waters Associates, Milford, MA), on the basis of a calibrated sugar standard composed of glucose, fructose, sucrose and 1-kestose (Fluka, Buchs, Switzerland) at 16 µg.ml<sup>-1</sup>.

### 3.3.13 Enzymatic quantification of sucrose and hexoses

Extracts were filtered and diluted 5 and 10 times with ultrapure deionised water for the sucrose assay while extracts were not diluted for the hexose assay. These dilutions were established on the basis of obtaining absorbance values within the range of the spectrophotometric linearity, over which absorbance and sample concentration are directly proportional. Sucrose and hexose in the extracts were quantified using an enzyme-coupled spectrophotometric assay (Bergmeyer and Bernt, 1974). The assay method was adapted for use in a UV resistant Costar® 96-well microtitre plate (Corning

Incorporated, New York, USA) with a final assay volume of 255  $\mu$ l. Changes in absorbance were measured at 340 nm using a Synergy HT™ Multi-detection microplate reader (Bio-tek® Instruments Incorporated, Vermont, USA) operated by KC4™ software version 3.1. Assays of each sample were performed in quadruplicate.

#### 3.3.14 Tissue moisture content determination

Moisture content was determined by weighing a central core taken from selected internodes (4, 5, 6, 7, 9, 13 and 16) immediately after harvest, and again after 3 d at 70 °C. The difference in mass was expressed as a percentage of the fresh mass (fm).

#### 3.3.15 Statistical analyses

A one-way Analysis of Variance (ANOVA) with a posthoc Tukey test was conducted to determine statistical differences between the different sugarcane lines and internodes with respect to the total amount of sugar present in each (including and excluding 1-kestose). For this purpose, SPSS v11.5 for Windows (SPSS Inc.) was utilised. The alpha value used for these tests was 0.05, such that p values less than this indicate statistical differences.

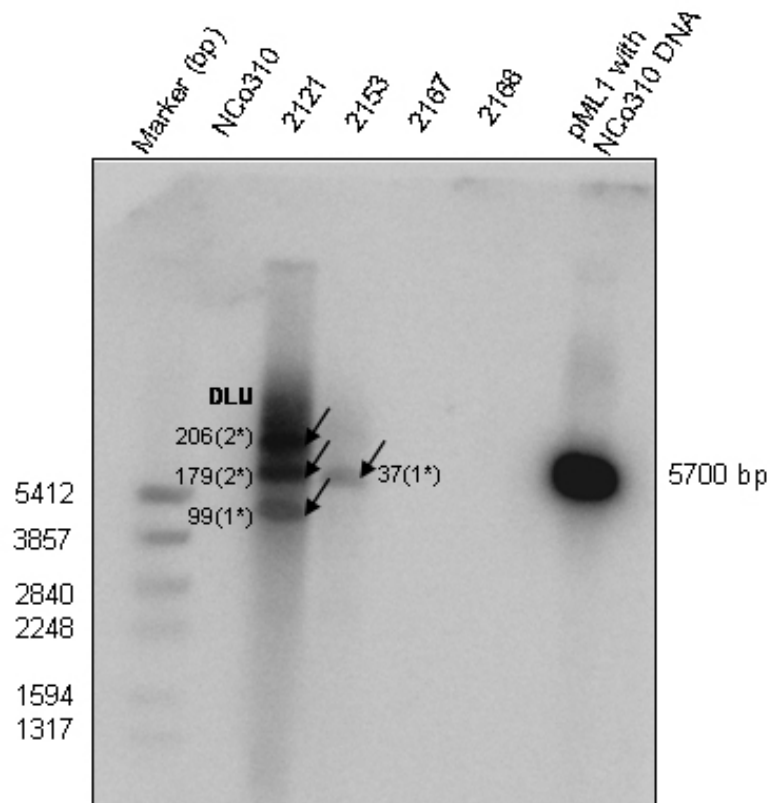
### 3.4 Results

#### 3.4.1 Molecular genetic analyses of the genotypes

##### *1-SST copy number*

Genomic DNA from all five sugarcane lines was cleaved with *Hind*III. Based on the position of *Hind*III sites within pML1 plasmid, cleavage with this enzyme yields two fragments of 3013 bp and 5770 bp. The larger fragment contains a complete and intact copy of the 1-SST gene. Therefore, *Hind*III-cleaved genomic DNA from transgenic lines will also yield fragments containing complete copies of the 1-SST gene within the genome.

Cleaved genomic DNA was Southern blotted and probed with a 609 bp region of 1-SST amplified from pML1 plasmid (Figure 3.1). No hybridisation was detected in the control line (NCo310) nor in lines 2167 and 2168. The other two lines, 2121 and 2153, showed hybridisation of a 5770 bp fragment that corresponds to a complete intact copy of the 1-SST gene. Based on the specificity of the probe, equal-sized fragments in 2121 and 2153 that hybridised to the probe confirmed the presence of an intact copy of 1-SST within these genomes (Figure 3.1).



**Figure 3.1** Southern blot of *Hind*III-digested genomic DNA from NCo310 and four lines that were regenerated following transformation with the pML1 plasmid (2121, 2153, 2167 and 2168). Plasmid pML1 with carrier NCo310 DNA was also digested using *Hind*III. A 609 bp region of 1-SST amplified from pML1 was used as a probe. Far left and right lanes contain the molecular marker and the positive control respectively. The intensity of the signal, representing a fragment of DNA that hybridised to the probe, was measured in Density Light Units (DLU). The numbers marked by asterisks denote the minimum number of 1-SST copies present.

Since, loading amounts of genomic DNA can differ between gel lanes, copy number of 1-SST in each sugarcane line (loaded per gel lane) was predicted individually. A minimum number of 1-SST copies was estimated on the basis of the hybridisation signal detected (Density Light Units). There was at least one intact copy (37554 DLU) of the 1-SST gene present in the 2153 genome. No rearrangement events occurred in this sugarcane line which is evident by the lack of other hybridised fragments (Figure 3.1).

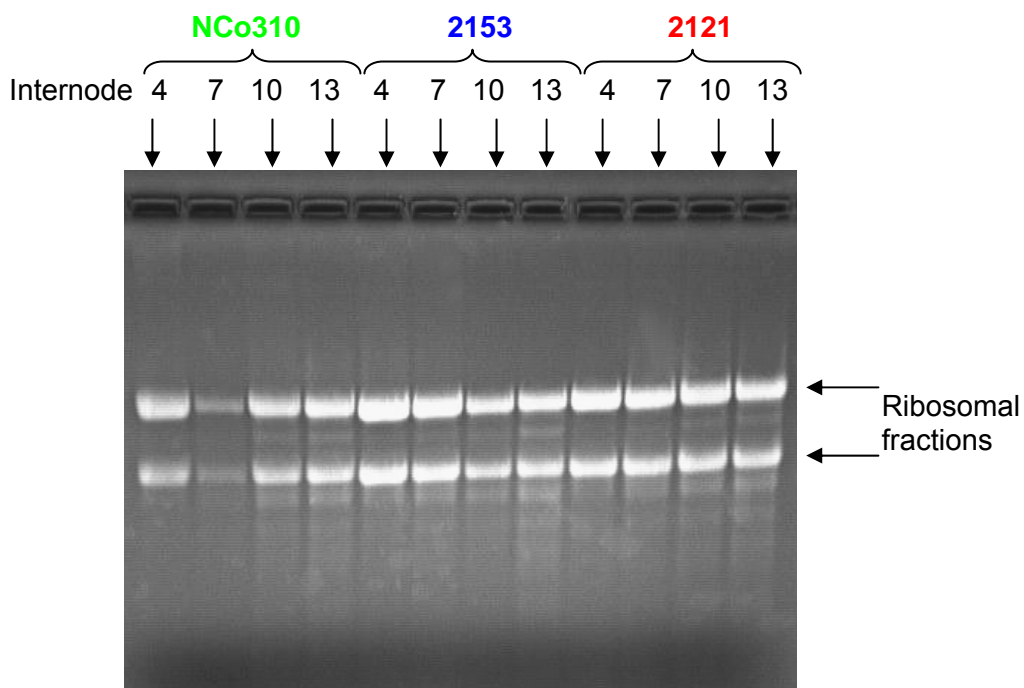
On the contrary, line 2121 showed two events of rearrangement (Figure 3.1). These are the result of the loss or replacement of *Hind*III cleavage sites in the plasmid, which disrupts cleavage. Hence, these fragments contain incomplete or rearranged stretches

of the 1-SST gene. There was at least one copy (99364 DLU) of the smaller fragment and a minimum of two copies of the larger fragment that resulted from rearrangement events (206239 DLU). This line also contained a minimum of two intact and complete copies (179250 DLU) of 1-SST. Therefore, a minimum of five 1-SST gene copies (or portions thereof) were present in line 2121.

These sugarcane lines were distinct in terms of their 1-SST gene copy number, but the presence of 1-SST in their genomes confirmed their transgenic status. On the basis of this molecular analysis, lines 2153 and 2121 were selected for further study.

#### *Transcription of 1-SST*

Following confirmation of the presence of 1-SST in the genomes of sugarcane lines 2121 and 2153, it was necessary to determine whether this gene was transcribed in these lines. RNA extracted from internodes 4, 7, 10 and 13 from the two transgenics and control line, NCo310, was separated by agarose gel electrophoresis (Figure 3.2), Northern blotted and probed with a 609 bp region of 1-SST.



**Figure 3.2** Separation of total RNA in an agarose formaldehyde gel. Total RNA was extracted from internodes 4, 7, 10 and 13 from a control (NCo310) and two transgenic lines (2153 and 2121). The two distinct ribosomal fractions are indicated by arrows.

The  $A_{260}/A_{280}$  ratio of the RNA extracts ranged between 1.5 - 1.7 which indicated that the extracted RNA was of a high purity. Two separate ribosomal RNA fractions were evident after staining with ethidium bromide, which suggested that no major degradation of RNA had occurred. However, no hybridisation was detected when the RNA was blotted and probed. Transcript levels may have been too low for detection using classical Northern blot analysis. Hence, copy DNA (cDNA) blot hybridisation was used as an alternative to determine transcription in the transgenic sugarcane lines.

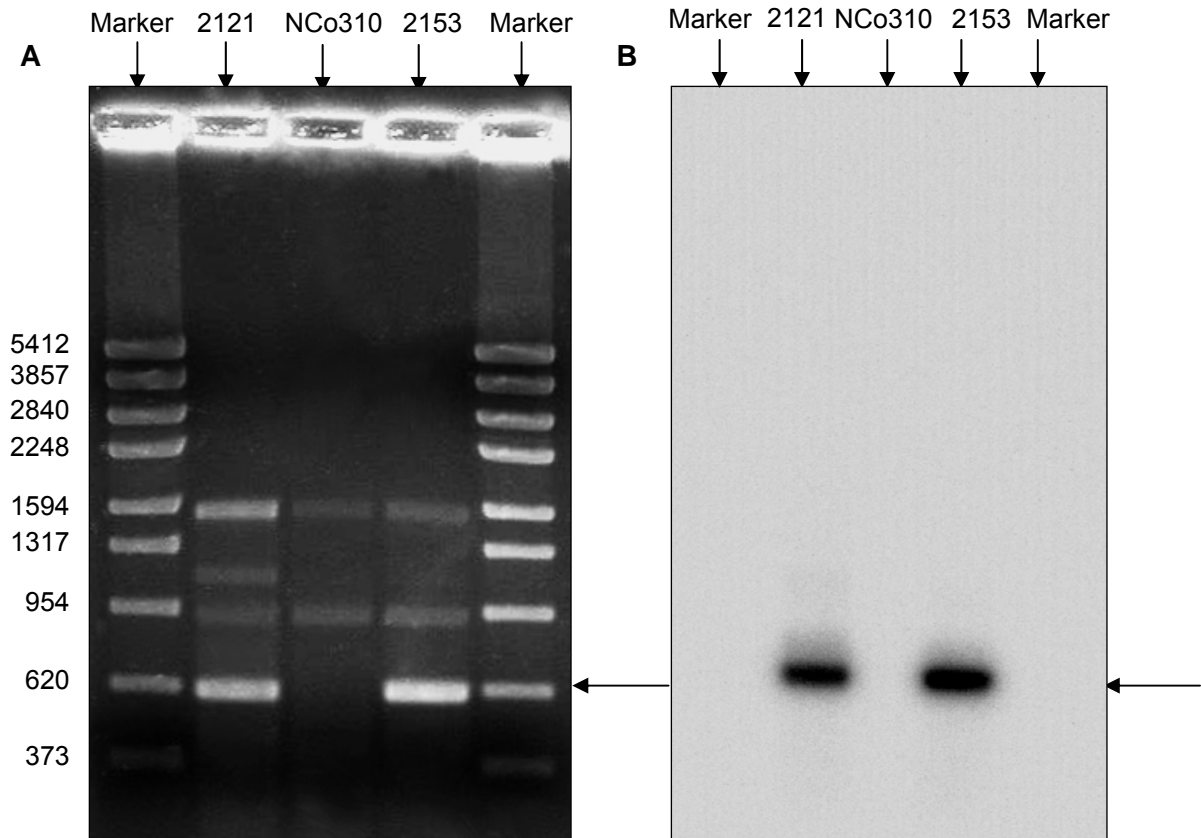
Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify cDNA reverse transcribed from total RNA of internode 4 of one control (NCo310) and two transgenic lines (2121 and 2153). The RT-PCR products were separated by agarose gel electrophoresis (Figure 3.3A). A 609 bp cDNA fragment was common to both transgenic sugarcane lines but was absent from the control line. Additional fragments from non-specific amplification were present in all three lines. cDNA blotting showed hybridisation of the 609 bp probe to one cDNA fragment amplified from both transgenic lines (Figure 3.3B). No hybridisation was evident in the control line NCo310. This confirmed the presence of the 1-SST transcript in sugarcane lines 2121 and 2153.

#### *3.4.2 System analysis: comparison of moisture content*

Based on their molecular characteristics and their production of 1-kestose, sugarcane lines 2153 and 2121 were selected for further study. To counter the possible confounding effects of moisture content on sugar concentration, moisture content down the culm was analysed, and compared to control line NCo310 (Table 3.1). The moisture content decreased down the culm of all three lines (Table 3.1). There was no statistical difference ( $p > 0.05$ ,  $N=28$ ) in the water content present in corresponding internodes between the three sugarcane lines.

#### *3.4.3 Sugar analysis*

Two methods, High Pressure Liquid Chromatography (HPLC) and enzymatic assays were used to measure 1-kestose in sugarcane. A highly significant correlation ( $R^2 = 0.99$ ) between the concentrations of glucose, fructose and sucrose determined by the two different methods was evident (Figure 3.4). Hence, either method could have been used with confidence for sugar quantification. For this study, HPLC was selected as the method of choice and used for all further sugar quantification.

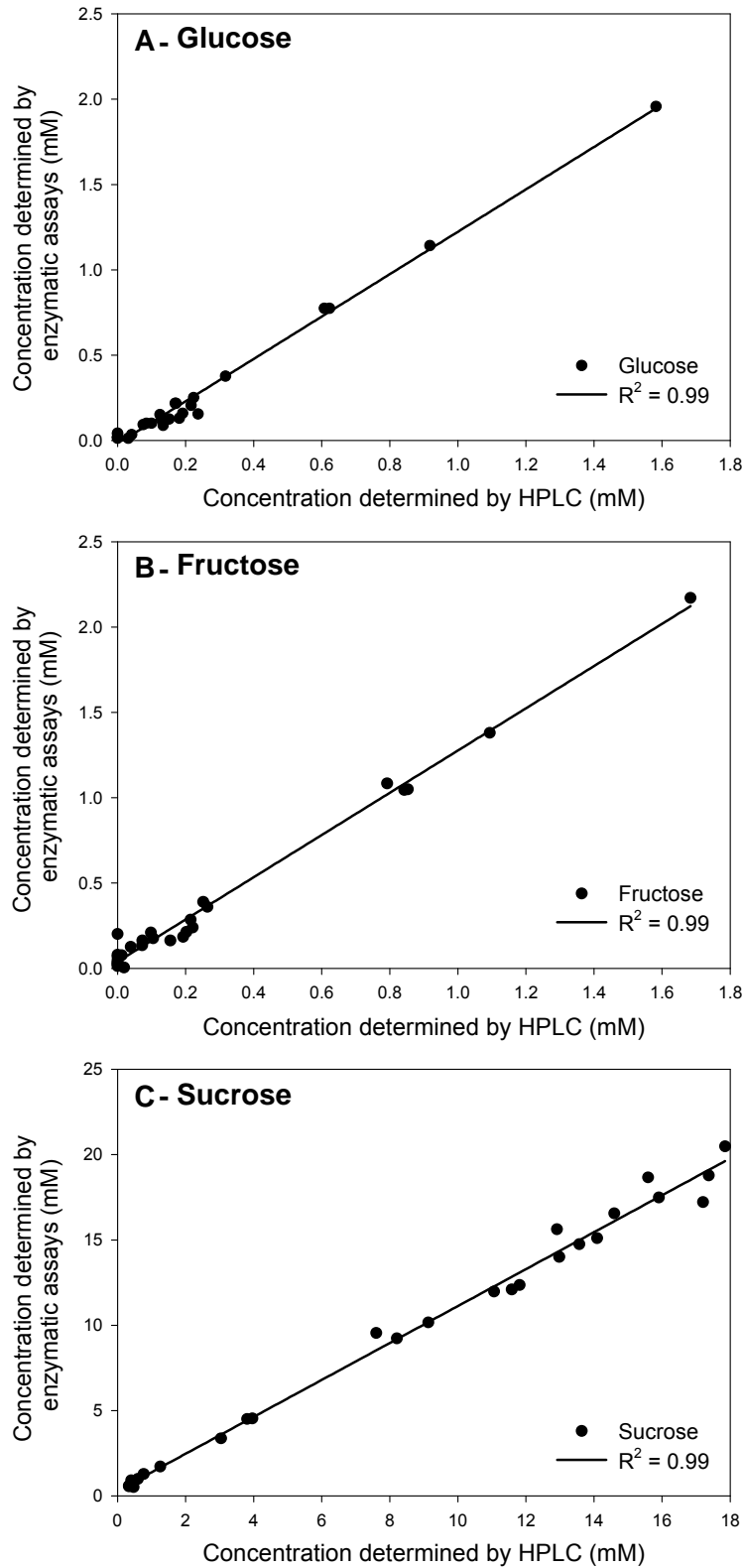


**Figure 3.3** Blot analysis of amplified cDNA. (A) Agarose gel showing PCR products amplified from cDNA that was reverse transcribed from RNA extracted from internode 4 of two transgenics (2121 and 2153) and one control (NCo310) line. The far left and right lanes contain molecular markers (bp). (B) cDNA blot that was probed with a portion of 1-SST. Arrows indicate fragments corresponding to the 609 bp portion of the 1-SST transcript that was contained in the genome of the transgenics but not in the control sugarcane line.

**Table 3.1** Percentage moisture present in seven selected internodes along the culm of three sugarcane lines, namely NCo310, 2153 and 2121. Values represent the mean  $\pm$  SE (N=4).

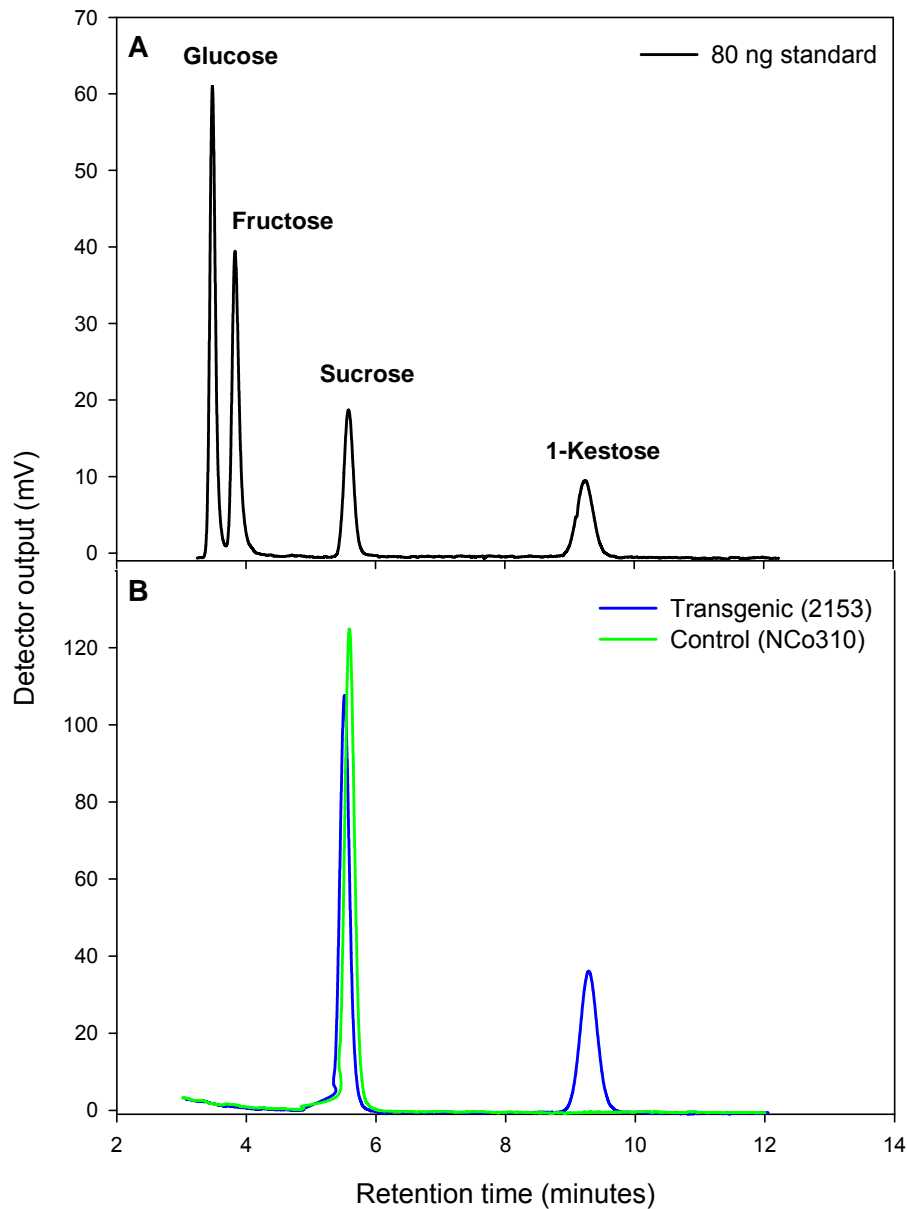
<i>Internode</i>	<i>Moisture Content (%)</i>							
	<b>NCo310</b>	<b>SE</b>	<b>2153</b>	<b>SE</b>	<b>2121</b>	<b>SE</b>		
4	91.41	$\pm$ 0.64	91.12	$\pm$ 0.42	91.72	$\pm$ 0.42		
5	92.38	$\pm$ 0.67	92.70	$\pm$ 1.01	91.74	$\pm$ 0.58		
6	89.96	$\pm$ 0.54	92.61	$\pm$ 0.40	91.97	$\pm$ 0.71		
7	85.89	$\pm$ 0.85	89.08	$\pm$ 0.62	90.28	$\pm$ 0.53		
9	81.63	$\pm$ 0.64	83.41	$\pm$ 0.95	87.31	$\pm$ 1.45		
13	78.60	$\pm$ 0.90	76.08	$\pm$ 0.70	78.03	$\pm$ 0.99		
16	78.95	$\pm$ 1.89	74.04	$\pm$ 0.40	77.85	$\pm$ 0.93		





**Figure 3.4** The concentration (mM) of glucose (A), fructose (B) and sucrose (C) determined by High Pressure Liquid Chromatography (HPLC) and enzymatic assays. In all three cases, a linear correlation ( $R^2 = 0.99$ ) between the two methods was obtained.

Sucrose was present in both the control and the transgenic line, but 1-kestose was also produced in line 2153 (Figure 3.5) and 2121. The presence of 1-kestose in the transgenic lines confirmed the expression and functionality of the introduced 1-SST gene in the transgenic sugarcane.



**Figure 3.5** Chromatographic separation of sugars. (A) Chromatogram showing the separation of glucose, fructose, sucrose and 1-kestose and an 80 ng sugar standard using HPLC. (B) Chromatogram of a sugar extract of internode 16 from a transgenic (2153) and a control (NCo310) line. Both lines produce sucrose but only the transgenic line produces 1-kestose.

*Sugar levels in the culm*

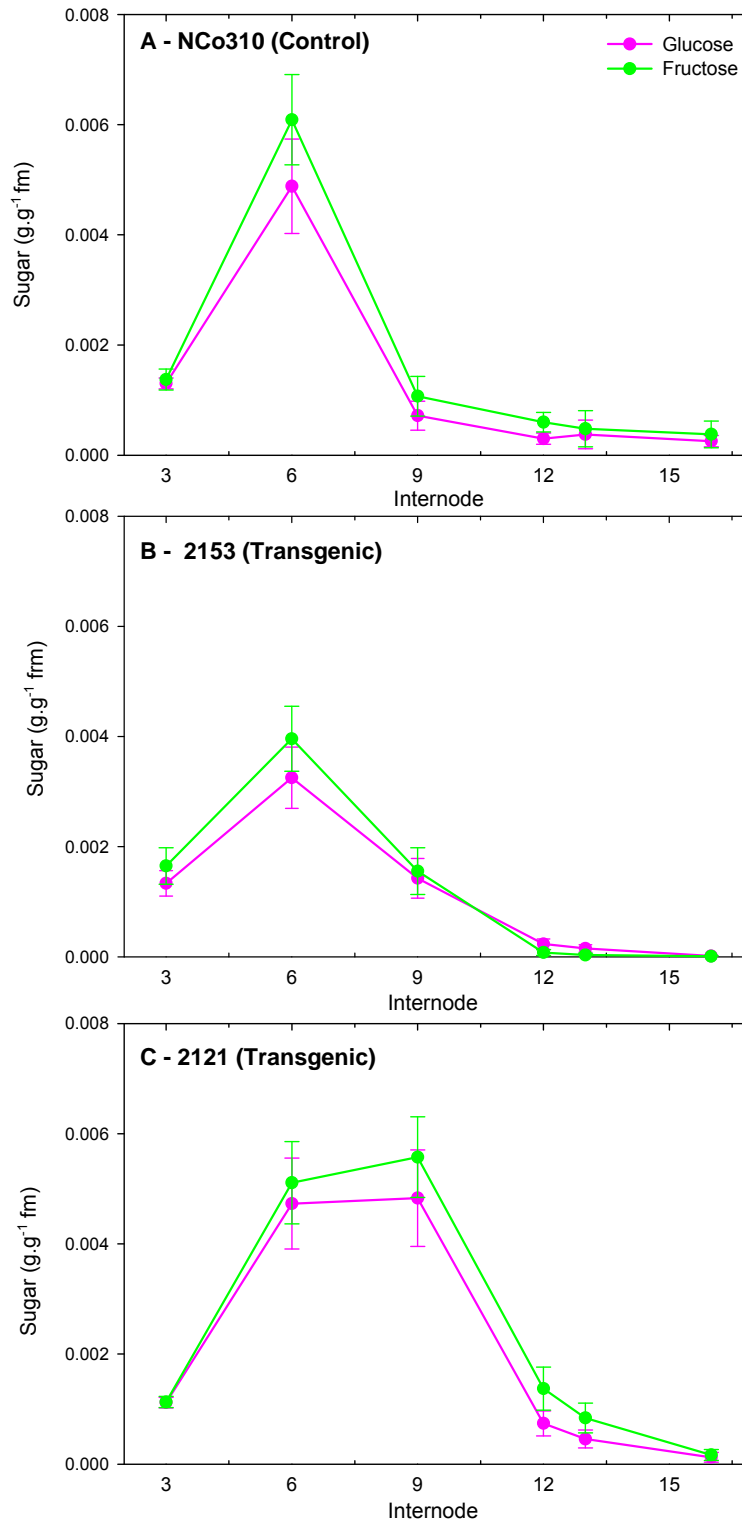
Sugar extracts from internodes 3, 6, 9, 12, 13, and 16 from the two transgenic lines and one control line were analysed to quantify the levels of glucose, fructose, sucrose and 1-kestose. In all three sugarcane lines fructose and glucose showed a similar trend in distribution down the culm, where hexose levels were highest in the young internodes, peaking at internode 6 (Figure 3.6). Sugarcane line 2121 showed increased hexose levels in both internode 6 and 9 which rapidly declined in internode 12.

Transgenic sugarcane line 2153 had approximately  $0.002 \text{ g.g}^{-1}$  fresh mass (fm) less fructose and glucose in internode 6 than the other lines (Figure 3.6). In all three sugarcane lines, the ratio of glucose to fructose is approximately one to one, despite the progressive increase in 1-kestose levels in 2153 from  $7.94 \pm 2.96 \text{ nmol.g}^{-1} \text{ fm}$  in internode 6 to  $112.01 \pm 17.42 \text{ nmol.g}^{-1} \text{ fm}$  in internode 16, and the slight increase of  $1.05 \pm 0.93 \text{ nmol.g}^{-1} \text{ fm}$  from the youngest to the oldest internode in sugarcane line 2121 (Table 3.2).

It was evident in all three sugarcane lines that sucrose levels increased with internodal maturity (Figure 3.7). Sucrose levels increased from internode 6 to internode 12 after which a plateau was reached. This trend was statistically verified with the old internodes (12, 13, 16) having significantly more sucrose than internode 9, which in turn contained significantly higher sucrose levels than the young internodes (3, 6) ( $p < 0.01$ ,  $N = 36$ ).

Comparison of sucrose levels between sugarcane lines highlighted that the transgenic lines 2153 and 2121 had 1.3- and 1.6-fold more sucrose respectively than control line NCo310. Moreover, the sucrose levels present in these transgenics were statistically verified to be significantly higher than NCo310 ( $p < 0.01$ ,  $N = 72$ ).

With respect to 1-kestose, this novel trisaccharide was not detected in the control line but it was present in both transgenic lines, where it appears to accumulate in the older internodes. There were notable differences between the transgenic lines in terms of 1-kestose. This trisaccharide was first detected from internode 6 in line 2153 but only from internode 9 in line 2121. Furthermore, sugarcane line 2153 contained 100 times more 1-kestose in internode 16 than sugarcane line 2121.



**Figure 3.6** Glucose and fructose levels (gram.gram<sup>-1</sup> fresh mass) down the culm of a control - NCo310 (A) and two transgenic, 2153 (B) and 2121 (C) sugarcane lines. Bars represent the mean  $\pm$  SE (N=12).

**Table 3.2** The concentration (nmol.g<sup>-1</sup> fm) of glucose (Glc), fructose (Fru), sucrose (Suc) and 1-kestose (1-Kes) present in six selected internodes spanning the culm of sugarcane lines NCo310 (control) and two transgenic lines, 2153 and 2121. Values represent the mean ± SE (N=12).

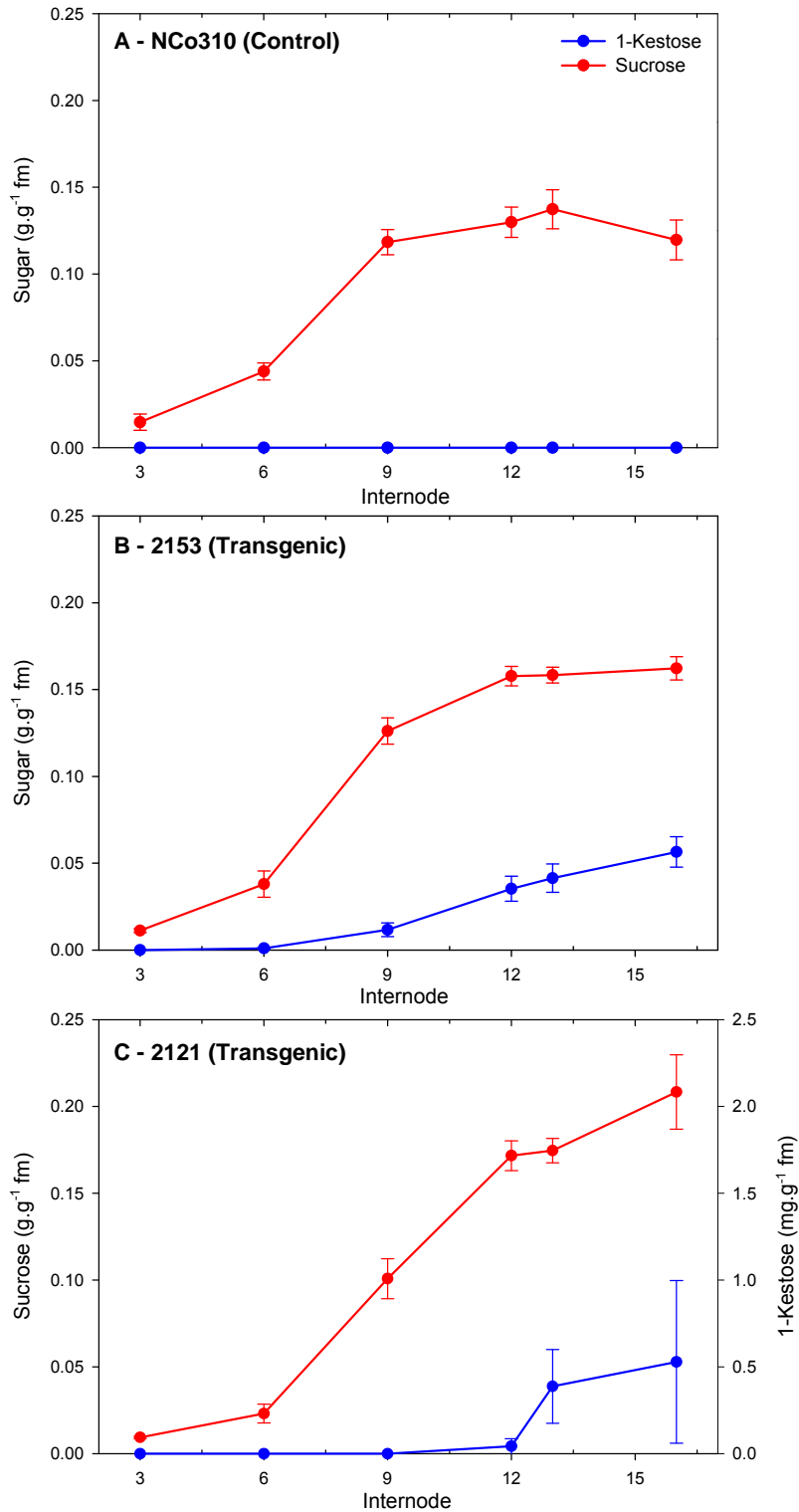
<i>Line</i>	<i>Internode</i>	<i>Sugar (nmol.g<sup>-1</sup> fm)</i>							
		<i>Glc</i>	<i>±SE</i>	<i>Fru</i>	<i>±SE</i>	<i>Suc</i>	<i>±SE</i>	<i>1-Kes</i>	<i>±SE</i>
<b>NCo310</b>	3	7.23	0.54	7.62	1.06	42.71	13.68	0.00	0.00
	6	27.09	4.54	33.79	4.36	128.16	14.27	0.00	0.00
	9	4.78	1.63	7.12	2.22	345.82	21.19	0.00	0.00
	12	3.99	0.72	7.98	2.19	379.36	30.89	0.00	0.00
	13	2.09	1.44	2.67	1.83	401.19	32.87	0.00	0.00
	16	1.42	0.58	2.10	1.34	349.54	33.56	0.00	0.00
<b>2153</b>	3	7.39	1.28	9.16	1.84	32.70	3.36	0.00	0.00
	6	18.04	3.10	21.96	3.27	110.96	22.15	7.94	2.96
	9	8.63	2.05	8.63	2.35	368.50	22.09	23.14	7.86
	12	3.08	0.58	2.55	0.04	460.88	16.33	69.96	14.34
	13	2.53	0.32	2.17	0.18	462.58	13.18	82.09	16.18
	16	0.95	0.08	0.65	0.05	474.01	19.63	112.01	17.42
<b>2121</b>	3	6.21	0.53	6.26	0.56	27.48	1.85	0.00	0.00
	6	26.25	4.57	28.36	4.15	67.48	15.81	0.00	0.00
	9	26.81	4.86	30.94	4.06	294.54	33.66	0.00	0.00
	12	5.48	1.40	10.15	2.33	501.35	25.19	0.08	0.08
	13	3.81	1.11	7.98	1.62	509.97	20.45	0.77	0.42
	16	2.79	1.53	3.74	1.14	608.66	62.63	1.05	0.93

#### *Total sugar content*

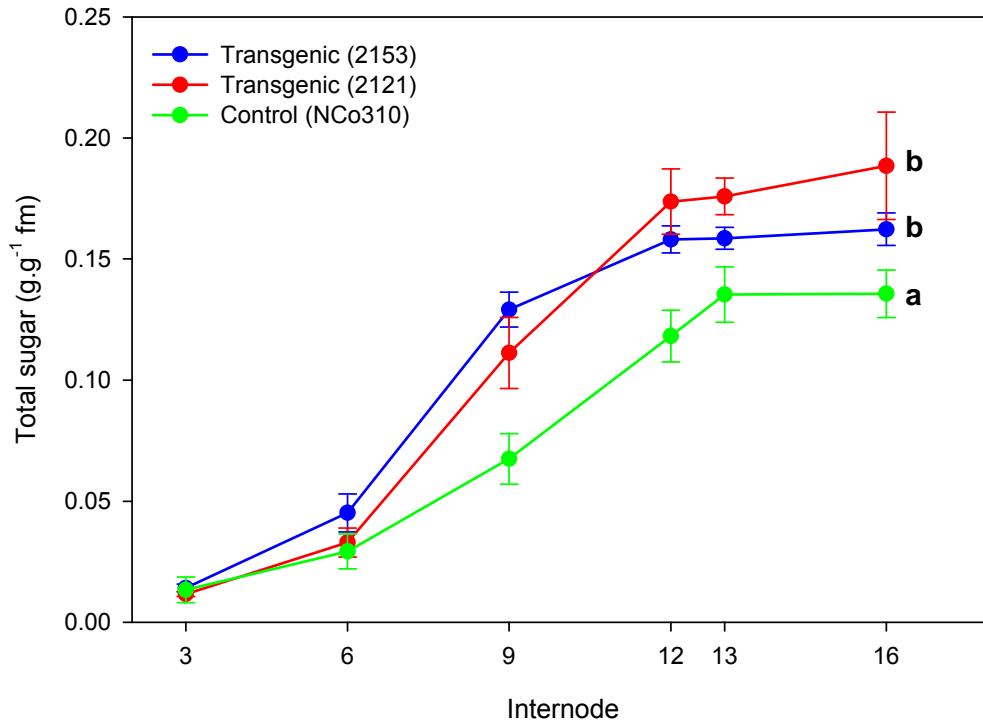
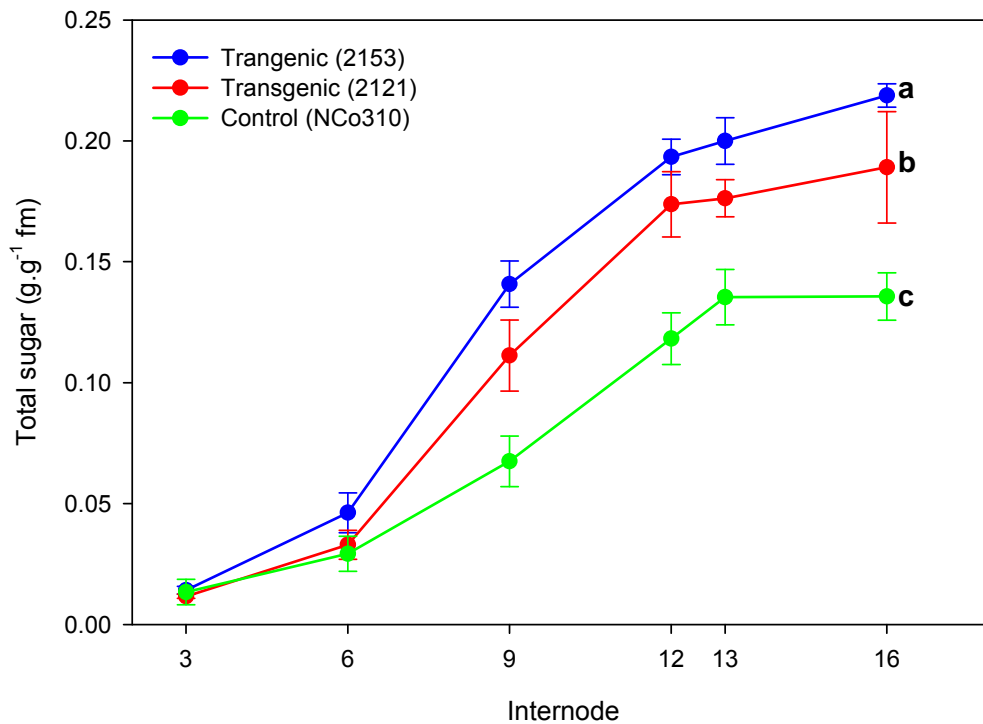
Following the quantification of the individual sugars down the culm of the three sugarcane lines, the total sugar levels in the transgenic lines were calculated to determine whether they had been affected by the genetic event. Total sugar present in internodes 3, 6, 9, 12, 13 and 16, excluding (Figure 3.8A) and including (Figure 3.8B) 1-kestose, was calculated and the control and transgenic lines analysed for differences.

Irrespective of sugarcane line, there was a significant difference ( $p < 0.01$ ,  $N = 36$ ) in the total sugar content present between internodes such that total sugar content increased with increasing internodal maturity (Figure 3.8). When glucose, fructose and sucrose were totalled for each internode from the three sugarcane lines, a statistical difference

between the transgenics and the control line was observed, with both transgenics having significantly ( $p < 0.01$ ,  $N = 72$ ) more total sugar than the control line (Figure 3.8A).



**Figure 3.7** Sucrose and 1-kestose levels ( $\text{gram}\cdot\text{gram}^{-1}$  fresh mass) down the culm of a control - NCo310 (A) and two transgenic - 2153 (B) and - 2121 (C) sugarcane lines. Bars represent the mean  $\pm$  SE ( $N = 12$ ).

**A – Excluding 1-kestose****B – Including 1-kestose**

**Figure 3.8** Total amount of sugars (A) excluding and (B) including 1-kestose present in a control (NCo310) and two transgenic (2153 and 2121) lines. Bars represent the mean  $\pm$ SE (N=12). Different lowercase letters denote an overall statistical significant difference ( $p < 0.01$ ) between sugarcane lines, where the alpha value is 0.05. A significant interaction ( $p < 0.01$ ) between line and internode was also noted.

When all measured sugars, including 1-kestose, were taken into account, the transgenic lines (2153 and 2121) had significantly more total sugar ( $p < 0.01$ ,  $N = 72$ ) than NCo310, the control line (Figure 3.8B). In turn, 2153, which is the line that produces the highest quantity of 1-kestose (Table 3.2, Figure 3.7), had significantly more ( $p < 0.01$ ,  $N = 72$ ) total sugar than 2121 (Figure 3.8B).

### 3.5 Discussion

Although 1-SST has been introduced into potato (Hellwege *et al.*, 1997; Hellwege *et al.*, 2000) and sugar beet (Sévenier *et al.*, 1998), this is the first report of 1-kestose production in transgenic sugarcane grown under field resembling conditions. This study centred on the molecular characterisation of four sugarcane lines that were regenerated after transformation with 1-SST. The ultimate aim of this work was to select the most appropriate sugarcane lines for future carbon partitioning studies (Chapter 5), determine 1-kestose production in these lines under field resembling conditions, and to assess the effect on sucrose levels.

*Non linear relationship between 1-SST copy number, expression and 1-kestose*  
Gene copy number was negatively correlated with levels of 1-kestose. Sugarcane line 2121 contained a minimum of five 1-SST gene copies or portions thereof (Figure 3.1), but it contained 100 times less 1-kestose in the oldest sampled internode (Figure 3.7) than 2153, which had at least one intact and complete copy of 1-SST in the genome (Figure 3.1). On the basis of the distinct difference in terms of quantity of 1-kestose produced in the two transgenic lines, they will be referred to as the high – (2153) and low – (2121) 1-kestose producers. These lines offer a valuable experimental system and were therefore used for further analyses. The genomic DNA of the other two analysed lines (2167 and 2168) did not include 1-SST, hence these two lines were not selected for any further testing.

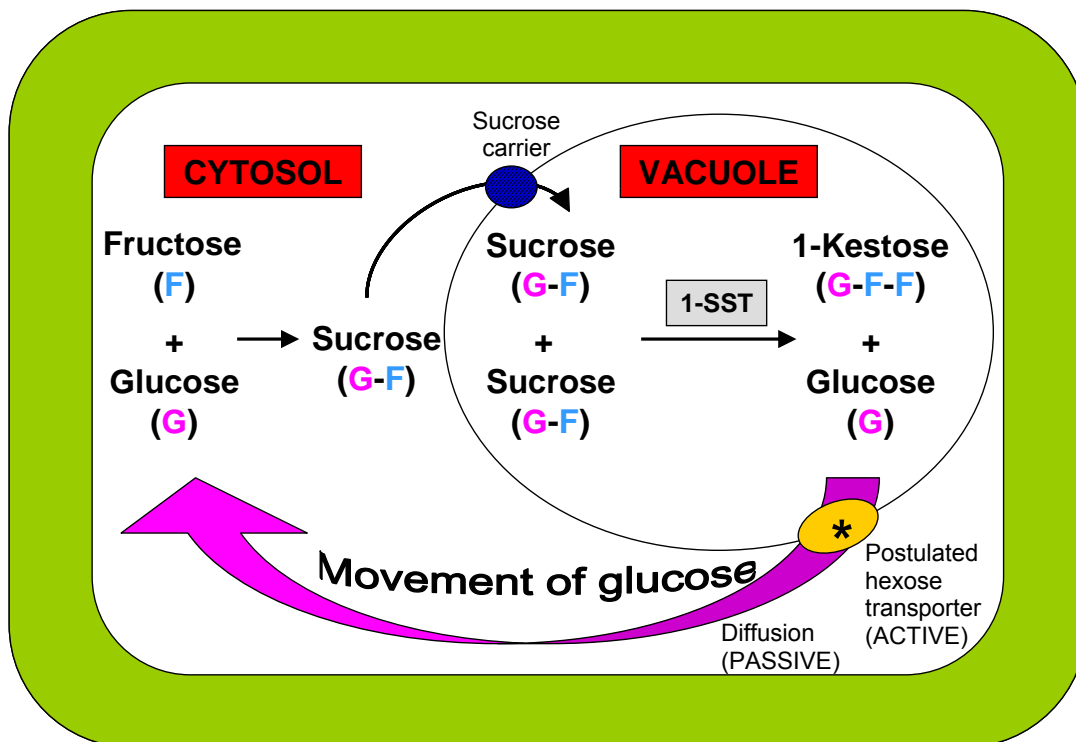
Copy DNA gel blot hybridisation, unlike Northern blot analysis, does not facilitate the quantification of transcriptional expression, however it is useful to confirm the presence of a particular transcript. Jaakola *et al.* (2001) successfully utilised cDNA blotting as an alternative to Northern blotting. These researchers maintain that cDNA gel blot hybridisation is more sensitive and reliable since it eliminates the concern of RNA degradation. Evidently the 1-SST transcript is present in both transgenic lines as reflected in the cDNA gel blot hybridisation (Figure 3.3B) and by the presence of 1-



kestose (Figure 3.5 and 3.8). Interestingly, the 1-SST gene was not silenced in the low 1-kestose line despite the high gene copy number in the genome.

*Indications of an efficient vacuolar glucose export mechanism*

Both transgenic sugarcane lines produced 1-kestose (Figure 3.5, Figure 3.7) which is composed of one glucose and two fructosyl moieties (Figure 3.9). Since 1-kestose is synthesised from sucrose by the action of 1-SST, and sucrose is composed of glucose and fructose in a one to one ratio, it follows that glucose should increase on a one to one basis with every molecule of 1-kestose produced. On the basis that vacuolar glucose is neither metabolised nor phosphorylated, glucose formed from this novel reaction is likely to accumulate. However, elevated glucose levels were not observed in either transgenic line (Figure 3.6, Table 3.2). The ratio of glucose to fructose was approximately 1:1 suggesting an efficient system capable of regulating hexose levels in the vacuole. This is likely to be by transporting glucose out of the vacuole (Figure 3.9).



**Figure 3.9** Schematic presentation of a transgenic sugarcane cell showing the vacuolar synthesis of 1-kestose from two sucrose molecules and the by-production of glucose. The yellow circle marked by an asterisk designates the possible position of a postulated transporter of glucose from the vacuole to the cytosol thereby regulating hexose levels between the cytosol and vacuole. It is speculated that transport could be either via an active symport located in the tonoplast or by means of passive diffusion.

A sucrose H<sup>+</sup>/ antiport mechanism has been postulated for vacuolar sucrose transport (Getz, 1991; Getz, 2000) but no mechanism of hexose movement out of the vacuole has been described. It has been proposed, however, that a hexose H<sup>+</sup>/ symport is present in the plasmalemma (Getz, 1991). On the basis that all biological membranes are composed of a phospholipid bilayer and associated proteins (Wolfe, 1995; Buchanan *et al.*, 2000), it is reasonable to postulate that a hexose H<sup>+</sup>/ symport proposed to be located in the plasmalemma (Getz, 1991) could also be present in the tonoplast. In support of this, Rae *et al.* (2005) inferred the expression of a hexose transporter in sugarcane parenchyma cells. Alternatively, a passive method of diffusion of hexoses out of the vacuole may be operational, thereby facilitating glucose movement between the cytosol and the vacuole. A passive route of equilibration implies that the hexose concentration in the cytosol and vacuole will equal the cytosolic concentration. Since the vacuole occupies approximately 90 % of the cellular volume (Komor, 1994; Komor, 2000), the distribution of hexoses will tend such that 90 % will be in the vacuole and only 10 % in the cytosol. Hence, it is likely that movement of glucose out of the vacuole is not passive.

*The transgenics have increased sucrose and total sugar levels*

No significant difference in moisture content was observed between corresponding internodes of the three tested sugarcane lines (Table 3.1). Therefore, the concentration of measured sugars is not due to differences in moisture content between sugarcane lines (Table 3.1). In all three sugarcane lines, sucrose levels increased with increasing internodal maturity (Table 3.2, Figure 3.7). This phenomenon of a progressive shift from active growth and elongation in young internodes to sucrose storage in older internodes has been widely documented (Moore, 1995; Whittaker and Botha, 1997; Zhu *et al.*, 1997; Botha and Black, 2000; Rose and Botha, 2000; Moore, 2005; Rae *et al.*, 2005). Furthermore, a multitude of literature report a gradient of maturation and sucrose accumulation down the culm until complete maturation and peak sucrose levels are reached (Moore, 1995; Whittaker and Botha, 1997; Zhu *et al.*, 1997; Botha and Black, 2000; Rose and Botha, 2000; Moore, 2005; Rae *et al.*, 2005).

The transgenic lines showed incremental accumulation of 1-kestose in the older internodes (Table 3.2, Figure 3.7). The hypothesis of this work was that since 1-kestose is produced from sucrose, sucrose levels would decline with the production of 1-kestose. However, the transgenic sugarcane lines did not show reduced sucrose levels. On the contrary, the high – and low – 1-kestose producers contained 1.3- and

1.6-fold more sucrose respectively than the control, and these differences were statistically significant (Figure 3.7). This outcome is particularly noteworthy on the basis of limited photosynthate entering the tissues.

The transgenic lines also had significantly higher total sugar content than NCo310, when all measured sugars were taken into account (Figure 3.8). This finding can be attributed to the additive production of 1-kestose coupled with elevated sucrose levels in the transgenics. This the first report of alternative sugar production in transgenic sugarcane grown under field resembling conditions. The only reported research on alternative sugar production in sugarcane is that conducted on glasshouse-grown cane (Birch and Wu, 2005; Wu and Birch, 2007). Sugarcane was transformed with bacterial sucrose isomerase which catalyses the conversion of sucrose to isomaltulose; another disaccharide. The genetic modification yielded high sucrose concentrations and in turn increased the soluble carbohydrate content (Birch and Wu, 2005; Wu and Birch, 2007).

### 3.6 Conclusion

Fifty percent of the sugarcane lines tested were confirmed to be transgenic by the presence of the 1-SST gene and transcript, as well as the production of 1-kestose. Remarkably, this trisaccharide was produced in addition to elevated sucrose levels, which increased total sugar content. The two transgenic lines were distinctive high- and low- 1-kestose producers and they afford the opportunity for further investigations into the compounding effect of 1-kestose production on the partitioning of carbon and sucrose metabolism (Chapter 5).

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## CHAPTER 4

### CHARACTERISATION OF THE KINETIC PROPERTIES OF SUGARCANE INVERTASES IN RELATION TO 1-KESTOSE

#### 4.1 Abstract

Soluble acid invertase (SAI) and neutral invertase (NI,  $\beta$ -fructofuranosidase EC 3.2.1.26) from non-transgenic sugarcane (*Saccharum* spp. hybrids) internodal tissues were partially purified, and the affinity of these sucrose hydrolysing enzymes for 1-kestose was tested. Partial purification resulted in the separation of SAI and NI. Kinetic analysis of the partially purified invertases revealed two isoforms of SAI eluting at approximately 100 mM KCl in a linear gradient while NI eluted at approximately 500 mM KCl. The final specific activities of SAI and NI were 88.57 pkat.mg<sup>-1</sup> protein and 92.31 pkat.mg<sup>-1</sup> protein, respectively. This implied a 16- fold purification of SAI, and 4- fold purification of NI. The pH optimum for NI was 7.0 and that for soluble acid invertase less than 5.0. Due to the broad pH activities of the invertases, activities significantly overlapped between pH 4.5 and 7.0. The enzymes displayed hyperbolic saturation kinetics for sucrose hydrolysis. Soluble acid invertase isoform 1, 2 and NI had  $K_m$  values of 11.73  $\pm$  1.31 mM, 8.36  $\pm$  1.26 mM and 18.83  $\pm$  2.68 mM for sucrose respectively. The sugarcane-extracted invertases had very low affinities for 1-kestose with  $K_m$  values ranging from 50 - 247 mM. Furthermore, the presence of 200 mM 1-kestose had an inhibitory effect on invertase-mediated sucrose hydrolysis reducing SAI activity to 51 % and 54 % for isoform 1 and 2, respectively. In the presence of 1-kestose, 97 % of NI activity remained, thus NI does not seem to be inhibited by 1-kestose.

#### 4.2 Introduction

Since 1-kestose is native to fructan-accumulating plants, the relationship between sugarcane invertases and this trisaccharide has not been previously investigated. Knowledge of the ability of sugarcane invertases to hydrolyse 1-kestose *in vitro*, and its relation to invertase-mediated sucrose hydrolysis, is essential for understanding future investigations of *in vivo* carbon partitioning (Chapter 5) in 1-kestose-producing sugarcane lines (Chapter 3).

The culm tissues of sugarcane (*Saccharum* spp. hybrids) accumulate high concentrations of sucrose (for review see Moore, 1995; Komor, 2000) and this is the nett result of continual hydrolysis and synthesis (Wendler *et al.*, 1990; Zhu *et al.*, 1997). Sucrose supplies energy to the plant, but in order for it to be used hydrolysis of the



glycosidic bond between the glucose and fructose moieties is required. This is mediated by two different enzymes. Sucrose synthase (SuSy, EC 2.4.1.13) catalyses the reversible cleavage of sucrose to UDP-glucose and fructose, while invertases ( $\beta$ -fructofuranosidase, EC 3.2.1.26) catalyse the irreversible hydrolysis to glucose and fructose. There are three forms of invertases and they are characterised according to their pH optima, subcellular localisations and biochemical properties namely cell wall (insoluble, acid), vacuolar (soluble, acid), and cytosolic (soluble, neutral) invertase (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999).

These enzymes have been purified from a number of species and their kinetic properties analysed with respect to sucrose hydrolysis. It has been widely reported that invertases also hydrolyse fructans (Cairns and Ashton, 1991; Pollock and Cairns, 1991; Cairns, 1993; Bonnett and Simpson, 1993; Simpson and Bonnett, 1993). Fructans are oligo- and poly-fructosyl sucrose and 12-15 % of higher plants use these as their primary carbohydrate (Pollock and Cairns, 1991). Fructan breakdown is mediated by fructan exohydrolase (FEH, EC 3.2.1.80) which sequentially hydrolyses the terminal fructose residues, thereby shortening the chain (Bonnett and Simpson, 1993; Simpson and Bonnett, 1993; Pollock *et al.*, 1999; Ritsema and Smeekens, 2003).

Although it has been reported that invertases are capable of fructan hydrolysis, there is controversy surrounding invertase-mediated hydrolysis of 1-kestose; the shortest fructan of the inulin type (Pollock, 1986; Cairns and Pollock, 1988). 1-Kestose is a trisaccharide ( $GF_2$ ) which is produced by the transfer of a fructosyl moiety from one sucrose molecule to another by sucrose: sucrose 1- fructosyltransferase (1-SST, EC 2.4.1.99) in fructan-accumulating plants. There have been reports that this trisaccharide is susceptible to hydrolysis by invertases (Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003) and that it is likely that invertase would have a similar affinity for 1-kestose as for sucrose. However, a recent publication reported almost no invertase activity on 1-kestose and in addition suggested an inhibitory effect of 1-kestose on sucrose hydrolysis by invertase (Nagaraj *et al.*, 2005). This suggested that plant invertases might not use 1-kestose as a substrate. To date, there have been no reports on the ability of sugarcane-extracted neutral- and soluble acid- invertase to hydrolyse 1-kestose. The aim of this study was therefore to clarify the relationship between 1-kestose and invertases. Neutral (NI) and soluble acid invertases (SAI) from sugarcane were extracted, partially purified and their

kinetic properties, using sucrose as a substrate, were characterised. Furthermore, the ability of these enzymes to hydrolyse 1-kestose was investigated.

### 4.3 Materials and methods

#### 4.3.1 Sugar extraction

Sugars were extracted from 100 mg of finely chopped core tissue from internode 17 of transgenic sugarcane (*Saccharum* spp. hybrid) line 2153 according to the method described in section 3.3.11.

#### 4.3.2 Chromatographic separation of the individual sugars

The soluble sugar extracts were concentrated 10 times by vacuum-drying (240 g, 1 bar) at 30 °C in an Eppendorf concentrator (Model 5301, Eppendorf, Hamburg, Germany) and their pellet resuspended in HM extraction buffer (30 % (v/v) 100 mM HEPES (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) pH 7.8 together with 20 mM MgCl<sub>2</sub> (Saarchem, Wadeville, South Africa) and 70 % (v/v) absolute ethanol), vortexed and centrifuged at 20200 g for 5 min. A semi automatic Thin Layer Chromatography (TLC) sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland) spotted 5 µl of each extract onto silica gel TLC plates (10 cm x 20 cm, Merck Chemicals Pty Ltd, Darmstadt, Germany). The individual sugars were separated using ethyl acetate (BDH Chemicals Limited, Poole, England), acetic acid (Saarchem, Wadeville, South Africa) and deionised water (2:1:1 v/v ratio) and the plate was developed to completion twice in a CAMAG Horizontal developing chamber (CAMAG, Muttenz, Switzerland), and dried for 2 min at 80 °C each time. Fructose-containing compounds were visualised by spraying the TLC plates with a urea reagent (Wise *et al.*, 1955) and heating the plates to 80 °C for 10 min. By comparison with a 50 µg 1-kestose standard (Fluka, Buchs, Switzerland) that was separated alongside the samples, spots corresponding to 1-kestose were scraped from the silica gel TLC plate, resuspended in 100 µl deionised water and solubilised at 4 °C overnight. Following brief vortexing and centrifugation at 20200 g for 10 min, the supernatant was retained.

#### 4.3.3 Hydrolysis of 1-kestose by yeast invertase

An aliquot of solubilised 1-kestose was incubated with 660 U β-fructofuranosidase from yeast (EC 3.2.1.26, Roche Diagnostics GmbH, Mannheim, Germany) in a 10:1 (v/v) ratio at room temperature for 20 min. Protein was removed from the solution by trickling it over the surface of Hybond™ -C extra membranes (Amersham Biosciences UK Limited, Buckinghamshire, England), prior to High Pressure Liquid Chromatography (HPLC) analysis. The resultant sugars were separated as described

in section 3.3.12. An 8  $\mu\text{g}\cdot\text{ml}^{-1}$  sugar standard containing glucose, fructose, sucrose and 1-kestose (Fluka, Buchs, Switzerland) was used.

#### 4.3.4 Extraction and partial purification of invertase from sugarcane

Internodes were numbered such that internode 3 was taken as the internode subtended by the leaf with the first exposed dewlap. Internodes 5 to 8 of field-grown sugarcane cultivar N19 were harvested, a core from each was chopped and the pooled tissue frozen in liquid nitrogen. The tissue was ground using a small IKA® All basic mill (IKA®-Werke, GmbH and company, Germany). Thereafter ice-cold extraction buffer (50 mM HEPES pH 7.2 containing 100  $\text{mL}\cdot\text{L}^{-1}$  glycerol, 1 mM EDTA pH 8.0, 10 mM DTT and cØmplete® protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Penzburg, Germany)) was added to the tissue in a 1:2 (m/v) ratio. The slurry was gently stirred for 5 min at 0 °C and then filtered through two layers of muslin cloth. The filtered solution was centrifuged at 12000  $g$  for 15 min at 4 °C and the soluble fraction retained and desalted at 4 °C on a Sephadex™ G-25M column (Amersham Biosciences UK Limited, Buckinghamshire, England) that was pre-equilibrated with the same buffer. The desalted sample was loaded onto a HiTrap™ Q HP 5 ml ion exchange column (Amersham Biosciences UK Limited, Buckinghamshire, England) that was connected to a Gilson FC 204 fraction collector (Gilson Medical Electronics Inc., WI, USA). The column was washed with 25 mM HEPES (pH 7.2) to remove unbound protein. Once  $A_{280}$  of the flow-through had decreased to zero, the bound proteins were eluted (2  $\text{ml}\cdot\text{min}^{-1}$ ) with a linear gradient of 0 – 500 mM KCl in 25 mM HEPES (pH 7.2) over 16 column volumes and collected in 2 ml fractions. Protein elution was monitored by  $A_{280}$  and fractions containing NI or SAI activity greater than 20 % of the maximum peak were pooled and stored at 4 °C for kinetic analysis.

#### 4.3.5 Enzyme assays

Neutral invertase and SAI were assayed in 50 mM HEPES (pH 7.2) and 50 mM citrate/phosphate (pH 5.0) respectively, with 125 mM sucrose at 28 °C for 16 h in a final volume of 125  $\mu\text{l}$ . An aliquot from each sample was taken prior to the incubation, flash-frozen in liquid nitrogen and used as assay blanks. Glucose production was determined by a modified method of Bergmeyer and Bernt (1974), (*Section 3.3.13*), but in this case an appropriate amount of partially purified NI or SAI was used and the reaction was incubated for 25 min after the addition of hexokinase/glucose-6-phosphate dehydrogenase (EC 2.7.1.1/ 1.1.1.49, 0.5 U per assay, Roche Diagnostics GmbH, Mannheim, Germany). Activity was linear with respect to enzyme concentration. For kinetic constant determination, a range of substrates namely

sucrose (0 – 125 mM), 1-kestose (0 – 125 mM) or sucrose (62.5 mM) plus 1-kestose (0 – 200 mM) were used and  $K_m$  and  $V_{max}$  determined by nonlinear fitting of the data to the Michaelis-Menten equation (Sigmaplot v7.0, with Enzyme Kinetics module v1.1, SPSS Inc.). The pH optima of the invertases were determined using the assay system described above with 125 mM sucrose and incubated with 50 mM citrate/phosphate/HEPES buffer (pH 4.5 – 8.0).

#### 4.3.6 Protein determination

Quantification of protein present in NI and SAI was performed according to Bradford (1976) using bovine serum albumin (BSA) (Bio-rad Life Science Group, Johannesburg, SA) as a standard. Samples were diluted 10 times in 25 mM HEPES (pH 7.2) and protein assay dye reagent concentrate (Bio-rad Life Science Group, Hercules, CA, USA) was added in a 1:4 (v/v) ratio. After exactly 8 min, absorbance at 595 nm was read using Synergy HT™ Multi-detection microplate reader (Bio-tek® Instruments Incorporated, Vermont, USA) operated by KC4™ software version 3.1.

#### 4.3.7 Statistical analyses

A two-tailed student t-test was used to determine statistical differences between treatments. These differences are indicated by different lowercase letters, where the alpha value was 0.05.

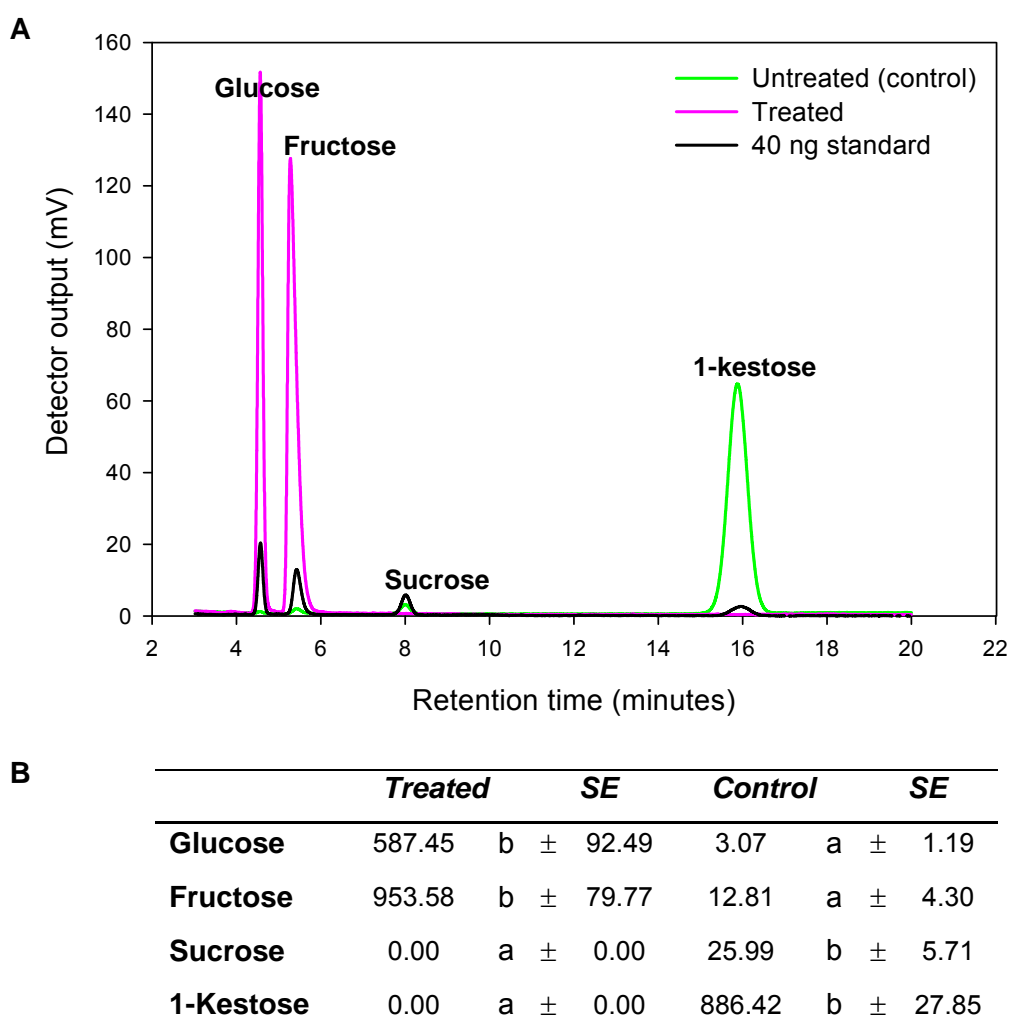
### 4.4 Results

#### 4.4.1 1-Kestose is hydrolysed by yeast invertase

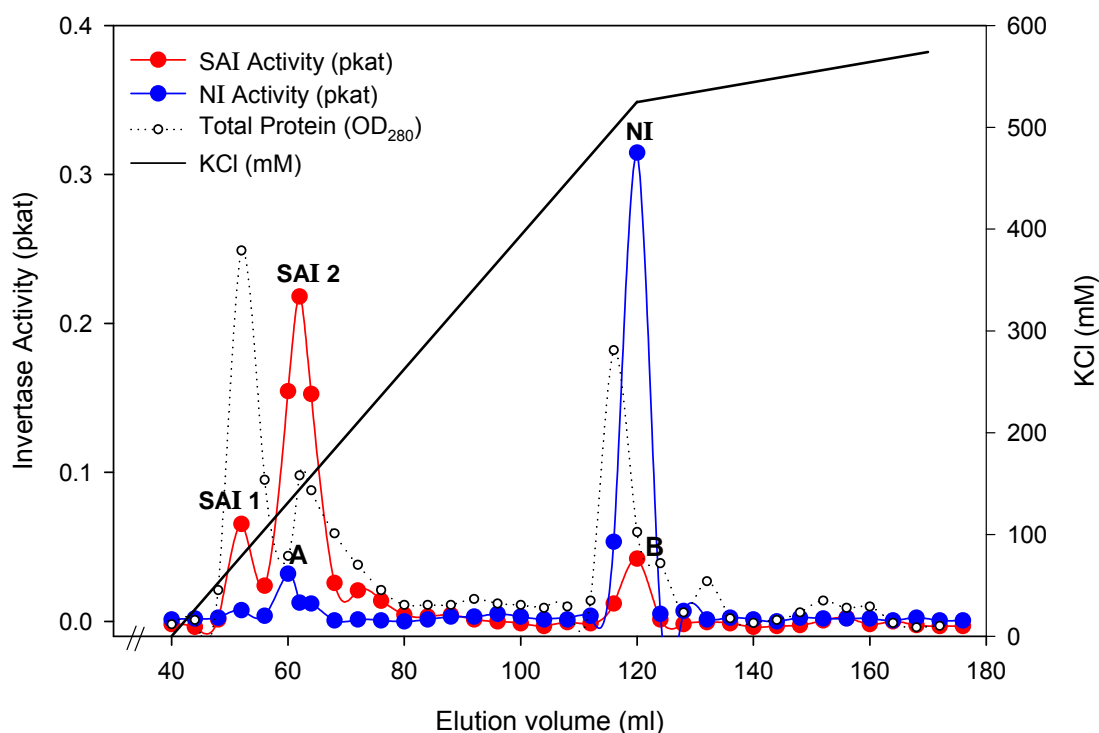
1-Kestose was incubated with yeast invertase and the resultant sugars were separated (Figure 4.1A) and quantified (Figure 4.1B) by HPLC. Treatment with yeast invertase resulted in the hydrolysis of 1-kestose to yield glucose and fructose. The untreated 1-kestose sample showed the sugar levels prior to treatment with yeast invertase. Since 1-kestose was extracted and separated from sugarcane that was genetically modified to produce this trisaccharide (Chapter 3), the untreated control was composed of 95 % 1-kestose and 5 % sucrose, glucose and fructose (Figure 4.1B). Furthermore, there was a highly significant difference ( $p < 0.01$ ,  $N=3$ ) between the individual sugars in the untreated control and the yeast treated sample with statistically less 1-kestose and significantly more hexoses present after exposure to yeast invertase than before (Figure 4.1B). The average fructose to glucose ratio of three independent treatments after incubation with yeast invertase was  $1.67 \pm 0.16$ . Hence, the composition of 1-kestose was confirmed to be two fructose moieties to one glucose moiety.

## 4.4.2 Chromatographic separation and purification of sugarcane-extracted invertases

Invertases extracted from young internodes from sugarcane line N19 were separated and partially purified by anion exchange chromatography (Figure 4.2). Soluble acid invertase and neutral invertase respectively eluted at ca 100 mM and 500 mM KCl in a linear gradient. Two soluble acid invertase peaks were detected which inferred the presence of two isoforms and these were designated SAI 1 and SAI 2. Peak A represents some NI activity at acidic pH (5.0) while peak B shows SAI activity at neutral pH (7.2).



**Figure 4.1** Treatment of 1-kestose with yeast invertase. **(A)** Chromatogram showing the glucose and fructose produced when 1-kestose was hydrolysed by yeast invertase (treated) in relation to an untreated 1-kestose (control). The  $8 \mu\text{g}\cdot\text{mL}^{-1}$  standard constituted 40 ng each of glucose, fructose, sucrose and 1-kestose and was used to quantify the sugars and denote their retention times. **(B)** The amount ( $\mu\text{M}$ ) of glucose and fructose produced when 1-kestose was treated with yeast invertase, compared to the sugars present in the untreated control. Values are the mean  $\pm$  SE (N=3). Different lowercase letters indicate statistical differences in terms of the amount of an individual sugar present before and after hydrolysis.



**Figure 4.2** Separation of soluble acid invertase (SAI) and neutral invertase (NI) by anion exchange chromatography (HiTrap™ Q HP). Bound proteins were eluted in a linear KCl gradient (0 - 500 mM) and invertase activity was determined by the glucose production after 16 h incubation with 125 mM sucrose. One peak of NI activity and two peaks displaying SAI activity eluted at approximately 500 mM and 100 mM KCl respectively. The two detected SAI peaks were designated isoform SAI 1 and SAI 2. Furthermore, activity of NI at an acidic pH (5.0) and that of SAI at a neutral pH (7.2) was noted as peak A and B.

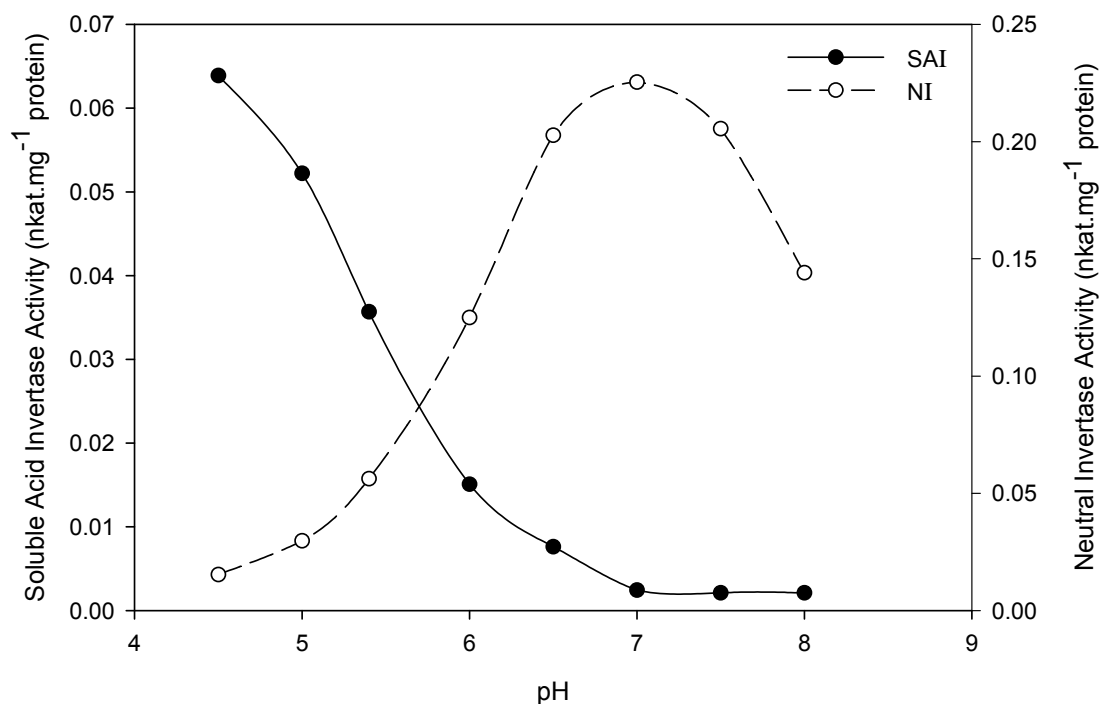
Details of purification are presented in Table 4.1. The SAI and NI activities were 88.57  $\text{pkat}\cdot\text{mg}^{-1}$  protein and 92.31  $\text{pkat}\cdot\text{mg}^{-1}$  protein respectively. This implied a 16- fold purification of SAI, and 4- fold purification of NI. Of the total crude extract activity, 32 % was recovered in SAI and 29 % in NI thereby contributing to 61 % of the total activity recovered by purification.

**Table 4.1** Purification of extracted sugarcane neutral and acid invertases. Activities were determined with 125 mM sucrose as a substrate.

Step		Total Protein (mg)	Total Activity (pkat)	Specific Activity ( $\text{pkat}\cdot\text{mg}^{-1}$ protein)	Purification (fold)	Yield (%)
Crude extract (desalted)	SAI	7.22	38.94	5.39	1	100
	NI		154.21	21.36		
Anion exchange chromatography (HiTrap™ Q HP)	SAI	0.14	12.40	88.57	16	32
	NI	0.49	45.23	92.31	4	29

#### 4.4.3 Soluble acid- and neutral- invertase function at a broad pH range

Partial purification separated SAI and NI and their activities were assessed over a series of pH values. Neutral invertase had a pH optimum of 7.0 and its maximal activity was halved at pH 5.9 (Figure 4.3). The activity of SAI was highest at a pH less than 5.0. Due to activity of these invertases at a broad pH range, the activity of the two invertases overlapped between pH 4.5 and 7.0 (Figure 4.3).

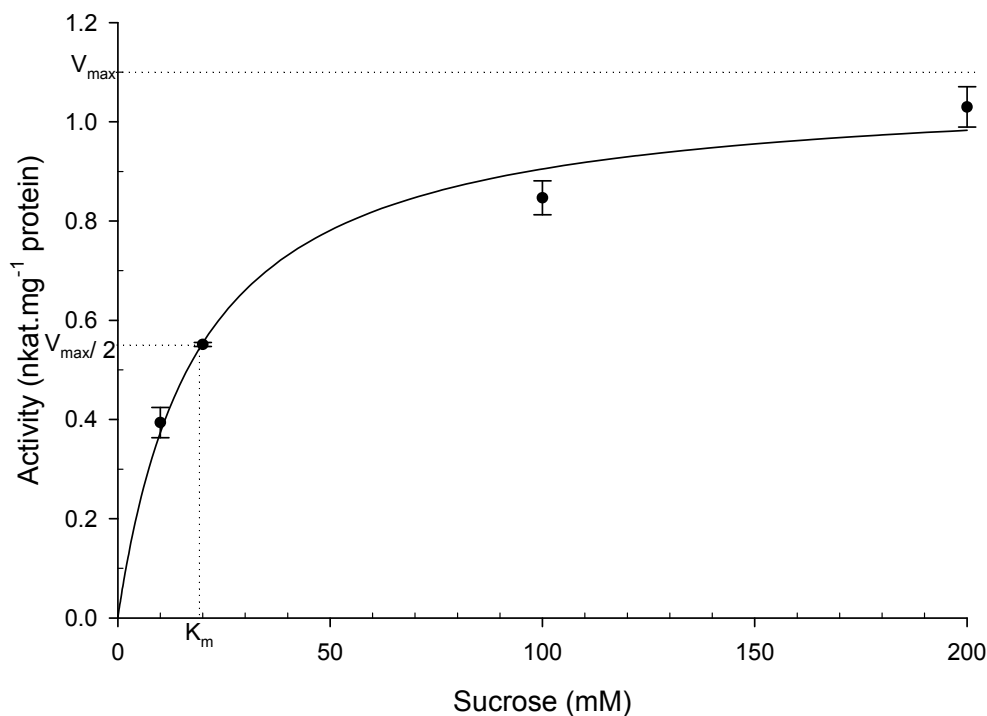


**Figure 4.3** The pH dependence of SAI 2 and NI separated by anion exchange chromatography. An overlap of activity is evident from pH 4.5 to pH 7.

#### 4.4.4 Kinetic properties of soluble acid- and neutral invertase

##### *Sucrose as a substrate for sugarcane invertases*

All three invertase enzymes exhibited typical hyperbolic saturation kinetics when sucrose was used as a substrate. The kinetic relationship between sucrose and neutral invertase activity is representative of this (Figure 4.4). Kinetic constants derived from fitting the data to Michaelis-Menten plot demonstrated  $K_m$  values of  $11.73 \pm 1.31$  mM,  $8.36 \pm 1.26$  mM and  $18.83 \pm 2.68$  mM for SAI 1, SAI 2 and NI respectively (Table 4.2). Isoforms 1 and 2 of SAI had specific activities of  $2.52 \pm 0.08$  and  $5.78 \pm 0.21$  nkat.mg<sup>-1</sup> protein respectively, while NI had a specific activity of  $1.08 \pm 0.04$  nkat.mg<sup>-1</sup> protein.



**Figure 4.4** Substrate saturation curve for sugarcane NI at pH 7.2. The curve was fitted to the mean of three independent replicates using sucrose (mM) as a substrate and is illustrative of the hyperbolic Michaelis-Menten kinetics displayed by all three invertases in relation to sucrose.

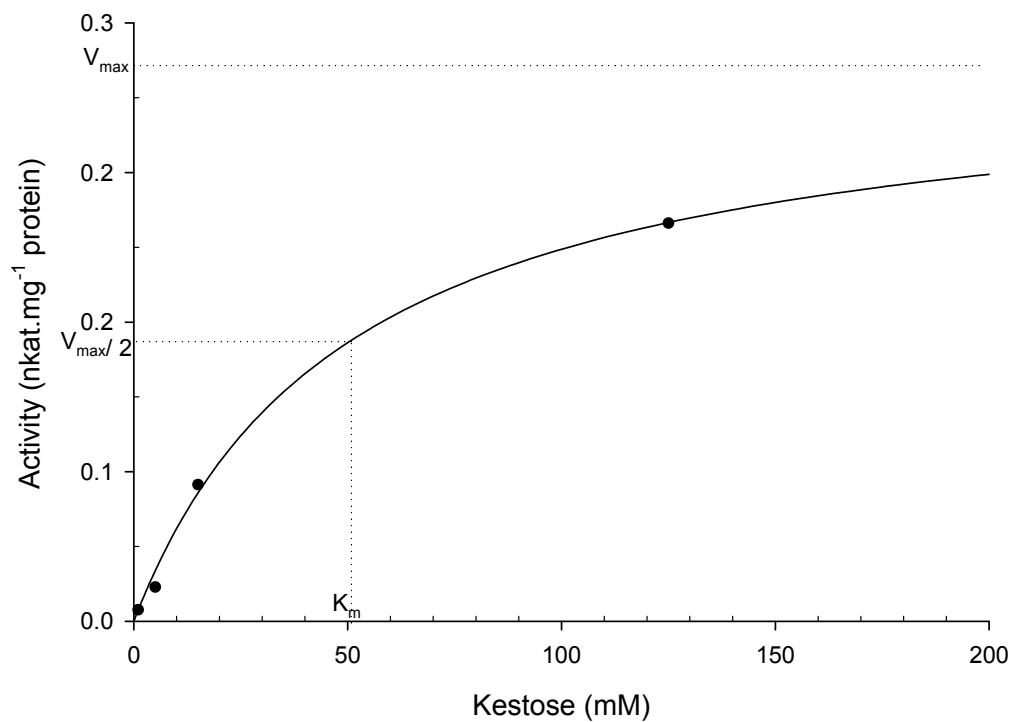
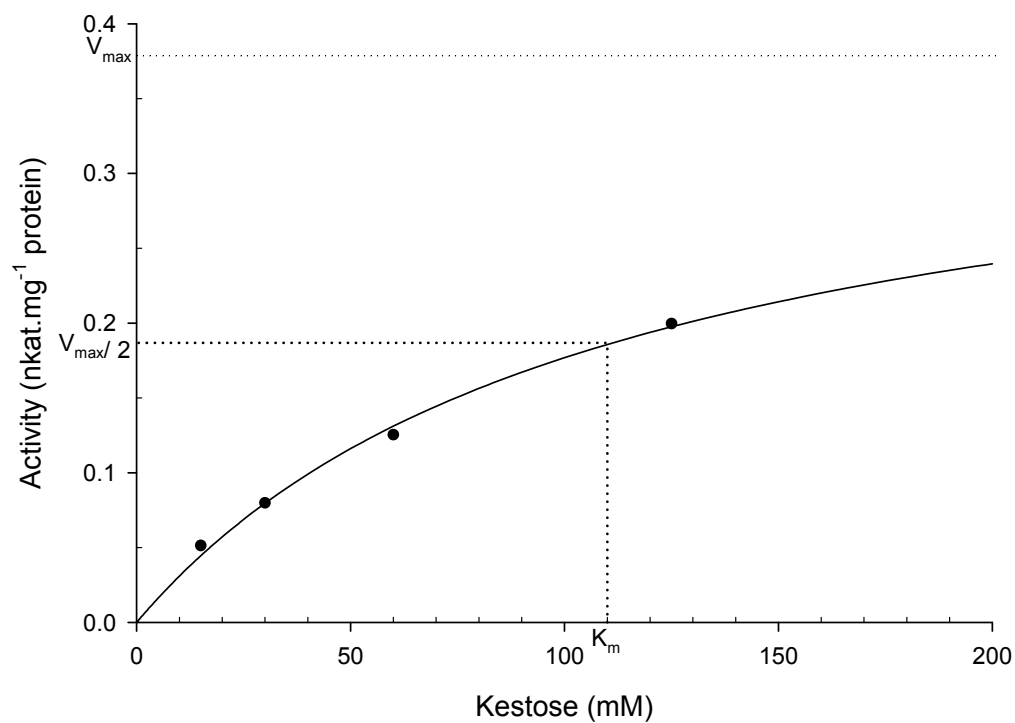
**Table 4.2** Kinetic constants for sugarcane acid and neutral invertase using sucrose as a substrate. The  $R^2$  co-efficient describes the fit of the data to a Michaelis-Menten hyperbolic curve.

Invertase	$V_{max}$ (nkat.mg <sup>-1</sup> protein)	$K_m$ (mM)	Fit ( $R^2$ )
<b>Soluble acid</b>			
<b>Isoform 1</b>	2.52 ± 0.08	11.73 ± 1.31	0.99
<b>Isoform 2</b>	5.78 ± 0.21	8.36 ± 1.26	0.99
<b>Neutral</b>	1.08 ± 0.04	18.83 ± 2.68	0.95

#### *1-Kestose as a substrate for sugarcane invertases*

When 1-kestose is used as a substrate, the substrate concentrations used did not saturate the enzyme (Figure 4.5). Instead, the reaction is still in the acceleration phase even beyond 100 mM 1-kestose. Curves for SAI are representative of the relationship of 1-kestose concentration and enzyme activity for both invertases (Figure 4.5A and 4.5B). Kinetic constants of SAI and NI are given in Table 4.3. The two SAI isoforms had  $K_m$  values of 50.43 ± 9.88 mM and 110.04 ± 22.36 mM respectively while NI had a  $K_m$  of 247.44 ± 91.78 mM 1-kestose.



**A – SAI isoform 1****B – SAI isoform 2**

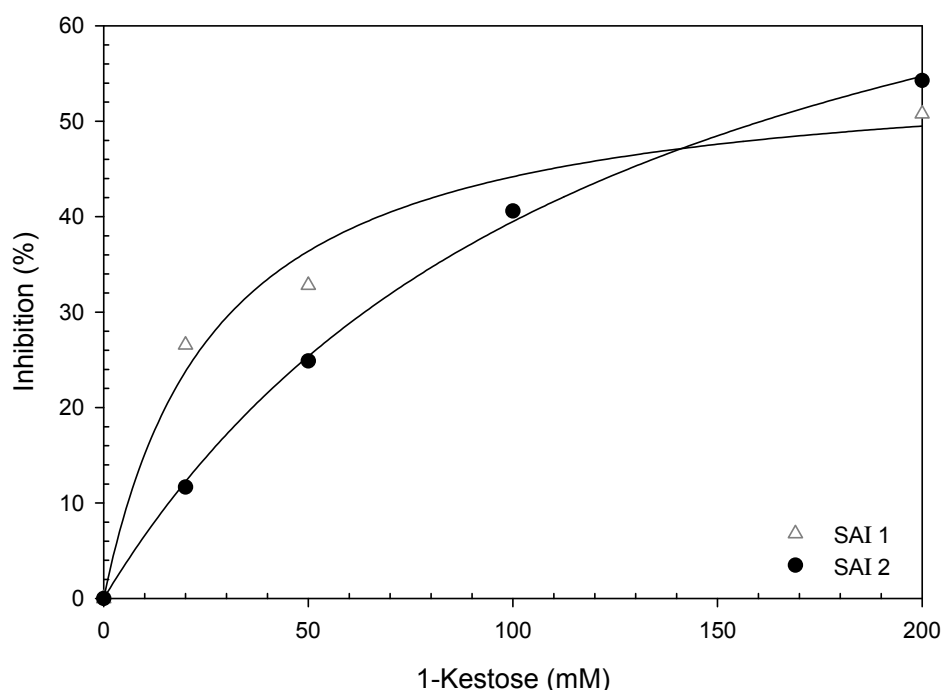
**Figure 4.5** The kinetic relationship between soluble acid invertases and 1-kestose. Substrate saturation curves for sugarcane (A) SAI 1 and (B) SAI 2 at pH 5.0. Substrate saturation of 1-kestose was not reached.

**Table 4.3** Kinetic constants for sugarcane acid and neutral invertase using 1-kestose as a substrate. The  $R^2$  co-efficient describes the fit of the data to a Michaelis-Menten hyperbolic curve.

Invertase	$V_{max}$ (nkat.mg <sup>-1</sup> protein)	$K_m$ (mM)	Fit ( $R^2$ )
<b>Soluble Acid</b>			
Isoform 1	0.281 ± 0.021	50.43 ± 9.88	0.996
Isoform 2	0.372 ± 0.043	110.04 ± 22.36	0.994
<b>Neutral Invertase</b>	0.029 ± 0.004	247.44 ± 91.78	0.966

#### *Sucrose hydrolysis in the presence of 1-kestose*

The ability of sugarcane invertases to hydrolyse sucrose was hindered by 1-kestose such that the higher 1-kestose concentrations decreased the efficiency of invertase hydrolysis of sucrose (Figure 4.6). At 200 mM 1-kestose, there was a respective 51 % and 54 % inhibition of SAI 1 and SAI 2 on sucrose hydrolysis. Furthermore  $I_{0.5}$  values were determined to assess the 1-kestose concentration at which 50 % inhibition of invertase sucrose hydrolysis occurs (Table 4.4). Ninety-seven percent of neutral invertase activity remained, thus the presence of 1-kestose does not seem to reduce the activity of sugarcane neutral invertase.



**Figure 4.6** Percentage inhibition of sugarcane soluble acid invertases on sucrose (62.5 mM) hydrolysis, by the presence of increasing 1-kestose concentrations.

**Table 4.4** The 1-kestose concentration (mM) at which 50 % of sucrose hydrolysis by the invertases is inhibited ( $I_{0.5}$  values).

Invertase	$I_{0.5}$ (mM)
<b>Soluble Acid</b>	
<b>Isoform 1</b>	27.32 ± 11.94
<b>Isoform 2</b>	125.41 ± 12.92
<b>Neutral</b>	2.37 ± 0.61

#### 4.5 Discussion

The purpose of this chapter was to characterise the kinetic properties of sugarcane invertases and test their ability to hydrolyse 1-kestose, a trisaccharide that is only synthesised by fructan-accumulating plants. This work provides insight into invertase-mediated hydrolysis of 1-kestose *in vitro* and contributes towards the understanding of 1-kestose hydrolysis in the transgenic sugarcane lines characterised in Chapter 3.

##### *Efficient separation of NI and SAI by anion exchange chromatography*

The pH optimum of NI was determined to be 7.0 while that of SAI was below 5.0. On the basis that invertases are classified according to their optimal pH and spatial location, this suggests a cytoplasmic and vacuolar location of NI and SAI respectively. These data are consistent with those reported previously (Hatch *et al.*, 1963, Gayler and Glasziou, 1972; Tymowska-Lalanne and Kreis, 1998; Vorster and Botha, 1998; Sturm, 1999; Hashizume *et al.*, 2003). The pH profile of the two sugarcane-extracted invertases are broad and overlapping (Figure 4.3). Slight activity of NI at an acidic pH (Figure 4.2, peak A) and of SAI at a neutral pH (Figure 4.2, peak B) emphasised that the approach of physically separating these invertases by anion exchange chromatography was necessary and more efficient than that by pH alone. On the basis of a broad pH range of the extracted invertases, the 4- and 16- fold purification of NI and SAI respectively and total yield of 61 % should be viewed as an estimate (Table 4.1).

##### *Identification of two soluble acid invertase isoforms in sugarcane*

The separation and partial purification of these enzymes revealed two distinguishable isoforms of SAI that were designated SAI 1 and SAI 2. Both eluted at approximately 100 mM in a linear KCl gradient (Figure 4.2). It is common for most plant species to contain at least two isoforms of soluble acid invertase (Sturm, 1999; Hashizume *et al.*, 2003). On the basis that each invertase isoform is encoded for by a different gene (Sturm, 1996; Tymowska-Lalanne and Kreis, 1998), and since sugarcane is a polyploid

(D'Hont *et al.*, 1998; Butterfield *et al.*, 2001), it is surprising that only two soluble acid isoforms were identified from sugarcane extracts. It has been suggested that multiple isoforms confer a physiological advantage in that they may allow for more flexibility in terms of control of sucrose metabolism (Tymowska-Lalanne and Kreis, 1998).

#### *Kinetic properties of the extracted invertases*

Sucrose hydrolysis by both sugarcane SAI isoforms and NI displayed classic Michaelis-Menten kinetics typified by a hyperbolic substrate saturation curve (Figure 4.4). The two soluble acid isoforms had distinct  $K_m$  values when sucrose was used as a substrate. These are within the range of 2 – 13 mM reported for sugarcane soluble acid invertases (Gayler and Glasziou, 1972; Del Rosario and Santisopasri, 1977). The  $K_m$  values for sugarcane SAI were higher than those reported for sugarbeet (2 - 3 mM, Giaquinta, 1979), Japanese pear fruit (3.33 mM and 4.58 mM, Hashizume *et al.*, 2003), and rice (6.6 mM, Isla *et al.*, 1995) but in line with those reported for barley (1 - 14 mM, Obeland *et al.*, 1993; Nagaraj *et al.*, 2005).

Neutral invertase has a  $K_m$  of  $18.83 \pm 2.68$  mM (Table 4.2) which is comparable to previous sugarcane NI determinations of 25 mM (Hatch *et al.*, 1963) and 9.8 mM (Vorster and Botha, 1998). The  $K_m$  determination of NI in this study also falls within the range of 10 – 20 mM reported for NI isolated from other sources (Lee and Sturm, 1996; Ross *et al.*, 1996). Since neutral invertase is in substrate-saturating conditions in the sugarcane culm which has symplastic sucrose concentrations between 110 mM and 616 mM (Welbaum and Meinzer, 1990), the variation of  $K_m$  values reported for NI will not affect sucrose hydrolysis.

#### *Sugarcane invertases have a low affinity for 1-kestose*

1-Kestose is amenable to hydrolysis by yeast invertase (Figure 4.1). Cairns (1993) also reports the degradation of 1-kestose and other oligofructans by yeast invertase. To date, there have been no previous reports on the affinity of sugarcane invertases for 1-kestose. This is because 1-kestose is not naturally found in sugarcane. This trisaccharide is the primary intermediate between sucrose and fructan in fructan synthesising plants (Pollock, 1986; Cairns and Pollock, 1988), which only form up to fifteen percent of higher plants (Pollock and Cairns, 1991). It has, however, been widely reported that some invertases hydrolyse fructans (Cairns and Ashton, 1991; Pollock and Cairns, 1991; Cairns, 1993; Bonnett and Simpson, 1993; Simpson and Bonnett, 1993). As the shortest fructan of  $\beta$ -1,2-linked inulin, these reports are inclusive

of 1-kestose (Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003).

In this study, three soluble invertases were extracted from sugarcane; two showing maximal activity at an acidic pH and one with a neutral pH optimum (Figure 4.3). All three enzymes did not reach substrate saturation having  $K_m$  values ranging from 50.43 – 247.44 mM (Table 4.3, Figure 4.5) far exceeding those for sucrose (Table 4.2, Figure 4.4). This was indicative of a low affinity for 1-kestose. Similarly, Nagaraj *et al.* (2005) cloned the cDNA encoding soluble acid invertase from barley (HvINV1) and measured very little activity when the enzyme preparation was incubated with 100 mM 1-kestose. Preferential hydrolysis of sucrose also occurred when a commercially available mix of  $\beta$ -1,2 tri-, tetra- and pentasaccharides (neosugar) was simultaneously incubated with sucrose (Cairns *et al.*, 1997). All plant invertases are  $\beta$ -fructofuranosidases that recognise and hydrolyse the bond adjacent to the fructose moiety. Since 1-kestose is comprised of one glucose moiety and two fructose moieties (GF<sub>2</sub>), and on the basis of the structural similarity to sucrose (GF), the low affinity of sugarcane invertases for this trisaccharide is surprising. This could imply molecular interference within 1-kestose, or that the enzymes have different binding sites for sucrose and 1-kestose.

Neutral invertase had a  $K_m$  for 1-kestose that was approximately 5 times higher than SAI 1 and double that of SAI 2 suggesting that it has even a lower affinity for 1-kestose (Table 4.3). Hence, 1-kestose is far less amenable to hydrolysis by sugarcane NI than SAIs. The difference between these invertases may lie in their structure. It has been reported that acid invertases have a NDPNG pentapeptide sequence (Sturm and Chrispeels, 1990; Tymowska-Lalanne and Kreis, 1998; Bosch *et al.*, 2004; Roitsch and González, 2004). Since this sequence is unique to acid invertase, it provides a basis upon which a distinction between acid and neutral invertases can be made. It has been suggested that the absence of this motif determines the specificity of neutral invertase for sucrose whilst enabling acid invertases to hydrolyse other sugars such as stachyose and raffinose (Obeland *et al.*, 1993, Nagaraj *et al.*, 2005), and this may be extended to 1-kestose albeit with a reduced affinity than for sucrose.

Fructan storage is naturally localised in the vacuole of fructan-accumulating plants (Simpson and Bonnett, 1993). On the basis that acid invertase has an acidic pH optimum and is located in this compartment, it is logical that acid invertase has a higher affinity for 1-kestose than neutral invertase that is located in the cytosol and does not come into contact with fructans.

*1-Kestose has an inhibitory effect on sucrose hydrolysis by SAI*

1-Kestose influences the maximum catalytic rate of sucrose hydrolysis such that sucrose hydrolysis by SAI is hindered with increasing concentrations of 1-kestose. This is evident for SAI 1 and SAI 2 which show a respective 51 and 54 % inhibition of sucrose hydrolysis in the presence of 200 mM 1-kestose (Figure 4.6). This could imply that the presence of 1-kestose allows for sucrose accumulation rather than degradation. The mechanism of inhibition is unknown. Incubation of soluble acid invertase cloned from barley with 50 mM 1-kestose in conjunction with 100 mM sucrose resulted in reduced invertase activity of sucrose showing 36 % inhibition of activity than when sucrose was incubated alone (Nagaraj *et al.*, 2005). This data confirms the inhibitory effect of 1-kestose on SAI-mediated hydrolysis of sucrose. This may have a functional role *in vivo* in fructan plants, whereby sucrose is likely to be channelled into 1-kestose thereby stimulating fructan accumulation. Sucrose hydrolysis by NI was not significantly inhibited by 1-kestose (Table 4.4).

**4.6 Conclusion**

Sugarcane-extracted invertases do not hydrolyse 1-kestose with the same efficacy as sucrose. Furthermore, 1-kestose was five-times less amenable to hydrolysis by NI than the two SAI isoforms. The trisaccharide is not a preferential substrate for sugarcane invertases and it had an inhibitory effect on SAI-mediated hydrolysis of sucrose. This is the first study to focus on the affinity of sugarcane invertases for 1-kestose *in vitro*. Genetic modification of sugarcane plants with 1-SST to produce 1-kestose as an alternative sugar (Chapter 3), facilitates the investigation of 1-kestose hydrolysis *in vivo* and allows for the study of carbon allocation in 1-kestose-producing sugarcane lines (Chapter 5).

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## CHAPTER 5

### CARBON PARTITIONING IN 1-KESTOSE- PRODUCING TRANSGENIC SUGARCANE LINES

#### 5.1 Abstract

Two genetically modified sugarcane (*Saccharum* spp. hybrids) lines were used as a tool for investigating carbon partitioning. The genetic modification involved expression of the gene encoding sucrose: sucrose 1-fructosyl transferase (1-SST) which facilitates the transfer of a fructosyl moiety from one sucrose molecule to another to produce the trisaccharide 1-kestose, plus glucose. To determine whether carbon allocation had been altered by this genetic event,  $^{14}\text{C}$  whole-plant radiolabelling experiments were conducted. Radiolabelled  $\text{CO}_2$  was fed to the leaf subtending internode 5 and the allocation of carbon to different parts of the culm was determined. There was no significant difference ( $p > 0.05$ ,  $N=72$ ) between the lines in the distribution of total radiolabel down the culm. However, the percentage of total radiolabel in the water-soluble fraction per internode in the high 1-kestose producer was significantly higher than the other two lines ( $p < 0.01$ ,  $N=72$ ). As a result, the percentage of radiolabel in the water-insoluble fraction in this transgenic line was concomitantly lower than in the other lines. No statistical differences between the lines were evident in terms of radiolabel present in sucrose ( $p > 0.05$ ,  $N=12$ ), and hexose levels were similar in all lines. The transgenics contained approximately 12 % of total radiolabelled solubles in 1-kestose and this difference between the control and the transgenics was statistically significant ( $p < 0.01$ ,  $N=4$ ). In turn, radiolabel present in the total sugar pool of both transgenics significantly exceeded that of the control ( $p < 0.01$ ,  $N=72$ ) emphasising the additive production of 1-kestose in the transgenics. The expression of 1-SST in sugarcane established an additional carbohydrate sink by the flow of carbon from the sucrose pool into 1-kestose. The redirection of carbon from the insoluble- to the soluble fraction in the high 1-kestose producing line allowed for the accumulation of high levels of 1-kestose over time. This study showed that carbon allocation was differentially altered by 1-kestose production.

#### 5.2 Introduction

With the exception of a recent study on the metabolic fate of sucrose in intact sugarcane internodal tissue of NCo376 (McDonald, 2000), carbon partitioning in intact culm tissue has been poorly studied. Furthermore, carbon partitioning in intact culm tissues of sugarcane plants producing 1-kestose has not been previously reported.

Having characterised the transgenic lines and established their production of 1-kestose (Chapter 3), and in light of the inefficiency of acid invertase in hydrolysing this trisaccharide with a similar affinity as it does sucrose *in vitro* (Chapter 4), the work presented in this chapter aims to assess the effect of the genetic event on the allocation of carbon *in vivo*.

Sugarcane (*Saccharum* spp. hybrids) has the ability to accumulate high quantities of sucrose in the culm tissues (for review see Moore, 1995; Komor, 2000). At any stage of development there is a gradient of increasing sucrose content down the culm of sugarcane (Moore, 1995; Whittaker and Botha, 1997; Zhu *et al.*, 1997; Botha and Black, 2000; Rose and Botha, 2000; Moore, 2005; Rae *et al.*, 2005). It is difficult to study sucrose accumulation in intact culm tissue since sampling of the culm requires destructive harvesting of the entire plant (Moore, 1995). As a result, tissue slices and cell suspension cultures have been used as alternative approaches for the formulation of sucrose accumulation models in intact sugarcane. Carbon partitioning studies in tissue slices from N19 (Bindon and Botha, 2001, 2002) and NCo376 varieties (Botha *et al.*, 1996; Whittaker and Botha, 1997) showed that maturation of internodal tissues corresponds to the redirection of carbon to sucrose at the expense of water-insoluble matter, respiration, amino acids, organic acids and phosphorylated intermediates (Botha *et al.*, 1996; Whittaker and Botha, 1997; Bindon and Botha, 2001, 2002). More carbon was allocated to sucrose than structural matter and respiration in sugarcane cell suspension cultures under nitrogen limiting conditions (Veith and Komor, 1993). Respiration and biosynthesis (water insoluble fraction) therefore compete with sucrose storage for incoming carbon.

Potato tubers expressing sucrose: sucrose 1- fructosyltransferase (1-SST, EC 2.4.1.99) from artichoke produced high levels of 1-kestose (G1-2F1-2F) and nystose (G1-2F1-2F1-2F1). In some lines, this was in excess of sucrose levels (Hellwege *et al.*, 1997). This study confirmed the successful synthesis of low molecular weight fructans (1-kestose and nystose) in the storage organs of a non-fructan plant. In a further study, potato (*Solanum tuberosum*) plants transformed with both 1-SST and fructan: fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) from globe artichoke (*Cynara scolymus*), led to substantial inulin (linear  $\beta(2\rightarrow1)$ -linked fructose moieties joined to sucrose) accumulation in the tubers (Hellwege *et al.*, 2000). However, the soluble carbohydrate composition of the tubers was not significantly altered by the genetic event and a slight reduction in starch content was observed. This implies that inulin synthesis occurs at the expense of starch in these lines.

This study investigates carbon partitioning, using whole plant radiolabelling of 1-kestose-producing transgenic sugarcane lines, to determine how the introduction of 1-SST and the corresponding production of 1-kestose altered carbon partitioning within the culm.

### 5.3 Materials and methods

#### 5.3.1 Plant material

Setts of two transgenic sugarcane (*Saccharum* spp. hybrid) lines (2121 and 2153) of the commercial cultivar NCo310 were obtained from Bayer BioScience GmbH (Potsdam, Germany). Details of their transformation, growth and maintenance are described in section 3.3.1 and 3.3.2. Twelve-month old cane was used for radiolabelling.

#### 5.3.2 Isotope

Radiolabelled sodium bicarbonate ( $\text{NaH}^{14}\text{C}\text{O}_3$ , Amersham Biosciences UK Limited, Buckinghamshire, England), with a specific activity of  $2.11 \text{ GBq.mmol}^{-1}$ , was used for this study.

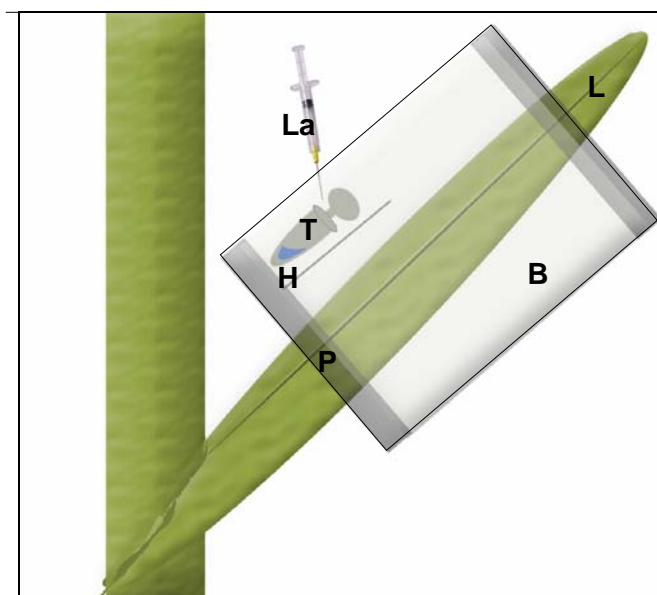
#### 5.3.3 Radiolabelling protocol

Sugarcane culms were radiolabelled using the following protocol (Figure 5.1): a portion (3 cm x 20 cm) of leaf 5 (Section 3.3.11, Figure 5.2) was enclosed within a polyethylene bag (0.75 L) that was double heat sealed along the length, and sealed to the leaf widthways using Prestik<sup>®</sup> (Bostik Ltd., Stafford, England). The bags were constructed to include a heat-sealed partition perpendicular to their base, which supported a 1.5 ml graduated microcentrifuge tube containing 9.25 GBq  $\text{NaH}^{14}\text{C}\text{O}_3$ . The bags were inflated with a manual pump and 1 ml of 10 % (v/v) lactic acid (Saarchem, Wadeville, South Africa) was added to the tube, through the bag via a needle (0.8 x 38 mm gauge) and syringe, which served to release [ $^{14}\text{CO}_2$ ]. The resultant hole in the bag was immediately sealed with Magic<sup>™</sup> tape (Scotch, Isando, South Africa). After 1 h, the bags were carefully removed and a cold chase period of either one, seven or 21 days (d) began. After each time point, 6 selected internodes from 4 replicate stalks per sugarcane line (2121, 2153, NCo310) were destructively harvested.

#### 5.3.4 Harvesting of radiolabelled plant material

Material from leaf 5, as well as tissue from internodes -2, +1, +4, +7, +8, +11 (Figure 5.2) were harvested from 4 replicate stalks per line 1, 7 and 21 d after labelling. The

total mass of each internode was recorded prior to the removal of the rind, after which 100 mg of central internodal tissue was finely chopped, flash-frozen in liquid nitrogen and stored at  $-85\text{ }^{\circ}\text{C}$ . In a preliminary study, 10 mg of leaf 5 was harvested immediately after labelling and stored at  $-85\text{ }^{\circ}\text{C}$ . Analysis of  $^{14}\text{C}$  in the soluble leaf extract, as well as the small amount of label (33 Bq) that remained in the tube after 1 h, confirmed successful labelling.



**Figure 5.1** Diagram showing the method used for whole plant labelling in carbon partitioning experiments. A portion of leaf five (L) was enclosed within an air-inflated polyethylene bag (B) that was sealed to the leaf with Prestik® (P). Labelling was initiated by the addition of a 10 % lactic acid (La) solution to a microcentrifuge tube (T) containing  $\text{NaH}[^{14}\text{C}]\text{O}_3$ , which was housed within the bag and supported by a heat-sealed partition (H). The bag was removed after 1 h.

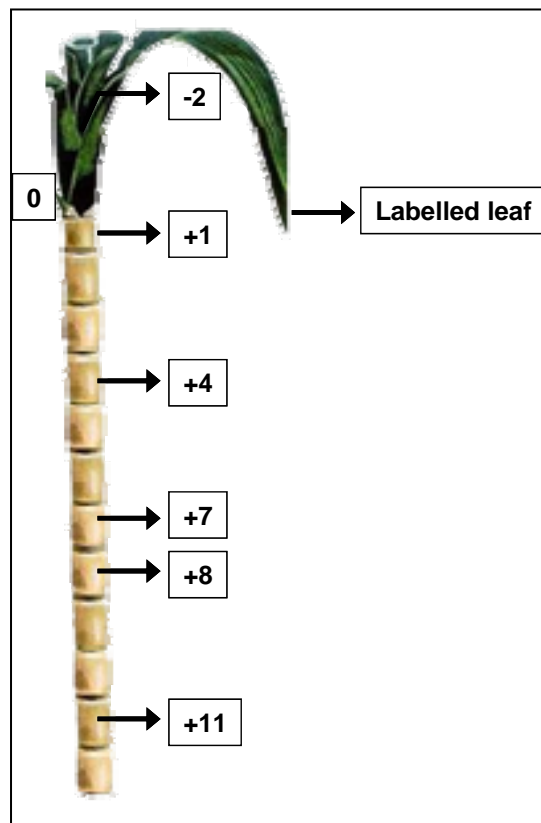
### 5.3.5 Sugar extraction

Soluble material was extracted from the above-mentioned tissues as described in section 3.3.11. The remaining insoluble material was allowed to dry overnight at room temperature, after which both the insoluble material and soluble extracts were stored at  $-20\text{ }^{\circ}\text{C}$ .

### 5.3.6 Separation of sugars

The individual sugars in the soluble fraction were separated by TLC as previously described in section 4.3.2. A range (7.8 Bq to 1000 Bq) of known  $^{14}\text{C}$ -glucose ( $11.7\text{ GBq}\cdot\text{mmol}^{-1}$  specific activity, Amersham Biosciences UK Limited, Buckinghamshire, England) and  $^{14}\text{C}$ -sucrose ( $22.2\text{ GBq}\cdot\text{mmol}^{-1}$  specific activity, Amersham Biosciences UK Limited, Buckinghamshire, England) activities were spotted, separated and visualised on silica gel TLC plates. Two sugar solutions of 25 mM sucrose (to mimic

endogenous sugar concentration in the stalk), 1 containing 7.8 Bq and the other 15.6 Bq of [ $^{14}\text{C}$ ]-glucose and [ $^{14}\text{C}$ ]-sucrose were used as  $^{14}\text{C}$  standards per TLC plate to quantify sample signals.



**Figure 5.2** Diagram showing the convention used to number internodes in this study. Internodes were numbered in relation to their position to the labelled leaf (leaf 5). The internode subtended by the labelled leaf was numbered internode 0 and all internodes above, and below this point were designated with sequential negative and positive numbers respectively. In this study, internodes -2, +1, +4, +7, +8 and +11 were sampled along with the labelled leaf. Diagram modified from <http://www.fiberfutures.org/showcase/sugarcane.html>.

### 5.3.7 Detection of radiolabel in the sugars

#### *Sucrose and hexoses*

Following their development, the TLC plates were individually sealed in polyethylene film and each exposed to a super resolution cyclone storage phosphorscreen (Packard Instrument Company, Meriden, CT, USA) overnight. Data were captured and quantitatively analysed using OptiQuant™ software v3.1 for the Cyclone storage phosphorscreen imager (Model A431201, Packard Instrument Company, Meriden, CT, USA).

### *1-Kestose*

All sugars containing fructose moieties (both radiolabelled and unlabelled) were visualised by spraying the TLC plates with a urea reagent (Wise *et al.*, 1955) and heating them to 80 °C for 10 min. The stained TLC plates were scanned and the images recorded. By comparison with a 50 µg 1-kestose standard (Fluka, Buchs, Switzerland) that was separated alongside the samples, spots corresponding to 1-kestose were scraped from the silica gel TLC plates, resuspended in 100 µl deionised water and solubilised at 4 °C overnight. Following brief vortexing and centrifugation at 20200 *g* for 10 min, 70 µl of the supernatant was added to 2 ml Insta-gel<sup>®</sup> Plus (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and vortexed. Carbon-14 activity was detected by liquid scintillation counting for 4 h per sample using a Tri-Carb 2100TR Liquid Analyser (Packard Instrument Company, Meriden, CT, USA) with Tri-Carb operating software for 2100TR. Radiolabelled background standards (Packard Instrument Company, Meriden, CT, USA) were used to calibrate the counter while a range of quenched standards adjusted for any chemical or colour quenching.

#### *5.3.8 Detection of radiolabel in the total soluble and insoluble fractions*

Approximately 100 mg of radiolabelled dry insoluble material was transferred to 6 ml polyethylene Pony<sup>™</sup> vials (Packard Instrument Company, Meriden, CT, USA) and the tissue homogenised in 1 ml deionised water for 2 min at 20000 rpm and 24000 rpm for 2 min, using an Ultra-Turrax vertical homogeniser (Janke & Kunkel, IKA-works, Inc., Willmington, NC). Thereafter, 2 ml Insta-gel<sup>®</sup> Plus was added and the vials were immediately vortexed to initiate gel formation, as well as to disperse the tissue in the gel. Activity of <sup>14</sup>C in the insoluble samples was detected by liquid scintillation counting for twenty min per sample using a Tri-Carb 2100TR Liquid Analyser with Tri-Carb operating software for 2100TR. In addition to this, total soluble counts were determined by combining 50 µl of soluble radiolabelled extracts with 2 ml Insta-gel<sup>®</sup> Plus scintillant and each sample counted for 20 min.

#### *5.3.9 Statistical analyses*

A one-way Analysis of Variance (ANOVA) with a posthoc Tukey test was conducted to determine statistical differences between the different sugarcane lines, internodes and time points. For this purpose, SPSS v11.5 for Windows (SPSS Inc.) was utilised. The alpha value used for these tests was 0.05, such that p values less than this indicate statistical differences. All data expressed as percentages were transformed to an arcsine distribution prior to statistical analysis.



## 5.4 Results

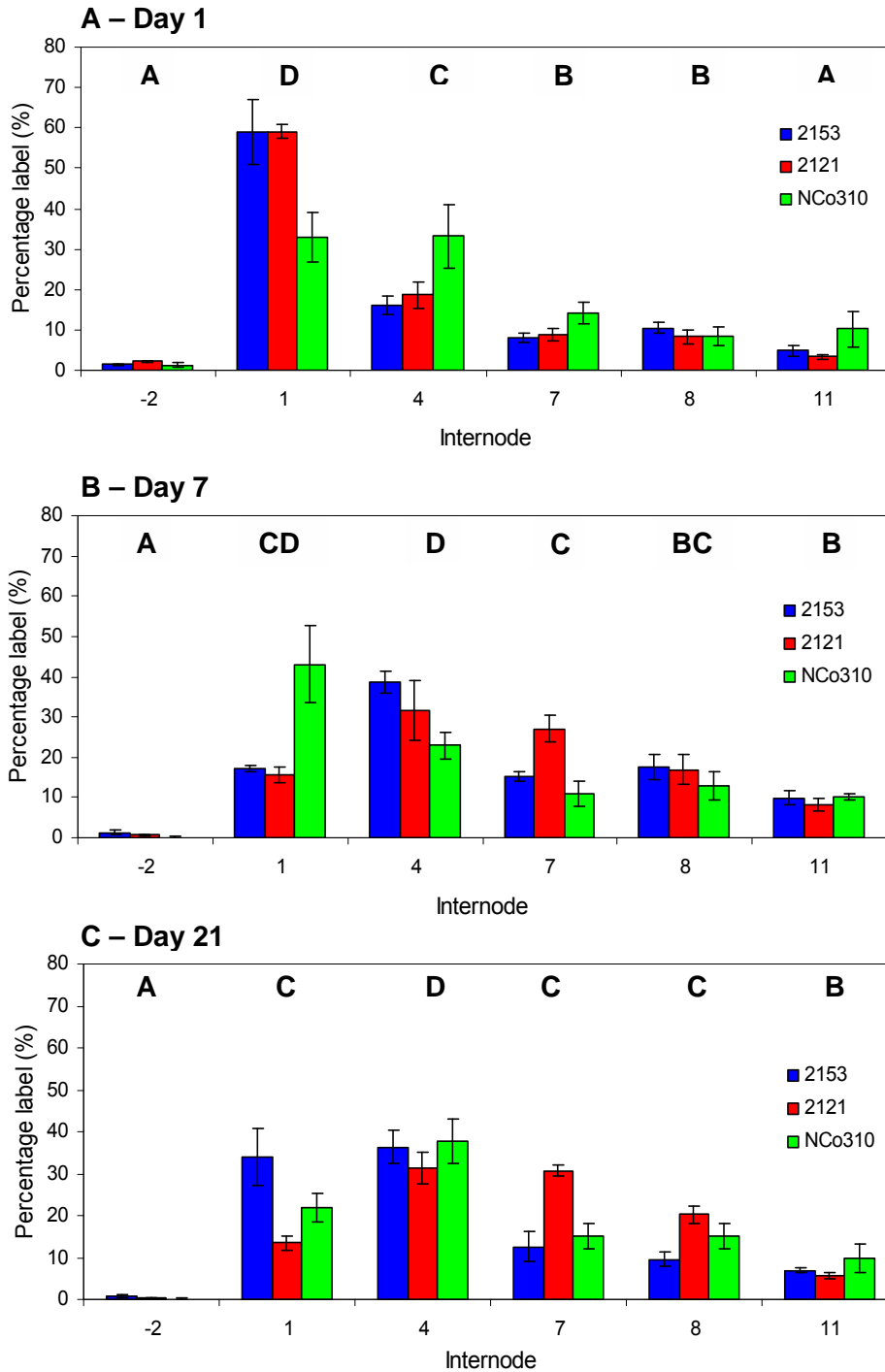
### 5.4.1 Internodal distribution of total radiolabel within the culm

Liquid scintillation counting facilitated the detection of radiolabel in the soluble and insoluble fractions, which together constituted the total label present. This was measured in each selected internode one, seven and 21 days after radiolabelling of leaf 5, and expressed as a percentage of the total radiolabel present in the culm. In this study, the culm was taken to be the sum of the selected internodes that were harvested, namely -2, +1, +4, +7, +8 and +11 (Figure 5.2). Irrespective of sugarcane line, there were statistical differences in terms of the percentage radiolabel present in these individual internodes ( $p < 0.01$ ,  $N = 12$ , Figure 5.3). At all three time points, internode -2 statistically had the least radiolabel in the culm (less than 2 %). This suggests that internode -2 is a weak sink as little label moves up to this internode from the labelling point. The first day after radiolabelling (Figure 5.3A), the most radiolabel in the culm was present in internode +1, which is situated immediately below the point of labelling, implicating it as a strong sink. With time, the peak of radiolabel shifted such that the most radiolabel in the culm was present in internode +4 after seven (Figure 5.3B) and 21 days (Figure 5.3C). The percentage label present in the subsequent internodes showed a progressive decline with internodal maturity.

These internodal differences evident at the three time points were shared by all three sugarcane lines. Therefore, there was no significant difference between the three sugarcane lines in the distribution pattern of radiolabel down the culm ( $p > 0.05$ ,  $N = 24$ ).

### 5.4.2 Partitioning of total label into the water-soluble and -insoluble fractions

Since the distribution pattern of total radiolabel down the culm was observed to be similar in all three sugarcane lines, the next logical step was to analyse the partitioning of the total label into the water-soluble and -insoluble fractions per internode. This analysis would elucidate any statistical differences in these fractions between internodes as well as sugarcane lines.



**Figure 5.3** Internodal distribution of total label in the culm of two 1-kestose producing transgenics (2153 and 2121) and a control sugarcane line (NCo310), one (A), seven (B) and 21 (C) days after radiolabelling. Radiolabel per internode was expressed as a percentage of the total radiolabel present in the culm. At each time point, there was no significant difference ( $p > 0.05$ ,  $N = 24$ ) between the distribution of radiolabel down the culm of the three sugarcane lines. Regardless of sugarcane line, there were significant differences ( $p < 0.01$ ,  $N = 12$ ) between the percentage label present in the individual internodes and different uppercase letters denote this difference at each time point. A significant ( $p < 0.01$ ) interaction between line and internode at each time point is noted. Bars are the mean  $\pm$  SE ( $N = 4$ ).

Radiolabel present in the water-soluble fraction was expressed as a percentage of the total radiolabel per internode at each time point after labelling. In all three sugarcane lines, there was no statistical difference between the percentage radiolabel in the water-soluble fraction in different internodes one day after radiolabelling ( $p > 0.05$ ,  $N = 12$ , Figure 5.4A). But, significant internodal differences were evident after seven days (Figure 5.4B) and were even more pronounced 21 days after radiolabelling (Figure 5.4C), with the older internodes (+4, +7, +8 and +11) having more radiolabel present in the water-soluble fraction than their younger counterparts ( $p < 0.01$ ,  $N = 12$ ).

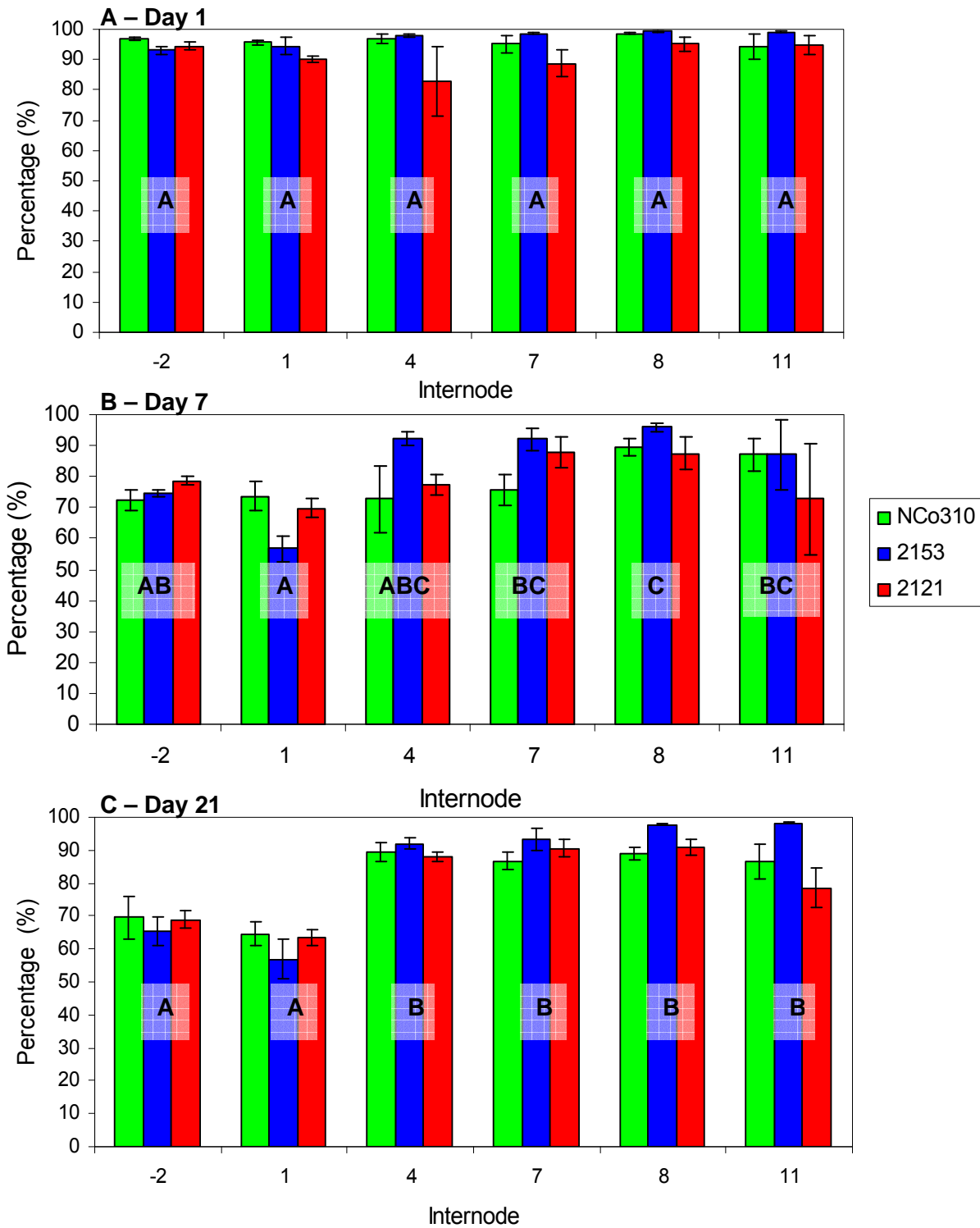
Irrespective of time point, transgenic sugarcane line 2153 contained statistically more radiolabel in the water-soluble fraction than the other two lines ( $p < 0.01$ ,  $N = 72$ ). Although this difference appears minimal on the first day after labelling, more notable increases in 2153 are evident seven and then 21 days after radiolabelling. Since the total radiolabel per internode is constituted by the radiolabel present in both the water-soluble and -insoluble fractions, it follows that an increase in the percentage radiolabel in the water-soluble fraction of line 2153 must be accompanied by a concomitant decrease in the percentage radiolabel present in the insoluble fraction in this transgenic line. Hence more carbon was directed to the water-soluble component in 2153 than the other sugarcane lines and this was at the expense of the water-insoluble fraction.

#### *5.4.3 Analysis of selected sugars within the water-soluble fraction*

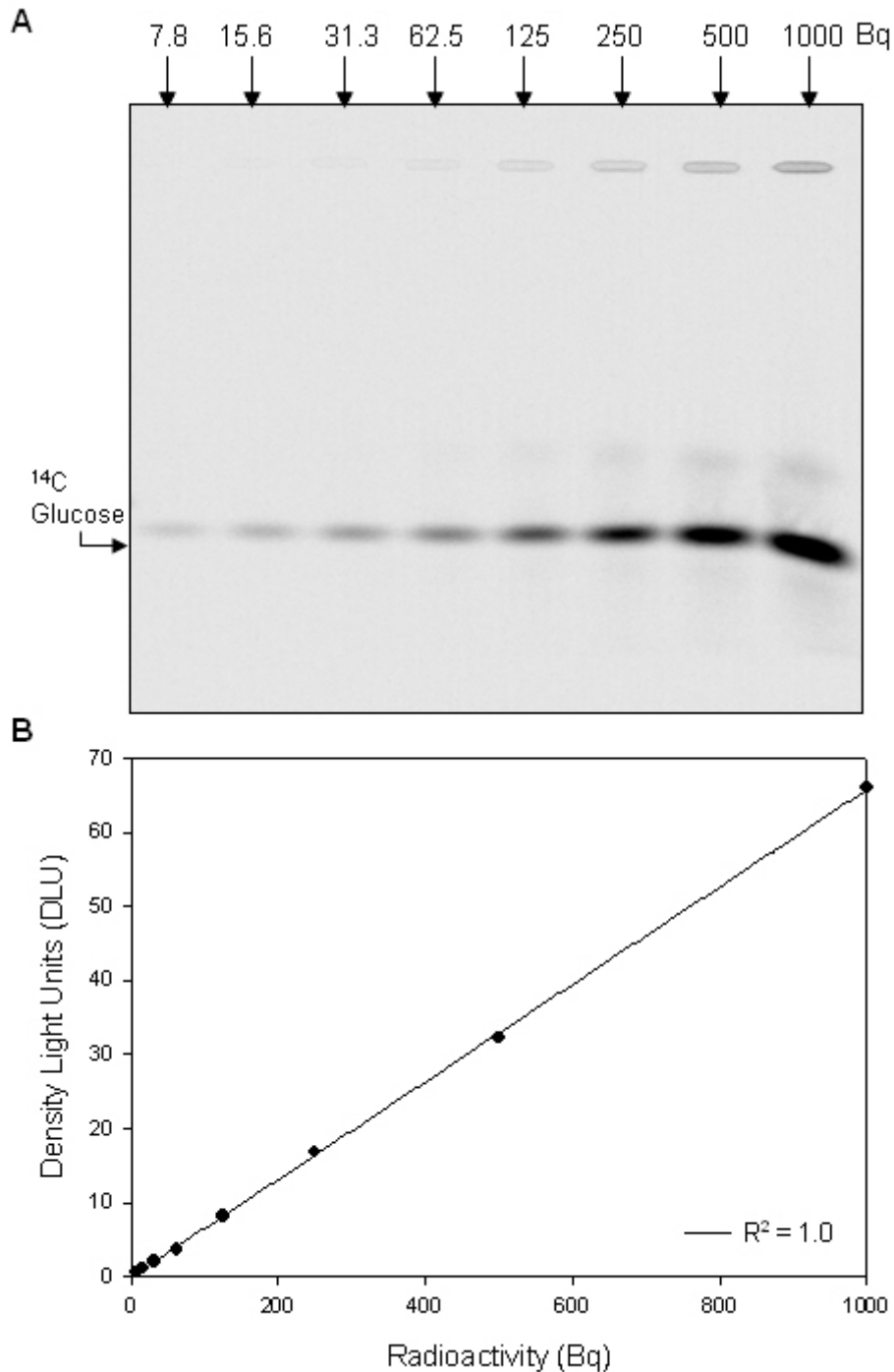
Since sugarcane line 2153 had significantly more radiolabel present in the water-soluble fraction than the other two lines, this fraction was further analysed. This entailed the observation of the glucose, fructose, sucrose and 1-kestose sugar pools of the three sugarcane lines to determine any differences. It was necessary to first establish the effectiveness of the techniques to be utilised.

##### *The relationship between radioactivity and density light units*

The use of thin layer chromatography (TLC) in conjunction with phosphorimaging for the respective separation and detection of  $^{14}\text{C}$  within the sugar pools required verification. It was essential to elucidate the relationship between radioactivity (Bq) and density light units (DLU). For this purpose, a range (7.8 Bq – 1000 Bq) of  $^{14}\text{C}$  glucose standards were separated by TLC, the radiolabel detected by phosphorimaging and the spots quantified in terms of DLU (Figure 5.5A). A linear relationship ( $R^2 = 1.0$ ) between Becquerel and DLU was evident (Figure 5.5B). This relationship was utilised as a calibration tool, as internal standards of 7.8 Bq and 15.6 Bq were spotted alongside the samples on each TLC plate and the sample data normalised against these standards.



**Figure 5.4** Radiolabel present in the water-soluble fraction as a percentage of the total label per internode, one (A), seven (B) and 21 (C) days after radiolabelling. Bars are the mean  $\pm$  SE (N=4). Irrespective of time point, the transgenic line 2153 had significantly ( $p < 0.01$ , N=72) higher levels of label in the water-soluble fraction than the other transgenic (2121) and the control line (NCo310). Different uppercase letters indicate a significant difference between the internodes at the specified time point. A significant ( $p < 0.01$ ) interaction between line and internode was evident at all three time points.



**Figure 5.5** Relationship between radioactivity and phosphorimaging units (DLU). **(A)** A phosphorimage of eight <sup>14</sup>C glucose standards, ranging from 7.8 Bq to 1000 Bq, that were separated using TLC. The resultant dark spots were quantified in terms of Density Light Units (DLU) and a **(B)** linear relationship between DLU and radioactivity (Bq) was established. DLU scale=  $1 \times 10^4$ .

For analysis of the radiolabel present in extracted sugar samples, no optical density beyond the linear standard curve was considered.

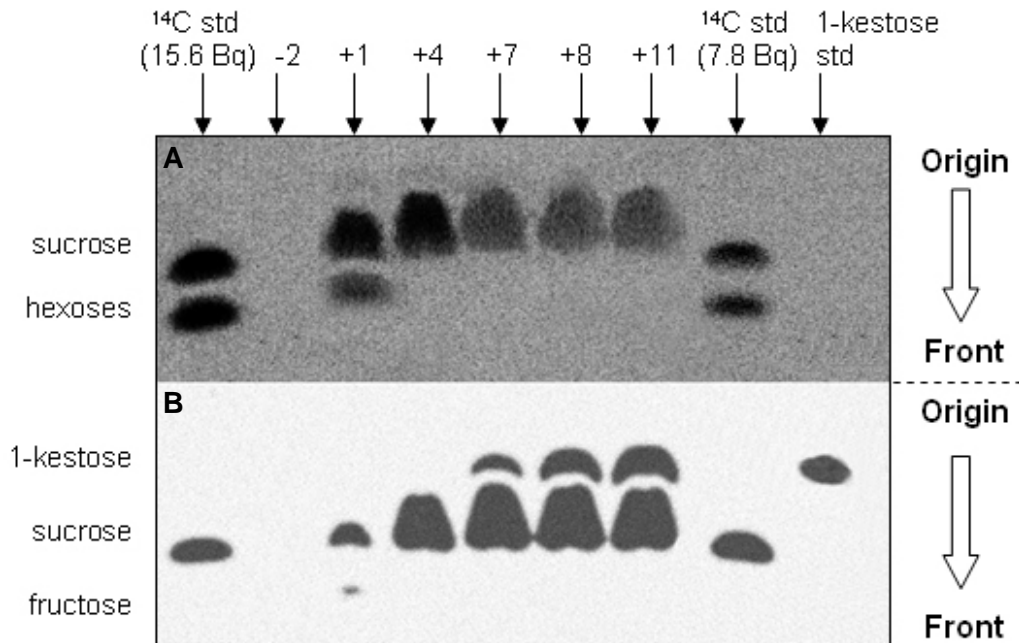
*Radiolabelled sugars detected by phosphorimaging and staining*

The individual sugars from the harvested internodes from each sugarcane line were separated by TLC and the radiolabel in each visualised by phosphorimaging. A phosphorimage of a section of a TLC plate from the transgenic line 2153 is shown in Figure 5.6A. Radiolabelled sucrose was detected in internodes +1, +4, +7, +8 and +11 while radiolabelled hexoses were only present in internode +1. Radiolabel in 1-kestose could not be visualised using this method. A fructose-specific stain was administered to the TLC plates which facilitated the visualisation of all sugars (labelled or unlabelled) containing fructose moieties. This allowed for the localisation of 1-kestose. It is evident from the stained image, that 1-kestose was present in the older internodes of this line and amounts of the trisaccharide increased with internodal maturity (Figure 5.6B). All spots corresponding to 1-kestose were subsequently scraped from the TLC plates and the amount of label within each solubilised spot quantified by liquid scintillation counting. Any count less than  $0.4 \text{ Bq.mg}^{-1}$  was regarded as background and not included in the analysis nor in subsequent graphical representation. No radiolabelled sugars were detected in internode -2 using phosphorimaging or the staining procedure.

*Partitioning within the sugar pool*

The radiolabel present in the individual sugars constituting the sugar pool was analysed in each harvested internode from the three lines, and expressed as a percentage of the total radiolabelled solubles in the culm one, seven and 21 days after radiolabelling (Figure 5.7). There were no statistical differences between the percentage radiolabel present in sucrose between the sugarcane lines at all three time points ( $p > 0.05$ ,  $N=12$ , Figure 5.7A, B, C). The percentage radiolabel present in the hexoses was similar for all three lines at the first two time points (Figure 5.7D, E). But, after 21 days, transgenic line 2153 contained significantly more radiolabel in the hexoses than the other two lines ( $p < 0.01$ ,  $N=4$ , Figure 5.7F). Radiolabel in 1-kestose was detected in the transgenic lines at all three time points but not in the control line (Figure 5.7G, H, I). The culm of line 2121 had significantly more radiolabel in this trisaccharide ( $p < 0.01$ ,  $N=4$ ) than 2153 on the first day after radiolabelling. However, after seven days the opposite trend was observed. Twenty-one days after radiolabelling, there was no significant difference between the transgenic lines in terms of the percentage radiolabel present in 1-kestose ( $p > 0.05$ ,  $N=4$ ). The transgenic lines contained approximately 12 % of the total radiolabelled solubles in 1-kestose and this difference between the transgenics and the control was statistically significant ( $p < 0.01$ ,  $N=4$ ). Internodal statistical differences were evident for each sugar and time point. After 21 days, the most radiolabel in sucrose and hexoses was present in internode +4,

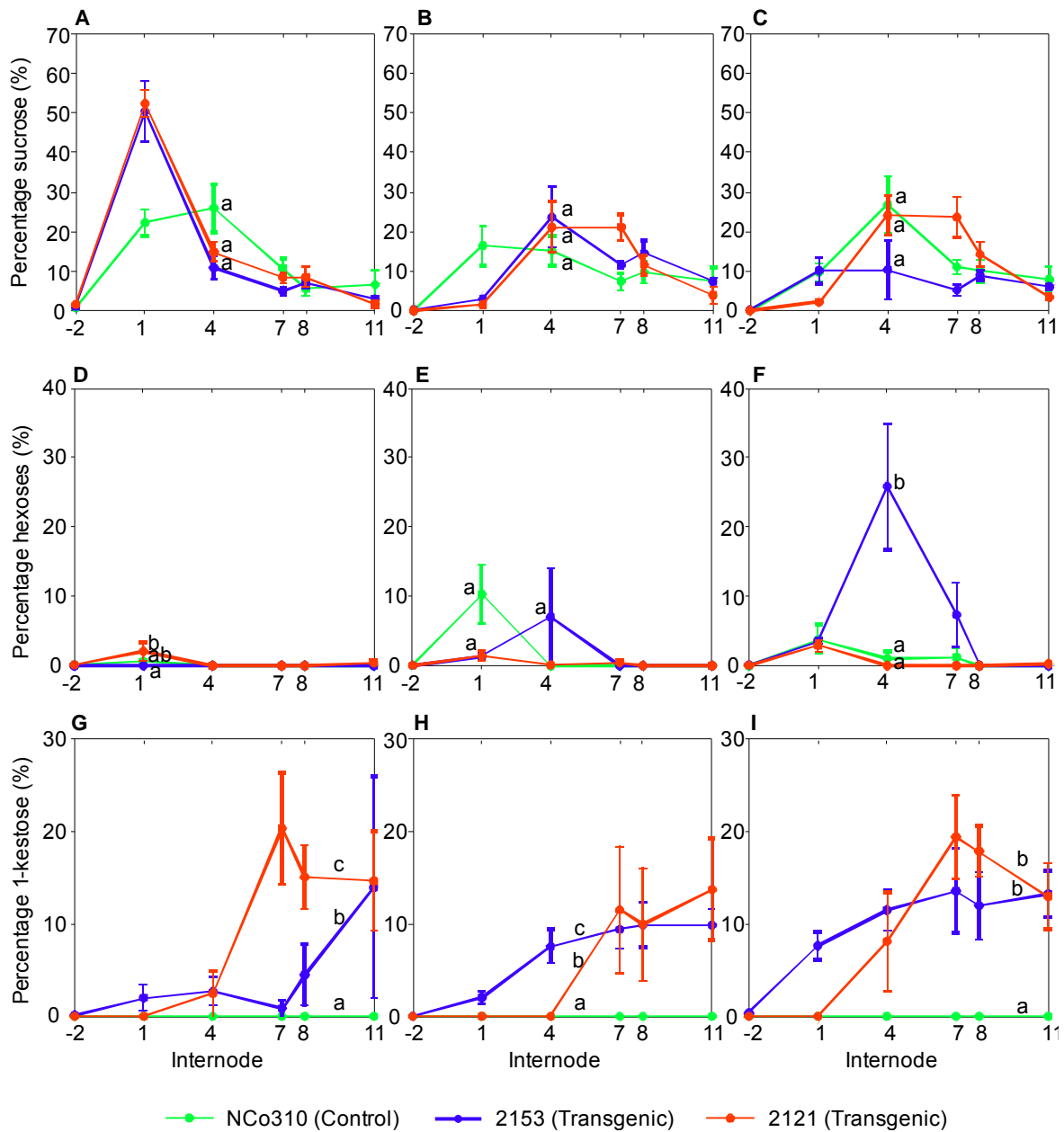
while internode +7, +8 and +11 contained the most radiolabel in 1-kestose ( $p < 0.01$ ,  $N = 24$ ).



**Figure 5.6** Visualisation and quantification of radiolabel present in individual sugars extracted from transgenic internodal tissue. **(A)** A phosphorimage of a section of TLC plate that was used to separate the individual sugars within extracts from internodes -2, +1, +4, +7, +8 and +11 of the transgenic line 2153. The dark spots represent radiolabel present in sucrose, glucose and fructose in the internodes, as well as two  $^{14}\text{C}$  standards namely 15.6 Bq and 7.8 Bq containing radiolabelled sucrose and glucose. The size of the spots indicate the amount of radiolabel present which was quantified by the correlation between DLU and Bq. **(B)** The same section of TLC plate after staining, which facilitated the visualisation of total (both labelled and unlabelled) sugars containing fructose moieties. Sucrose, fructose and 1-kestose were visualised using this technique.

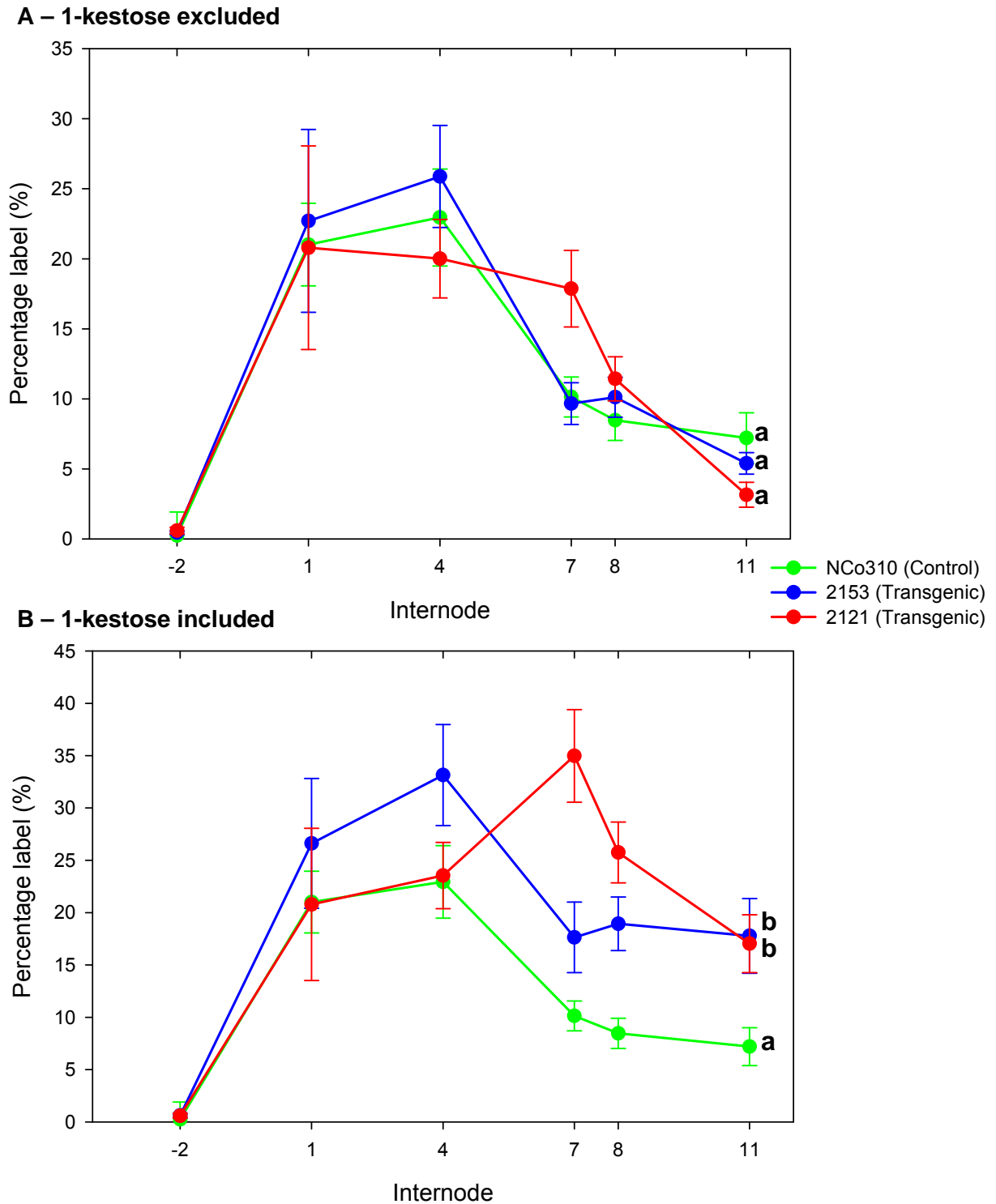
#### *Analysis of radiolabel in the total sugar pool*

The percentage radiolabel present in the individual sugars was totalled and the sugarcane lines analysed for differences. The exclusion of 1-kestose in this total resulted in all three lines having similar levels of radiolabel present in their sugar pools ( $p > 0.01$ ,  $N = 72$ , Figure 5.8A). However, when 1-kestose was taken into account, a highly significant difference was observed between the lines, with both transgenics (2153 and 2121) having statistically more radiolabel present in their total sugar pools than the control line (NCo310) ( $p < 0.01$ ,  $N = 72$ , Figure 5.8B). Therefore the difference in the percentage radiolabel between the control and the transgenics can be attributed to the additive production of 1-kestose in the transgenics.



**Figure 5.7** Amount of radiolabel present in the measured individual sugars down the culm of a control (NCo310) and two transgenic (2153 and 2121) sugarcane lines at three time points. The amount of label in sucrose (A, B, C), hexoses (D, E, F) and 1-kestose (G, H, I) in six selected internodes is expressed as a percentage of the total radiolabelled solubles in the culm one (A, D, G), seven (B, E, H) and twenty-one days (C, F, I) after labelling. Bars represent the mean  $\pm$  SE (N=4). Different lowercase letters denote statistical significance between sugarcane lines at a particular time point. A significant interaction ( $p<0.05$ ) between line and internode is noted in all figures except A.





**Figure 5.8** Comparison of radiolabel present in the sugar pool of two transgenics and one control line. **(A)** Amount of radiolabel in glucose, fructose and sucrose totalled per internode and expressed as a percentage of the total soluble label in the culm of the three lines. **(B)** Amount of label in glucose, fructose, sucrose and 1-kestose totalled per internode expressed as a percentage of the total soluble label in the culm of the three lines. In both A and B, bars represent the total of the mean  $\pm$  SE (N=72). Different lowercase letters denote statistical significant difference between the lines. A significant interaction ( $p < 0.01$ ) between line and internode is evident.

#### 5.4.4 Sugar turnover

The turnover of the individual sugars was determined in all three sugarcane lines to gain insight into the increased carbon content present in the soluble fraction of 2153. The sugar concentration and total radiolabel of hexose, sucrose and 1-kestose were measured in six internodes spanning the culm of one control sugarcane line and two transgenic lines at three time points after radiolabelling and specific activities of each was determined.

##### *Concentration of unlabelled sugars*

Hexose levels were highest in internode +1 and +4 and progressively declined with increasing internodal maturity, such that concentrations in the old internodes were below the detection limit of the method used (Table 5.1). The hexose pool size in all sampled internodes remained constant over time ( $p>0.05$ ,  $N=4$ ), with the exception of internode +1 of NCo310, which showed a statistically significant decline of hexose concentration with time ( $p<0.01$ ,  $N=4$ , Table 5.2).

In all three sugarcane lines, the concentration of unlabelled sucrose increased down the stalk, with the oldest sampled internodes containing the most sucrose and the youngest internode, the least sucrose (Table 5.1). Internodes +1 and +4 of both transgenic lines showed a statistical change in sucrose concentration over time (Table 5.2). In internodes +1 from both lines the sucrose concentration increased over time ( $p<0.01$ ,  $N=4$ ), while internode +4 had similar sucrose levels on the first and last day of harvesting ( $p>0.05$ ,  $N=4$ ) but had lower sucrose levels at the 7 day time point ( $p<0.01$ ,  $N=4$ ). Most internodes of all three sugarcane lines contained similar sucrose levels across time points ( $p>0.05$ ,  $N=4$ ).

1-Kestose was not present in any internodes of the control line NCo310, but was detected in both transgenic lines (Table 5.1). Sugarcane line 2121 contained low levels of 1-kestose that were evident from internode +7 and showed a slight increase with internodal maturity, peaking at approximately  $3 \mu\text{mol sugar.g}^{-1} \text{fm}$ . In 2153, 1-kestose was first detected in internode +4 and levels increased to more than  $100 \mu\text{mol sugar.g}^{-1} \text{fm}$  in the oldest internode; much higher than those detected in 2121. Levels of this trisaccharide were not statistically different across time points ( $p>0.05$ ,  $N=4$ , Table 5.2). From this point on, line 2153 and 2121 will be referred to as the high- and low 1-kestose transgenics respectively.

**Table 5.1** Hexose, sucrose and 1-kestose concentrations present in six internodes spanning the culm of two transgenics (2121 and 2153) and one control sugarcane line (NCo310), at three time points after radiolabelling. Values represent the mean  $\pm$  SE (N=4). N.D. = sugar concentration not detected.

Line	Day	Internode	Sugar concentration ( $\mu\text{mol sugar.g}^{-1}\text{fm}$ )							
			Hexoses	SE	Sucrose	SE	1-Kestose	SE		
2121	1	-2	11.61	$\pm$ 2.42	27.14	$\pm$ 2.43	N.D.			
	1	1	70.63	$\pm$ 15.43	51.62	$\pm$ 7.79	N.D.			
	1	4	74.27	$\pm$ 15.54	340.67	$\pm$ 32.06	N.D.			
	1	7	10.74	$\pm$ 2.04	530.57	$\pm$ 19.86	1.02	$\pm$ 0.25		
	1	8	6.92	$\pm$ 2.61	532.39	$\pm$ 18.73	2.77	$\pm$ 0.88		
	1	11	N.D.		532.88	$\pm$ 47.51	3.14	$\pm$ 0.46		
	7	-2	14.73	$\pm$ 0.91	23.52	$\pm$ 0.94	N.D.			
	7	1	25.20	$\pm$ 2.49	17.30	$\pm$ 3.48	N.D.			
	7	4	68.82	$\pm$ 12.62	174.24	$\pm$ 28.23	N.D.			
	7	7	17.02	$\pm$ 3.06	422.34	$\pm$ 26.26	N.D.			
	7	8	11.07	$\pm$ 4.55	468.10	$\pm$ 35.82	3.69	$\pm$ 0.92		
	7	11	1.94	$\pm$ 0.16	524.70	$\pm$ 38.18	N.D.			
	21	-2	11.08	$\pm$ 1.77	31.79	$\pm$ 4.41	N.D.			
	21	1	68.00	$\pm$ 12.38	133.53	$\pm$ 17.27	N.D.			
	21	4	30.15	$\pm$ 7.35	368.71	$\pm$ 45.42	N.D.			
	21	7	7.41	$\pm$ 0.48	551.14	$\pm$ 51.98	N.D.			
	21	8	N.D.		529.42	$\pm$ 45.68	N.D.			
	21	11	N.D.		605.43	$\pm$ 14.85	N.D.			
	2153	1	-2	16.91	$\pm$ 1.67	34.43	$\pm$ 4.23	N.D.		
		1	1	51.51	$\pm$ 8.30	124.65	$\pm$ 26.84	N.D.		
		1	4	14.92	$\pm$ 4.93	407.69	$\pm$ 33.00	31.06	$\pm$ 11.93	
1		7	N.D.		491.01	$\pm$ 40.63	97.55	$\pm$ 31.77		
1		8	N.D.		461.67	$\pm$ 22.54	101.97	$\pm$ 31.44		
1		11	N.D.		497.72	$\pm$ 45.04	129.47	$\pm$ 40.04		
7		-2	11.80	$\pm$ 2.31	29.46	$\pm$ 1.06	N.D.			
7		1	24.97	$\pm$ 6.86	33.14	$\pm$ 6.97	N.D.			
7		4	27.94	$\pm$ 6.15	287.63	$\pm$ 20.49	12.08	$\pm$ 4.30		
7		7	4.27	$\pm$ 1.13	424.87	$\pm$ 17.35	50.85	$\pm$ 18.04		
7		8	2.55	$\pm$ 0.80	439.71	$\pm$ 27.47	60.20	$\pm$ 16.30		
7		11	N.D.		470.94	$\pm$ 34.68	103.72	$\pm$ 27.53		
21		-2	20.92	$\pm$ 8.80	34.21	$\pm$ 6.60	N.D.			
21		1	43.52	$\pm$ 13.98	175.08	$\pm$ 34.29	5.95	$\pm$ 2.88		
21		4	6.76	$\pm$ 1.16	410.17	$\pm$ 24.28	26.28	$\pm$ 2.87		
21		7	1.63	$\pm$ 0.41	466.77	$\pm$ 15.29	61.46	$\pm$ 9.58		
21		8	N.D.		486.37	$\pm$ 16.90	84.11	$\pm$ 35.18		
21		11	N.D.		453.37	$\pm$ 25.97	102.84	$\pm$ 28.71		
NCo310		1	-2	16.29	$\pm$ 2.22	64.80	$\pm$ 38.66	N.D.		
		1	1	90.22	$\pm$ 8.69	141.45	$\pm$ 22.55	N.D.		
		1	4	9.72	$\pm$ 1.76	294.58	$\pm$ 44.33	N.D.		
	1	7	10.21	$\pm$ 3.99	277.05	$\pm$ 43.25	N.D.			
	1	8	10.40	$\pm$ 9.72	309.58	$\pm$ 58.27	N.D.			
	1	11	6.91	$\pm$ 1.45	258.15	$\pm$ 24.54	N.D.			
	7	-2	14.25	$\pm$ 2.90	24.12	$\pm$ 1.15	N.D.			
	7	1	57.90	$\pm$ 14.44	88.00	$\pm$ 9.96	N.D.			
	7	4	15.78	$\pm$ 3.68	347.25	$\pm$ 32.41	N.D.			
	7	7	4.75	$\pm$ 1.96	427.78	$\pm$ 30.90	N.D.			
	7	8	2.95	$\pm$ 0.28	432.37	$\pm$ 42.48	N.D.			
	7	11	2.64	$\pm$ 0.98	361.45	$\pm$ 72.62	N.D.			
	21	-2	14.36	$\pm$ 1.99	28.52	$\pm$ 3.36	N.D.			
	21	1	34.51	$\pm$ 6.86	155.04	$\pm$ 23.84	N.D.			
	21	4	4.25	$\pm$ 1.28	395.62	$\pm$ 14.83	N.D.			
	21	7	N.D.		433.24	$\pm$ 18.31	N.D.			
	21	8	0.92	$\pm$ 0.23	461.64	$\pm$ 31.71	N.D.			
	21	11	N.D.		420.76	$\pm$ 35.23	N.D.			

**Table 5.2** Statistical differences ( $p < 0.01$ ,  $N=4$ ), represented by different lowercase letters between radiolabelling time points in terms of sugar concentration, total label and specific activity of the hexose (hex), sucrose (suc) and 1-kestose (1-kes) pools in individual internodes of three sugarcane lines namely 2121, 2153 (transgenics) and NCo310 (control). Highlighted internodes designate significant differences between time points. A dash (–) indicates the absence of statistical data due to the lack of 1-kestose in the control line.

Line	Internode	Day	Sugar concentration			Total label			Specific activity		
			Hex	Suc	1-Kes	Hex	Suc	1-Kes	Hex	Suc	1-Kes
2121	-2	1	a	a	a	a	b	a	a	b	a
	-2	7	a	a	a	a	a	a	a	a	a
	-2	21	a	a	a	a	a	a	a	a	a
	1	1	a	a	a	a	b	a	a	b	a
	1	7	a	a	a	a	a	a	a	a	a
	1	21	a	b	a	a	a	a	a	a	a
	4	1	a	b	a	a	a	a	a	a	a
	4	7	a	a	a	a	a	a	a	b	a
	4	21	a	b	a	a	a	a	a	ab	a
	7	1	a	a	a	a	a	ab	a	a	a
	7	7	a	a	a	a	a	a	a	a	a
	7	21	a	a	a	a	a	b	a	a	a
	8	1	a	a	a	a	a	a	a	a	a
	8	7	a	a	a	a	a	a	a	a	a
	8	21	a	a	a	a	a	a	a	a	a
	11	1	a	a	a	a	a	a	a	a	a
	11	7	a	a	a	a	a	a	a	a	a
	11	21	a	a	a	a	a	a	a	a	a
2153	-2	1	a	a	a	a	b	a	a	b	a
	-2	7	a	a	a	a	a	a	a	a	a
	-2	21	a	a	a	a	a	a	a	a	a
	1	1	a	ab	a	a	b	a	a	b	a
	1	7	a	a	a	a	a	a	a	ab	a
	1	21	a	b	a	a	ab	a	a	a	a
	4	1	a	b	a	a	a	a	a	a	a
	4	7	a	a	a	a	a	b	a	a	a
	4	21	a	b	a	a	a	b	a	a	a
	7	1	a	a	a	a	a	a	a	a	a
	7	7	a	a	a	a	a	b	a	a	a
	7	21	a	a	a	a	a	b	a	a	a
	8	1	a	a	a	a	a	a	a	a	a
	8	7	a	a	a	a	a	a	a	a	b
	8	21	a	a	a	a	a	a	a	a	ab
	11	1	a	a	a	a	a	a	a	a	a
	11	7	a	a	a	a	a	a	a	a	a
	11	21	a	a	a	a	a	a	a	a	a
NCo310	-2	1	a	a	–	a	b	–	a	b	–
	-2	7	a	a	–	a	a	–	a	ab	–
	-2	21	a	a	–	a	a	–	a	a	–
	1	1	b	a	–	a	a	–	a	a	–
	1	7	ab	a	–	a	a	–	a	a	–
	1	21	a	a	–	a	a	–	a	a	–
	4	1	a	a	–	a	a	–	a	a	–
	4	7	a	a	–	a	a	–	a	a	–
	4	21	a	a	–	a	a	–	a	a	–
	7	1	a	a	–	a	a	–	a	a	–
	7	7	a	a	–	a	a	–	a	a	–
	7	21	a	a	–	a	a	–	a	a	–
	8	1	a	a	–	a	a	–	a	a	–
	8	7	a	a	–	a	a	–	a	a	–
	8	21	a	a	–	a	a	–	a	a	–
	11	1	a	a	–	a	a	–	a	a	–
	11	7	a	a	–	a	a	–	a	a	–
	11	21	a	a	–	a	a	–	a	a	–

*Total radiolabel and calculated specific activity of each measured sugar*

Despite each sugarcane plant being exposed to equal amounts of radioactivity for the same time period, the uptake of radiolabel by the individual plants could not be controlled or normalised for. This variation in feeding is reflected by standard error values greater than 10 %. The observed trends will be reported. Specific activity refers to the ratio of radiolabel to cold sugar concentration. A change in specific activity over time may be indicative of sugar turnover or accumulation.

In all three sugarcane lines, radiolabel in hexose was present in the internode subtended by the radiolabelled leaf (+1, Table 5.3). Hexoses in the internode above this labelling point were also labelled indicating movement of radiolabel up the culm. The two transgenic lines also contained label in hexose in some internodes below the labelling point. However, in the older internodes radiolabel could not be detected in hexoses, which indicates a slow breakdown of sucrose. The high 1-kestose line showed very high levels of radiolabel in hexose in internode +4 21 days after labelling. No statistical differences in the total label in hexoses present between the first, seventh or twenty-first day after radiolabelling in any of the sugarcane lines or internodes ( $p > 0.05$ ,  $N=4$ , Table 5.2), but the trend suggests that total label in hexoses decreased over time. Hexose specific activity was determined in internodes -2 and +1 in all three sugarcane lines and for internode +4 and +7 of the high 1-kestose producer (Table 5.4). Specific activity increased with increasing internodal maturity due to decreased hexose concentrations and constant radiolabel. No statistical differences in the specific activity of hexoses over time was noted in any of the three sugarcane lines ( $p > 0.05$ ,  $N=4$ , Table 5.2), however it appeared that specific activity decreased with time due to.

The most radiolabel in sucrose was present in internode +1 while a considerable amount of radiolabelled sucrose was present in the internode above the labelling point on the first day after labelling (Table 5.3). Radiolabel in sucrose declined down the culm, as the distance from the labelling point increased. This trend was evident for all three sugarcane lines. In terms of statistical differences, internode -2 in all three lines as well as internode +1 in both transgenics, showed a statistically significant decline from the first time point to the second ( $p < 0.01$ ,  $N=4$ ), after which label in sucrose remained constant until the harvest on the twenty-first day after radiolabelling (Table 5.2). There was a consistent decrease in specific activity of sucrose down the culm of all sugarcane lines and at all time points (Table 5.4). A decrease in specific activity over time is reflected statistically in internode -2 of all three lines as well as in internodes +1 and +4 in both transgenics ( $p < 0.01$ ,  $N=4$ , Table 5.2).

Radiolabelled 1-kestose was detected in all internodes in 2153 (Except +7 on day 1) at all time points (Table 5.3), while sugarcane line 2121 first showed radiolabel in 1-kestose in internode +7. Internodes +4, +7, +8 and +11 of line 2153 showed a trend of increasing radiolabel in 1-kestose over time but this trend was only statistically significant for internodes +4 and +7 ( $p < 0.01$ ,  $N=4$ ). Both transgenic lines showed a trend of declining specific activity of 1-kestose with increasing internodal maturity (Table 5.4), which can be attributed to higher 1-kestose concentration in older internodes which apparently dilutes the total label present. However, a statistical difference in the specific activity of 1-kestose over time was evident in internode +8 of line 2153 (Table 5.2,  $p < 0.01$ ,  $N=4$ ). No statistically significant relationship over time existed for any internode of 2121.

**Table 5.3** Total radiolabel present within the hexose, sucrose and 1-kestose pools located in six internodes spanning the culm of two transgenics (2121 and 2153) and one control sugarcane line (NCo310), at three time points after radiolabelling. Values represent the mean  $\pm$  SE (N=4). N.D. = total label in sugar not detected.

Line	Day	Internode	Total label ( $Bq.g^{-1} fm$ )								
			Hexoses	SE	Sucrose	SE	1-Kestose	SE			
2121	1	-2	610.93	$\pm$	277.18	6597.32	$\pm$	1071.63	N.D.		
	1	1	314.29	$\pm$	193.66	7611.58	$\pm$	1410.78	N.D.		
	1	4	N.D.			1185.66	$\pm$	410.15	N.D.		
	1	7	N.D.			625.50	$\pm$	123.65	1263.94	$\pm$ 189.00	
	1	8	N.D.			667.37	$\pm$	163.70	1085.68	$\pm$ 33.83	
	1	11	N.D.			293.94	$\pm$	168.64	1311.40	$\pm$ 101.67	
	7	-2	120.47	$\pm$	74.31	154.72	$\pm$	92.48	N.D.		
	7	1	557.80	$\pm$	277.04	519.68	$\pm$	205.85	N.D.		
	7	4	N.D.			1973.04	$\pm$	539.67	N.D.		
	7	7	N.D.			1572.57	$\pm$	555.75	540.74	$\pm$ 312.20	
	7	8	N.D.			879.85	$\pm$	369.90	510.45	$\pm$ 295.02	
	7	11	N.D.			297.82	$\pm$	97.17	791.46	$\pm$ 264.07	
	21	-2	N.D.			121.79	$\pm$	70.32	N.D.		
	21	1	559.47	$\pm$	279.70	374.34	$\pm$	101.07	N.D.		
	21	4	N.D.			2267.17	$\pm$	627.66	604.63	$\pm$ 349.70	
	21	7	N.D.			1616.91	$\pm$	320.90	1256.43	$\pm$ 33.36	
	21	8	N.D.			1073.38	$\pm$	361.50	1224.44	$\pm$ 88.53	
	21	11	12.43	$\pm$	10.76	297.82	$\pm$	30.54	1146.87	$\pm$ 287.45	
	2153	1	-2	N.D.			6050.59	$\pm$	1502.37	898.49	$\pm$ 703.08
		1	1	N.D.			11678.88	$\pm$	4074.66	423.55	$\pm$ 277.99
		1	4	N.D.			906.54	$\pm$	307.86	251.18	$\pm$ 145.24
1		7	N.D.			678.51	$\pm$	237.13	N.D.		
1		8	N.D.			849.83	$\pm$	302.81	508.00	$\pm$ 347.94	
1		11	N.D.			416.39	$\pm$	173.30	485.31	$\pm$ 283.32	
7		-2	N.D.			403.21	$\pm$	192.74	427.94	$\pm$ 259.18	
7		1	780.93	$\pm$	414.02	1315.92	$\pm$	316.40	788.97	$\pm$ 345.08	
7		4	N.D.			3376.22	$\pm$	1242.85	1025.27	$\pm$ 73.90	
7		7	N.D.			1424.12	$\pm$	328.53	985.30	$\pm$ 64.11	
7		8	N.D.			1889.96	$\pm$	831.18	941.00	$\pm$ 139.66	
7		11	N.D.			889.94	$\pm$	189.41	1120.96	$\pm$ 197.68	
21		-2	101.63	$\pm$	60.82	113.21	$\pm$	70.21	375.39	$\pm$ 225.66	
21		1	611.47	$\pm$	187.57	2059.14	$\pm$	1050.81	1125.93	$\pm$ 70.62	
21		4	3501.71	$\pm$	1524.51	1044.30	$\pm$	702.12	1280.65	$\pm$ 162.22	
21		7	568.24	$\pm$	122.24	615.72	$\pm$	299.59	1089.21	$\pm$ 27.63	
21		8	N.D.			926.38	$\pm$	141.54	1093.52	$\pm$ 24.30	
21		11	N.D.			555.23	$\pm$	131.92	1069.49	$\pm$ 49.70	
NCo310		1	-2	710.04	$\pm$	310.57	10595.22	$\pm$	2120.33	N.D.	
		1	1	363.31	$\pm$	191.86	12462.98	$\pm$	3100.13	N.D.	
		1	4	N.D.			10493.16	$\pm$	6628.49	N.D.	
	1	7	N.D.			3602.96	$\pm$	2384.94	N.D.		
	1	8	N.D.			1887.32	$\pm$	1091.74	N.D.		
	1	11	N.D.			1166.91	$\pm$	513.40	N.D.		
	7	-2	296.90	$\pm$	110.50	791.85	$\pm$	130.41	N.D.		
	7	1	3303.29	$\pm$	441.83	6745.62	$\pm$	2064.48	N.D.		
	7	4	N.D.			4504.64	$\pm$	1487.35	N.D.		
	7	7	N.D.			2447.55	$\pm$	1114.96	N.D.		
	7	8	N.D.			2555.84	$\pm$	991.29	N.D.		
	7	11	N.D.			1971.96	$\pm$	1109.29	N.D.		
	21	-2	N.D.			0.00	$\pm$	0.00	N.D.		
	21	1	2071.60	$\pm$	1313.58	4355.25	$\pm$	898.42	N.D.		
	21	4	N.D.			9729.62	$\pm$	3872.58	N.D.		
	21	7	N.D.			2666.49	$\pm$	586.82	N.D.		
	21	8	N.D.			2267.81	$\pm$	694.92	N.D.		
	21	11	N.D.			1697.07	$\pm$	511.02	N.D.		

**Table 5.4** The ratio of radiolabelled to unlabelled sugars (specific activity) within the hexose, sucrose and 1-kestose pools located in six internodes spanning the culm of one control and two transgenic sugarcane lines at three time points after radiolabelling. Values represent the mean  $\pm$  SE (N=4). N.D. = specific activity could not be determined. This is based upon an undetectable concentration of sugar and/or undetectable amount of radiolabel within the sugar.

Line	Day	Internode	Specific activity ( $\text{MBq.mol}^{-1}$ sugar)								
			Hexoses	SE	Sucrose	SE	1-Kestose	SE			
2121	1	-2	52.63	$\pm$	38.35	243.08	$\pm$	51.30	N.D.		
	1	1	4.45	$\pm$	4.86	147.45	$\pm$	36.27	N.D.		
	1	4	N.D.			3.48	$\pm$	0.66	N.D.		
	1	7	N.D.			1.18	$\pm$	0.19	1241.59	$\pm$ 277.80	
	1	8	N.D.			1.25	$\pm$	0.31	392.26	$\pm$ 131.03	
	1	11	N.D.			0.55	$\pm$	0.25	417.00	$\pm$ 197.80	
	7	-2	8.18	$\pm$	4.94	6.58	$\pm$	3.86	N.D.		
	7	1	22.13	$\pm$	11.33	30.04	$\pm$	11.90	N.D.		
	7	4	N.D.			11.32	$\pm$	2.11	N.D.		
	7	7	N.D.			3.72	$\pm$	1.19	N.D.		
	7	8	N.D.			1.88	$\pm$	0.77	138.50	$\pm$ 66.98	
	7	11	N.D.			0.57	$\pm$	0.31	N.D.		
	21	-2	N.D.			3.83	$\pm$	2.00	N.D.		
	21	1	8.23	$\pm$	3.30	2.80	$\pm$	0.78	N.D.		
	21	4	N.D.			6.15	$\pm$	1.59	N.D.		
	21	7	N.D.			2.93	$\pm$	0.50	N.D.		
	21	8	N.D.			2.03	$\pm$	0.50	N.D.		
	21	11	N.D.			0.49	$\pm$	0.07	N.D.		
	2153	1	-2	N.D.			175.75	$\pm$	24.03	N.D.	
		1	1	N.D.			93.69	$\pm$	15.27	N.D.	
		1	4	N.D.			2.22	$\pm$	0.69	8.09	$\pm$ 2.97
1		7	N.D.			1.38	$\pm$	0.44	1.47	$\pm$ 0.97	
1		8	N.D.			1.84	$\pm$	0.62	4.98	$\pm$ 2.20	
1		11	N.D.			0.84	$\pm$	0.40	3.75	$\pm$ 3.50	
7		-2	N.D.			13.69	$\pm$	6.00	N.D.		
7		1	31.27	$\pm$	12.66	39.70	$\pm$	13.29	N.D.		
7		4	N.D.			11.74	$\pm$	3.72	84.84	$\pm$ 99.69	
7		7	N.D.			3.35	$\pm$	0.76	19.38	$\pm$ 12.98	
7		8	N.D.			4.30	$\pm$	2.16	15.63	$\pm$ 4.58	
7		11	N.D.			1.89	$\pm$	0.45	10.81	$\pm$ 2.91	
21		-2	4.86	$\pm$	2.08	3.31	$\pm$	1.32	N.D.		
21		1	14.05	$\pm$	8.82	11.76	$\pm$	4.12	189.14	$\pm$ 111.02	
21		4	517.73	$\pm$	447.61	2.55	$\pm$	1.99	48.74	$\pm$ 36.56	
21		7	349.36	$\pm$	0.00	1.32	$\pm$	0.58	17.72	$\pm$ 6.85	
21		8	N.D.			1.90	$\pm$	0.32	13.00	$\pm$ 4.46	
21		11	N.D.			1.22	$\pm$	0.22	10.40	$\pm$ 2.56	
NCo310		1	-2	43.58	$\pm$	27.08	163.50	$\pm$	72.68	N.D.	
		1	1	4.03	$\pm$	2.28	88.11	$\pm$	21.03	N.D.	
		1	4	N.D.			35.62	$\pm$	20.56	N.D.	
	1	7	N.D.			13.00	$\pm$	4.76	N.D.		
	1	8	N.D.			6.10	$\pm$	2.34	N.D.		
	1	11	N.D.			4.52	$\pm$	1.08	N.D.		
	7	-2	20.83	$\pm$	21.03	32.83	$\pm$	8.17	N.D.		
	7	1	57.05	$\pm$	71.48	76.65	$\pm$	56.44	N.D.		
	7	4	N.D.			12.97	$\pm$	20.13	N.D.		
	7	7	N.D.			5.72	$\pm$	2.63	N.D.		
	7	8	N.D.			5.91	$\pm$	1.96	N.D.		
	7	11	N.D.			5.46	$\pm$	2.42	N.D.		
	21	-2	N.D.			1.22	$\pm$	2.41	N.D.		
	21	1	60.04	$\pm$	120.90	28.09	$\pm$	23.21	N.D.		
	21	4	N.D.			24.59	$\pm$	12.52	N.D.		
	21	7	N.D.			6.15	$\pm$	1.34	N.D.		
	21	8	N.D.			4.91	$\pm$	1.49	N.D.		
	21	11	N.D.			4.03	$\pm$	1.41	N.D.		



## 5.5 Discussion

Introduction and expression of 1-SST in the sugarcane genome led to the synthesis of 1-kestose. The production of this trisaccharide in sugarcane, a non-fructan accumulating plant, facilitated investigations of carbon allocation using whole-plant radiolabelling.

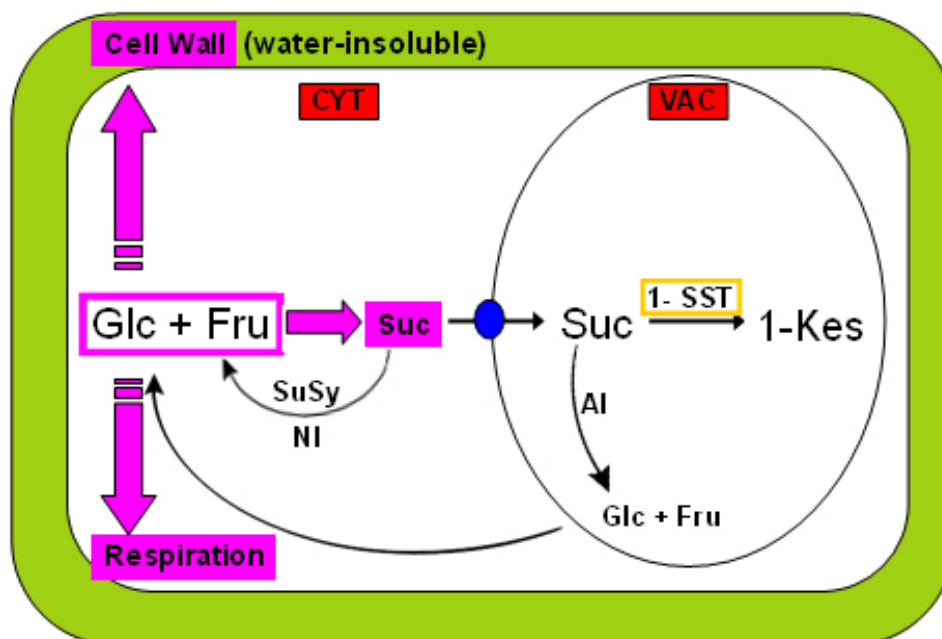
### *Redirection of carbon to the soluble fraction in the high-kestose transgenic*

The expression of 1-SST led to a major change in carbon partitioning in the water-soluble and -insoluble fractions. The high 1-kestose producing transgenic contained significantly more radiolabel in the water-soluble fraction than the other sugarcane lines (Figure 5.4). Since total radiolabel is constituted by both water-soluble and -insoluble fractions, increased radiolabel in the water-soluble fraction in this line was accompanied by a concomitant decrease in radiolabel present in the water-insoluble fraction. Noticeably, carbon was redirected from the water-insoluble fraction into sucrose synthesis in the high-1-kestose producing line (Figure 5.4). The genetic modification did not result in any visual phenotype such as decreased girth or size of the stalks, as well as no loss in physical sturdiness. However, future investigations should include more detailed fibre analyses.

Uridine 5'-diphosphate glucose (UDP-glucose) is involved in numerous metabolic pathways and is the precursor for sucrose synthesis (water-solubles), cell wall/fibre/protein synthesis (water-insolubles) as well as respiratory substrates (Whittaker and Botha, 1997). Hence, there is competition between these processes for carbon (Figure 5.9) (Bindon and Botha, 2002). The measurement of CO<sub>2</sub> production, organic acids, amino acids and proteins infer respiration, while fibre and cell wall constituents compose the majority of the water-insoluble component, along with starch and protein (Bindon and Botha, 2002). A redirection of carbon to sucrose at the expense of the water-insoluble fraction, respiration, organic acids, amino acids and phosphorylated intermediates was observed in mature sugarcane internodes in studies using tissue slices (Botha *et al.*, 1996; Whittaker and Botha, 1997; Bindon and Botha 2001, 2002). Furthermore, sugarcane cell suspension cultures under nitrogen-limiting conditions showed that more carbon was allocated to sucrose than to structural material and respiration (Veith and Komor, 1993).

Irrespective of sugarcane line, the mature internodes contained more radiolabel in the water-soluble fraction than internodes -2 and +1, 21 days after radiolabelling. This data confirms reports that more carbon is allocated to the water-soluble fraction in maturing

internodal tissues (Botha *et al.*, 1996; Whittaker and Botha, 1997; Bindon and Botha 2001, 2002).



**Figure 5.9** A simple schematic of a sugarcane cell showing competition between respiration, sucrose synthesis and fibre synthesis for incoming carbon. The transgenic lines in this study have an additional step after sucrose synthesis that is 1-kestose production by the action of 1-SST.

*1-Kestose production stimulates carbon flow through sucrose*

Both transgenic lines had statistically more radiolabel present in their total sugar pool than the control line and this was attributed to the additive production of 1-kestose in the transgenic lines which stimulated the flow of carbon through sucrose and increased the total sugar content of the transgenic lines (Figure 5.8). Similarly, sugarcane transformed with bacterial sucrose isomerase also had a higher sugar content (Birch and Wu, 2005; Wu and Birch, 2007). However, unlike the current study, carbon partitioning between sugar and fibre was not altered. Rather, transgenic lines showed increased photosynthesis and sink strength, and the increased photosynthate was stored as sugar (Birch and Wu, 2005; Wu and Birch, 2007).

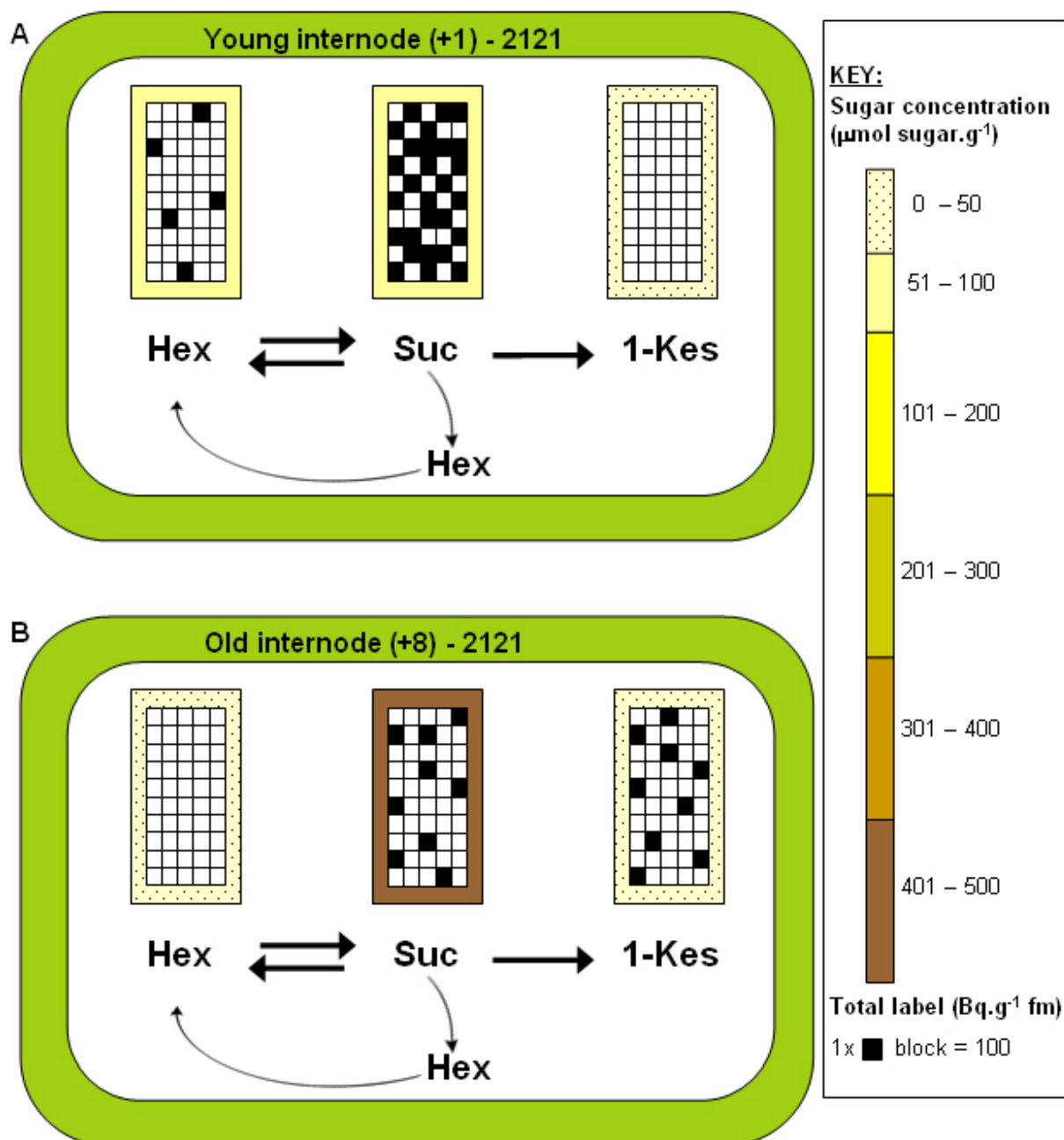
*'Snapshot' schematic enables comparison between sugars and sugarcane lines*

A diagram showing the average concentrations, radiolabel and specific activities of hexose, sucrose and 1-kestose in a young (+1) and old (+8) internode of the low - (Figure 5.10) and high -1-kestose transgenics (Figure 5.11) was constructed. The rectangles represent the individual sugar pools, while their shading denotes the average sugar concentration ( $\mu\text{mol sugar.g}^{-1}$ ) of the pool over a 21 day period. The

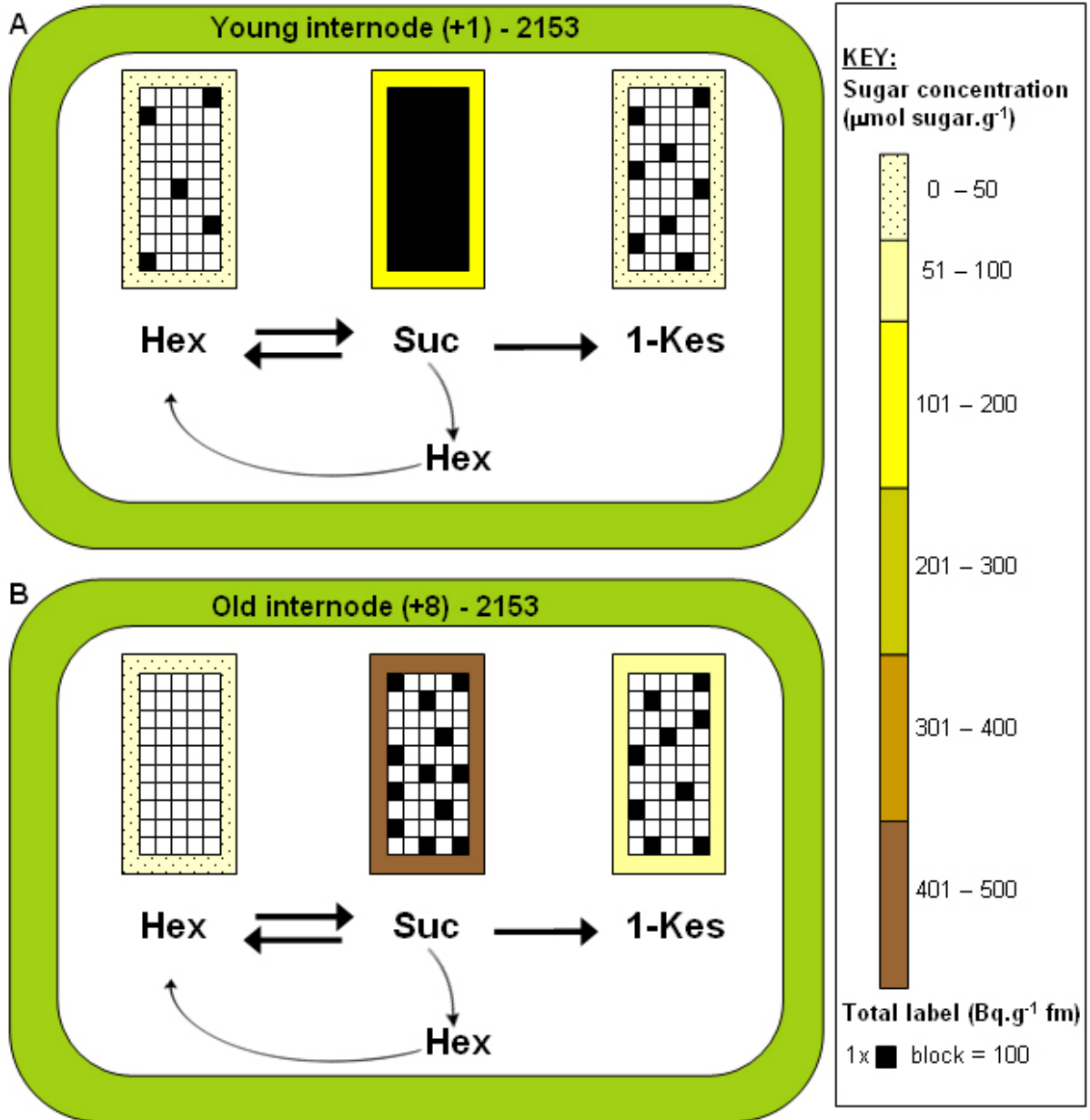
cytosolic and vacuolar sucrose pools could not be differentiated, hence one combined sucrose pool is depicted in the diagrams. Total radiolabel present within each sugar pool is represented by black squares, each equivalent to  $100 \text{ Bq.g}^{-1} \text{ fm}$ . Analysis of both sugar concentration and total label present within each pool indicates the specific activity of the pool. The 'snapshot' does not provide insight into sugar turnover but it facilitates comparisons between the sugar pools in young and old internodes of two sugarcane lines to be visualised.

*Cellular sugar uptake is likely to be in the form of sucrose*

Whole plant radiolabelling was conducted by feeding leaf 5 with  $^{14}\text{CO}_2$  with the ultimate aim of forming a radiolabelled sucrose pool within the culm tissues. The concentration of sucrose and hexose is similar in a young internode (+1) of both transgenic lines (Figure 5.10A and Figure 5.11A). But, the sucrose pool contains more radiolabel than the hexose pool. Bearing in mind that specific activity refers to the ratio of labelled to unlabelled sugars, the specific activity of sucrose exceeds that of hexose. Sucrose is transported symplastically, by moving via plasmodesmata through the mesophyll cells, to the sieve tubes in the phloem tissue and into the parenchyma cells (Moore, 1995). Sugarcane culm tissues can take up sucrose (Lingle, 1989) and it's reducing monomers; glucose and fructose (Sacher *et al.*, 1963; Hawker and Hatch, 1965; Glasziou and Gayler, 1972). However, it is uncertain whether radiolabel is taken up directly as sucrose or whether it is first cleaved into the hexoses and taken up into the sink tissues as monosaccharides. It has been suggested that sugar uptake is likely to be both as sucrose and hexoses (Moore, 1995). On the basis of sucrose having a higher specific activity than the hexoses, this data suggests that radiolabel is predominantly taken up as sucrose and the hexose pool therefore derives radiolabel largely from sucrose that had been hydrolysed in the cytosol.



**Figure 5.10** A schematic of a sugarcane cell from a (A) young internode (+1) and (B) old internode (+8) from line 2121 (low-1-kestose transgenic). The rectangles represent the hexose, sucrose and 1-kestose pools and the shading of each denotes the average sugar concentration present over the 21 day period ( $\mu\text{mol sugar.gram}^{-1}$ ). The black squares represent the total radiolabel present within the sugar pool such that one black square represents  $100 \text{ Bq.g}^{-1}$  fresh mass. Hence sugar concentration coupled with the total label present within the pool is indicative of the specific activity of the pool.



**Figure 5.11** A schematic of a sugarcane cell from a (A) young internode (+1) and (B) old internode (+8) from line 2153 (high 1-kestose transgenic). The rectangles represent the hexose, sucrose and 1-kestose pools and the shading of each denotes the average sugar concentration present over the 21 day period ( $\mu\text{mol sugar.g}^{-1}$ ). The black squares represent the total radiolabel present within the sugar pool such that one black square represents  $100 \text{ Bq.g}^{-1}$  fresh mass. Hence sugar concentration coupled with the total label present within the pool is indicative of the specific activity of the pool.

*Increased sugar concentrations apparently dilute specific activity*

The specific activity of hexose could not be determined in the old internode of the low - (Figure 5.10B) and high- 1-kestose producer (Figure 5.11B), as hexose concentrations were undetectable. Internode +8 had less radiolabel in sucrose than +1 but a larger sucrose concentration was present in the older internodes in both lines. Such a gradient of sucrose accumulation down the culm has been widely reported (Moore, 1995; Whittaker and Botha, 1997; Zhu *et al.*, 1997; Botha and Black, 2000; Rose and Botha, 2000; Moore, 2005). The increased concentration of sucrose apparently diluted the amount of label within the pool, which was less than in internode +1, and this yielded a lower specific activity of sucrose in the older internode. Although the transgenic lines differed in terms of the concentration of hexose and sucrose as well as the amount of radiolabel present within these pools, the specific activities of both hexose and sucrose were within the same range for both sugarcane lines.

*Radiolabel in 1-kestose is derived from a highly labelled sucrose pool*

This is the first reported research on carbon allocation in transgenic sugarcane producing 1-kestose, using whole plant radiolabelling studies to infer 1-kestose synthesis and possible hydrolysis. The specific activity of 1-kestose exceeds that of sucrose in all internodes of both transgenics. Since 1-SST uses sucrose to synthesise 1-kestose, the specific activity of 1-kestose should be less than sucrose; its source. The only explanation for this is the presence of two cellular sucrose pools; one with high specific activity and the majority of 1-kestose derived from this pool. Farrar and Farrar (1985) report kinetic evidence for the existence of multiple sucrose pools that have different turnover rates. It is assumed that the 1-kestose pool with a high specific activity was derived from the highly labelled sucrose pool. The cytosolic sucrose pool is at least nine times smaller than that in the vacuole but it is also the initial pool into which radiolabel enters the cell. The sucrose pool in the vacuole derives all its label from the cytosolic pool. Hence the specific activity of the cytosolic sucrose pool will always exceed that of the vacuolar pool. Because both these pools are collectively measured, and the vacuolar pool contributes the majority of sucrose, the calculated specific activity of sucrose will always understate the specific activity of the cytosolic pool with almost an order of magnitude. Therefore the 1-kestose pool which is smaller than that of sucrose, if synthesised from the highly labelled cytosolic sucrose pool, will have a specific activity higher than sucrose. On the contrary, 1-kestose derived from the vacuolar sucrose pool will have even less label and thus a lower specific activity than sucrose.

*Is 1-SST exclusively vacuolar?*

Although the construct delivered to the plant during transformation contained a signal sequence targeting expression of 1-SST to the vacuole (Hellwege *et al.*, 2000), the construct could have been altered on delivery to the plant. Therefore, 1-SST may not be located in the cell compartment to which it was assigned. Although the concentration of 1-kestose cannot be measured in the different cellular compartments, it is possible to calculate these concentrations by using some well-established parameters and by making a few assumptions. The tissue used in the present study contained on average 77 % moisture (Section 3.4.2), and it is known that the vacuole represents 90 % of the total cellular volume (Komor, 1994; Komor, 2000). From this the concentration of 1-kestose present in an intermediate and a mature internode from the two transgenic lines, 1-kestose could be calculated assuming either an exclusive cytosolic localisation or vacuolar localisation. The apoplastic volume was not considered in these calculations hence the reported concentrations of 1-kestose are an underestimation. If 1-kestose was exclusively cytosolic in the high-1-kestose producer (2153) the concentration will be in the range of 0.90 M to 1.45 M in the intermediate and mature internodes respectively. The solubility of 1-kestose would not allow such high concentrations and would also have serious consequences for regulation of water potential in the tissue. Based on the fact that the specific activities of 1-kestose exceeded that of sucrose, it can be concluded that 1-kestose production cannot be exclusively in the vacuole. In the case of the low 1-kestose transgenic line, the calculated cytosolic concentrations of 1-kestose in the intermediate (0.01 M) and mature internodes (0.04 M) are feasible. The high specific activity of 1-kestose confirms that synthesis is not exclusively vacuolar. This implies that 1-kestose synthesis in the low-1-kestose producing line could be exclusively cytosolic. Based on these calculations it would be plausible to suggest that, 1-SST activity might be present in both the vacuole and cytosol. Future investigation into pinpointing the cellular location of 1-SST in these transgenic lines, with the use of antibodies, would clarify these speculations.

*Implications of changing specific activities over time*

Analysis of the concentration, amount of radiolabel and the resultant specific activity of a sugar pool can infer either sugar turnover (decreasing specific activity), accumulation (increasing specific activity) or an apparent steady state (constant specific activity) of a sugar pool. A decrease in the specific activity over time can be a result of either a) a decrease in the amount of radiolabel in that pool despite a constant concentration of unlabelled sugar or b) an increase in the concentration of unlabelled sugars coupled

with a constant amount of radiolabel within the pool. The first scenario implies sucrose turnover. On the contrary, an increase in specific activity over time can suggest that the pool is accumulating sugar. This trend is due to an increase in the amount of radiolabel within a pool despite a stable pool of unlabelled sugar. An increase in specific activity can also result from a decline in the entrance of unlabelled sugars into the pool coupled with a constant level of radiolabel present. However, the latter is not indicative of sugar accumulation.

*Turnover of hexoses and sucrose in the young internodes*

At numerous time points and internodes from all three sugarcane lines, specific activity could not be determined due to undetectable levels of unlabelled or labelled hexoses. There were no statistical differences between the time points in terms of specific activity, concentration or total radiolabel present. However, the data that were obtained for hexoses showed a trend in all three sugarcane lines of decreasing specific activity with time. It appears that this is due to a decrease in the total label over time within a fairly constant concentration of hexoses, which implies turnover of hexoses. Since the highest concentration of hexoses is located in the younger internodes, hexose turnover in these internodes is likely. The young internodes are actively growing and metabolising and approximately 30 % of incoming carbon is burned off as carbon dioxide (CO<sub>2</sub>) while 35 % is used for the synthesis of sucrose (Bindon and Botha, 2002).

In all three sugarcane lines, the specific activity of sucrose shows statistically significant decreases over the 21 day time period. In particular, this is evident in internodes -2 of all three sugarcane lines and internode +1 of both transgenics. The decrease in sucrose specific activity in these internodes is due to a concomitant decrease in total label present within a relatively stable concentration of sucrose. During the chase period, unlabelled sucrose continues to enter the pool which apparently dilutes the radiolabel already present thereby lowering the specific activity. Over time a decrease in specific activity is indicative of sucrose turnover. Young internodes contain high levels of soluble acid invertases which cleave sucrose into hexoses (Hatch and Glasziou, 1963; Gayler and Glasziou, 1972; Zhu *et al.*, 1997). Furthermore, these young tissues are actively metabolising and one third of incoming carbon in young internodes is respired (Bindon and Botha, 2002), thus the sucrose pool will be turning over.



The older internodes of the two transgenic and control line show no marked turnover of sucrose over time. The pool size remains relatively constant over time while label also remains constant. Although a cycle of continual hydrolysis and synthesis occurs, even in mature internodes (Sacher *et al.*, 1963; Hawker and Hatch, 1965), the specific activities of sucrose in the mature internodes suggests that the rate of synthesis exceeds or equals hydrolysis. This implies sucrose accumulation in the mature internodes.

*The production of 1-kestose occurs slower than sucrose*

The rate of 1-kestose synthesis was calculated such that comparisons with the reported rate of sucrose synthesis could be drawn. Sugarcane line 2153 retained radiolabel in the 1-kestose pool, suggesting accumulation of this trisaccharide. Data from internode +11, which contained the highest concentration of 1-kestose, was utilised to calculate the rate of 1-kestose synthesis. Pulse-chase whole plant labelling experiments produce a surge of radiolabelled sugars which are subsequently replaced by unlabelled sugars. The pools do not reach equilibrium in the source. Hence, the specific activity of sucrose averaged for day 1 and day 7 was used for the calculation and this value was assumed to be constant over the 21 day time period. For the purpose of the calculation, it was assumed that there was only one radiolabelled sucrose pool. In addition, the rate of 1-kestose synthesis does not compensate for any 1-kestose that may exit the pool in this line and internode. For all these reasons, the rate of 1-kestose synthesis may be underestimated.

The change in the total radiolabel in 1-kestose from the first to the seventh day after radiolabelling was divided by the average specific activity of sucrose for the same time period. For internode +11 of the high 1-kestose producer, the rate of 1-kestose synthesis was  $0.046 \text{ nmol.g}^{-1} \text{ fm.min}^{-1}$ . Using protein values reported by Bindon and Botha (2001), the sucrose synthesis rate in an old (+11) internode was  $8.51 \text{ nmol.g}^{-1} \text{ fm.min}^{-1}$  while in a young internode (+1) it was approximately double at  $16.99 \text{ nmol.g}^{-1} \text{ fm.min}^{-1}$  (Bindon and Botha, 2002). Other reported rates of sucrose synthesis are approximately half those reported by Bindon and Botha (2002) for similarly-aged internodes (Whittaker and Botha, 1997; Botha and Black, 2000). Nevertheless, the use of the lowest sucrose synthesis rate, still yielded a rate 100 times higher than that obtained for 1-kestose synthesis. Hence 1-kestose is synthesised far slower than sucrose in transgenic sugarcane.

## 5.6 Conclusion

1-Kestose synthesis in the characterised transgenic lines (Chapter 3) acts as an additional sink which stimulates carbon flow through sucrose. As a consequence, carbon was redirected from the water-insoluble fraction to synthesise sucrose and 1-kestose in the high-1-kestose transgenic line. The genetic modification therefore differentially altered carbon allocation in the two transgenic lines. The specific activity of 1-kestose was higher than sucrose, and showed no turnover with time, which correlates with the low affinity of invertases for 1-kestose (Chapter 4). Hence, 1-SST appears to be localised primarily in the cytosol in the low 1-kestose producer but in both the vacuole and cytosol in the high-1-kestose line.

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## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSIONS

Knowledge of sucrose metabolism in sugarcane culm tissues has experienced a recent and rapid expansion. The study of genetically modified sugarcane where attempts were made to enhance the sucrose content (Grof and Campbell, 2001; Birch and Wu, 2005; Groenewald and Botha, 2007; Wu and Birch, 2007), as well as non-transgenic studies (Whittaker and Botha, 1997; Bindon and Botha, 2002), have furthered our understanding of carbon partitioning in sugarcane. However, serious gaps in the understanding of the metabolic control of sucrose accumulation and carbon partitioning in the culm remain a major obstacle, particularly when attempting to manipulate sugarcane metabolism. The work described in this study, is the first to report on carbon partitioning in 1-kestose-producing sugarcane grown under field resembling conditions. It contributes significantly to an improved understanding of carbon partitioning in the culm, and also demonstrates that an alternative product can be produced under field resembling conditions.

#### *Stable production of 1-kestose in genetically modified sugarcane*

This study utilised sugarcane lines that had been transformed with sucrose: sucrose 1-fructosyltransferase (1-SST) (Nell, 2006). Two of the four lines tested contained the gene encoding 1-SST in their genome (*Section 3.4.1*). On this basis these two lines were selected for further experimentation and their ability to produce 1-kestose under field conditions was evaluated. Despite the presence and stable expression of the transgene in both sugarcane lines, their levels of 1-kestose were distinct with the one line producing one hundred times more than the other (*Section 3.4.3*). These two lines were classified as high- and low- 1-kestose producers and offered a valuable experimental system. Although these lines accumulated very different amounts of the trisaccharide, the pattern of accumulation was similar. The highest levels of 1-kestose were always present in the mature internodal tissue (*Section 3.4.3*). This is important as it demonstrates that the new sugar follows the same accumulation pattern as sucrose with a concentration gradient down the culm that reflects the increasing maturity of internodal tissue.

There can be two possible explanations for this phenomenon. Firstly, it could indicate that 1-kestose accumulation can only occur after considerable accumulation of sucrose such that there is adequate substrate availability for 1-kestose production. Such a threshold of sucrose has been proposed as a trigger for fructan accumulation (Koops and Jonker, 1996). Alternatively, 1-kestose could mimic the accumulation pattern of sucrose down the

sugarcane culm *if* exposed to the same mechanism (invertase-mediated hydrolysis) that rapidly degrades sucrose in the young tissues but allows accumulation in mature tissues due to reduced enzymatic activity.

*1-Kestose production is not at the expense of sucrose*

Based on knowledge of resource limits, it was expected that 1-kestose would be produced at the expense of sucrose. On the contrary, 1-kestose was produced in addition to sucrose (*Section 3.4.3*). Even more surprising is that both transgenic lines had significantly higher sucrose levels than the control line. The production of this alternative sugar in addition to elevated sucrose levels increased the total sugar content in the transgenics.

Sugarcane transformed with bacterial sucrose isomerase, which catalyses the conversion of sucrose to another disaccharide - isomaltulose, also showed elevated sucrose concentrations as well as doubled sugar content (Birch and Wu, 2005; Wu and Birch, 2007). That approach (production of isomaltulose) as well as the one used in this study (production of 1-kestose) both draw on the sucrose pool for the production of an alternative sugar and show the same phenotype of enhanced sugar content. Since the studies differed in their approach, it is likely that it is not the new alternative sugar that results in elevated sucrose levels, but rather a commonality in both systems such as sucrose or one of its precursors.

In the current study, more radiolabelled carbon was present in the total sugar pool in both transgenics than in the control line (*Section 5.4.3*). There is therefore a larger investment of total carbon in the sugar pool to account for 1-kestose production and increased total sugar content in the transgenics (*Section 3.4.3*). Such a phenotype could be due to increased photosynthesis or a re-allocation of carbon within the internode with more carbon channelled into sugars. Although the impact of this transgene on sugarcane biomass yield has not yet been determined, no obvious increase in biomass in the transgenics was evident, as the culms were of a similar girth and height. Furthermore, there was no significant difference in the total amount of label per internode in the transgenics and the control line after labelling with  $^{14}\text{CO}_2$  (*Section 5.4.1*). Collectively these observations indicate that net photosynthesis was not significantly altered by the production of 1-kestose in these genetically modified lines.

The principle product of photosynthesis is sucrose, which is also the major translocated form of carbon and the primary storage carbohydrate in sugarcane. Due to the pivotal role

of sucrose in plant metabolism (Sonnewald *et al.*, 1991), and the strong association between sucrose synthase (SuSy) and sink strength (Xu *et al.*, 1989), uridine 5' diphosphate glucose (UDP-glucose) is central to carbon partitioning in sink tissues. UDP-glucose is also intimately associated with cytosolic pyrophosphate (PPi) in plant tissues. The importance of UDP-glucose and the vital role that it plays in sucrose metabolism was demonstrated by altering PPi metabolism in tobacco plants. This resulted in an aberrant phenotype which could be removed and the original phenotype restored by by-passing the UDP-glucose dependent flow of carbon into respiration (Lerchl *et al.*, 1995; Geigenberger *et al.*, 1996). Respiration, cell wall/fibre synthesis (water-insolubles) and sucrose synthesis (water-solubles) are the three major processes in which UDP-glucose plays an integral part and these pathways compete for available UDP-glucose.

In this study, the high-1-kestose transgenic (2153) contained significantly more radiolabel in the water-soluble fraction of the internode than the other two sugarcane lines, and this was at the expense of the insoluble fraction (*Section 5.4.2*). Hence, carbon was redirected from the water-insoluble fraction to the water-soluble fraction to account for the additive production of 1-kestose. The expression of 1-SST in sugarcane therefore established an additional carbohydrate sink by the flow of carbon from the sucrose pool into 1-kestose. This did not lead to a depletion of the sucrose pool, but rather stimulated carbon channelling into this pathway, increasing the non-structural carbohydrate content of the plant. The redirection of carbon to the water-solubles at the expense of the water-insoluble fraction emphasises the extreme importance placed on sucrose synthesis as a process, such that it is favoured over cell wall synthesis and respiration when required.

The reason for sucrose elevation and increased flux into sugar synthesis could be a result of enhanced sucrose synthesis, reduced breakdown or a combination of both. The experimental design used did not allow differentiation between sucrose synthesis and breakdown. Reduced breakdown could be a result of inhibition by 1-kestose on invertase-mediated hydrolysis of sucrose as proposed in Chapter 4 (*Section 4.4.4*). However, the low-1-kestose producing line also shows elevated sucrose levels (*Section 3.4.3*) and it is unlikely that the presence of low levels of 1-kestose can lead to sucrose accumulation over and above that evident in the control line. Irrespective of the reason for the elevated sucrose levels, this study shows that there is an enhanced flux of carbon entering the sugar pool (*Section 5.4.2*).

Interestingly, the 1-kestose-producing transgenics did not have an increased glucose content, which would be expected since glucose is a by-product of 1-kestose synthesis

(Section 3.4.3). This suggests that the system efficiently controls the pool size of glucose, which prevents the accumulation of this monosaccharide in any of the internodal tissues, even the most mature internodes that contain the highest concentration of 1-kestose. Rather, glucose is re-synthesised into sucrose in the cytosol, suggesting that the enzymes responsible for sucrose synthesis are continually active even in mature internodes.

*Breakdown of 1-kestose is less efficient than sucrose in sugarcane*

The kinetic properties of sugarcane invertases measured in this study indicated that both neutral invertase (NI, cytosolic) and soluble acid invertase (SAI, vacuolar) efficiently hydrolyse sucrose (Section 4.4.4). Breakdown of sucrose therefore occurs in both the cytosol and vacuole. The specificity of NI and SAI for sucrose are different. It has been suggested that this distinction is based upon a structural difference namely the presence of a NDPNG pentapeptide sequence in acid invertases, which may facilitate the enzyme to break down other sugars, whilst its absence in NI may result in the enzyme's specificity for sucrose (Tymowska-Lalanne and Kreis, 1998).

On the basis of the distinct affinities of these two enzymes for sucrose in the two cellular compartments, it is highly likely that their hydrolysis and affinity for 1-kestose, an alternative substrate, will also differ. The kinetic data shows that the invertases had a far lower affinity for 1-kestose than sucrose (Section 4.4.4). Regardless of the compartment in which 1-kestose is present, it seems that this trisaccharide is protected from hydrolysis by both SAI (vacuolar) and NI (cytosolic). This is indicative of an inefficient system of breakdown for this substrate.

This research was based upon the assumption that 1-kestose would be hydrolysed by sugarcane invertases with a similar affinity to sucrose and therefore give an indication of sucrose turnover in the vacuole. Numerous reports suggest that invertase is capable of 1-kestose hydrolysis (Cairns and Ashton, 1991; Cairns *et al.*, 1997; Van der Meer *et al.*, 1998; Ritsema and Smeekens, 2003). Sucrose turnover was confirmed by the specific activity data shown in Chapter 5 (Section 5.4.4). A significant decline in the specific activity of sucrose over time is evident. This is due to a concomitant decrease in the amount of label present in a relatively constant unlabelled sucrose pool, and the apparent dilution of the radiolabel present by unlabelled sucrose that enters the pool during the chase period. This trend was noted in the young internodes, where invertase levels are highest. 1-Kestose, on the other hand, had a relatively constant specific activity over the twenty-one day period because the total label present in the pool as well as the concentration of 1-kestose did not significantly change over time (Section 5.4.4). Hence



the initial assumption that the cytosolic and vacuolar reactions could be distinguished on the basis of 1-kestose hydrolysis was partially flawed.

*Production of 1-kestose probably occurs in more than one cellular compartment*

The specific activity of 1-kestose exceeded that of sucrose (*Table 5.4*). It has therefore been suggested that 1-kestose is likely to be synthesised from a highly labelled sucrose pool located in the cytosol. If 1-SST is expressed only in the cytosol, 1-kestose would be largely restricted to this compartment since there appears to be no tonoplast transport activity for 1-kestose in plants (Pontis *et al.* 2002). Because 1-kestose is not amenable to hydrolysis by neutral invertases resident in the cytosol (*Section 4.4.4*), this trisaccharide would accumulate over time. The kinetic properties of 1-SST (Koops and Jonker, 1996), allows synthesis of 1-kestose in the cytosol. Although there is no technology available to differentiate between cytosolic and vacuolar 1-kestose in the sugarcane tissue, theoretical levels of 1-kestose in the two compartments could be estimated on the basis of current understanding of the cellular volume occupied by the different compartments. These calculations, suggest that 1-SST activity may be present in both the vacuole and cytosol in the high- 1-kestose transgenic and exclusively in the cytosol in the low-1-kestose transgenic (*Section 5.5*). Although a signal sequence targeting the introduced 1-SST gene for vacuolar expression was included (Hellwege *et al.*, 2000), the data suggests that targeting was not efficient. Other types of targeting signals need to be considered in the future.

*Possible regulation of carbon flow*

A fixed amount of carbon enters the plant and the resultant UDP-glucose is made available to three competing processes namely respiration, cell wall/fibre synthesis (water-insolubles) and sucrose synthesis (water-solubles). This occurs normally in maturing sugarcane internodal tissue, with more carbon being allocated to sucrose synthesis at the expense of the other two processes (Whittaker and Botha, 1997; Bindon and Botha, 2002).

In addition to the natural increase of carbon entering the sucrose synthetic pathway upon internodal maturation, the genetic introduction of 1-SST and subsequent production of 1-kestose resulted in a further reallocation of carbon. The high 1-kestose transgenic showed significant channelling of carbon from the water-insoluble fraction to sucrose synthesis (*Section 5.5*). Although no other enzymes or pathways were intentionally altered by transformation with 1-SST, respiration and structure synthesis were impacted by a reduction in the amount of carbon channelled into these two pathways. This is suggestive

of feedback, 'cross-talk' or signalling between pathways. Sugars have been implicated as signalling molecules that initiate and regulate changes in gene expression (Turk and Smeekens, 1999) and the particular importance of sucrose and glucose in signal transduction pathways has been emphasised. It is likely that either sucrose or its precursors, including UDP-glucose, may be involved. Furthermore, changes in the sugar status of plant cells has been reported to alter the expression of sugar-responsive genes resulting in physiological responses (for review see Koch, 1996; Koch, 2004).

It is suggested that future investigations focus on the mechanisms by which sugar signalling occurs in the culm, and the extent to which it controls carbon partitioning.

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