TREHALOSE AND CARBON PARTITIONING IN SUGARCANE

Susan Bosch

Dissertation presented for the Degree of Doctor of Philosophy (Plant Biotechnology)

at the University of Stellenbosch

Promoter: Prof. F.C. Botha

Institute for Plant Biotechnology and South African Sugar Research Institute

Co-Promoter: Prof. J.M. Rohwer

Department of Biochemistry

December 2005

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation 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.

Signature: ……..

Date: ……..

ABSTRACT

The current understanding of the regulation of sucrose accumulation is still incomplete even though many scientists have investigated this subject. Components of trehalose metabolism have been implicated in the regulation of carbon flux in bacteria, yeast and more recently in plants. With a view to placing trehalose metabolism in the context of cytosolic sugarcane sucrose metabolism and carbon partitioning we have investigated the metabolites, transcripts and enzymes involved in this branch of carbohydrate metabolism in sugarcane internodal tissues.

Sugarcane internodal trehalose levels varied between 0.31 ± 0.09 and 3.91 ± 0.99 nmol.g^{-1} fresh weight (FW). From statistical analysis of the metabolite profile it would appear that trehalose does not directly affect sucrose accumulation, although this does not preclude involvement of trehalose-6-phosphate in the regulation of carbon partitioning. The metabolite data generated in this study demanded further investigation into the enzymes (and their transcripts) responsible for trehalose metabolism.

Trehalose is synthesised in a two step process by the enzymes trehalose-6-phosphate synthase (EC 2.4.1.15, TPS) and trehalose-6-phosphate phosphatase (EC 3.1.3.12, TPP), and degraded by trehalase (EC 3.2.1.28). Two novel sugarcane partial cDNAs that coded for trehalase (*tre*) and *actin* (required for normalisation in profiling experiments) were isolated and used along with partial transcripts for TPS and TPP to determine transcript levels in different tissue- and genotypes. A putative full-length *SugTPS* cDNA was isolated and characterised. Enzyme activities for TPS, TPP and trehalase were measured at levels of 2.7 nmol.min^{-1}.mg^{-1}protein, 8.5 nmol.min^{-1}.mg^{-1}protein and 6.2 nmol.min^{-1}.mg^{-1}protein respectively, from young internodal protein extracts of sugarcane, variety N19. TPP enzyme activity and transcript levels were higher in *S. spontaneum* than *Saccharum* interspecific hybrids.

Kinetic analysis of TPP and trehalase activities were performed with the purpose of providing parameters for an *in silico* kinetic model of trehalose and sucrose metabolism. Three isoforms of TPP were identified and desingated TPPAI, TPPAII and TPPB. Both TPPA isoforms had pH optima of 6.0, and TPPB of pH 6.5. Apparent K_m values were determined as 0.447 ± 0.007 mM for TPPAI, 13.82 ± 1.98 mM for TPPAII and 1.387 ± 0.18 mM for TPPB. Partial purification and characterisation of trehalase demonstrated dual pH optima of 3.5 and 6.0, with K_m values between 0.345 and 0.375 mM. These data were used as the basis for a kinetic model of trehalose metabolism.

A previously described kinetic model of cytosolic sucrose metabolism has been expanded to include the trehalose pathway (TPS, TPP and trehalase). The aim was to supplement the

available information on cytosolic metabolism in sugarcane storage parenchyma, identify points of control between sucrose and trehalose metabolism, and provide a platform from which further experimental and *in silico* modelling can be launched. The model predicted trehalose in the same order of magnitude as those determined in the metabolite profiling experiments. The majority of control of flux over the trehalose pathway resided in the TPS step, with flux control coefficients > 70% of the total pathway. Incorporation of the trehalose branch into the original sucrose model showed that reactions from the original model significantly affected the steady-state attributes of the trehalose pathway.

Due to the relatively low flux through the trehalose branch of the expanded model, complete recycling of trehalose, and the lack of allosteric regulation by trehalose-6-phosphate or trehalose on any of the reactions from the original sucrose model, incorporation of the trehalose branch had no significant effect on either steady-state cytosolic sucrose concentration or flux of sucrose into the vacuole. The expanded model affords a basis from which to further investigate trehalose metabolism in the context of plant sucrose accumulation.

OPSOMMING

Die proses van sukrose akkumulering word nie volledig verstaan nie, ten spyte van uitgebreide navorsing oor hierdie onderwerp. Aspekte van trehalose-metabolisme is al geïmpliseer in die regulering van koolstofverdeling in bakterieë, giste en meer onlangs in plante. Teen die agtergrond van sitosoliese sukrose-metabolisme en koolstofverdeling in suikerriet, het ons trehalose-metabolisme ondersoek deur die metaboliete, transkripte en ensieme te bestudeer wat by hierdie gedeelte van koolhidraatmetabolisme in suikerriet betrokke is.

Suikerrietstingel trehalose-vlakke het tussen 0.31 ± 0.09 en 3.91 ± 0.99 nmol.g^{-1} vars massa gewissel. Statistiese analise van die metabolietprofiel dui aan dat trehalose nie sukrose-akkumulering direk beïnvloed nie. Die betrokkenheid van trehalose-6-fosfaat by die regulering van koolstofverdeling kan egter nie uitgesluit word nie. Die analise van metabolietdata het vereis dat verdere ondersoek gedoen word op die ensieme wat verantwoordelik is vir trehalose metabolisme en op hulle transkripte.

Trehalose word gesintetiseer in 'n twee-stap proses deur die ensieme trehalose-6-fosfaat sintase (EC 2.4..1.14, TPS) en trehalose-6-fosfaat fosfatase (EC 3.1.3.12, TPP), en word afgebreek deur trehalase (EC 3.2.1.28). Twee nuwe suikerriet geen-fragmente wat kodeer vir trehalase (*tre*) en *aktien* (benodig vir normalisering in uitdrukkings-eksperimente) is geïsoleer en saam met geen-fragmente vir TPS en TPP gebruik om transkripsievlakke in verskillende weefsel- en genotiepes te bepaal. 'n Kandidaat vollengte *SugTPS* geenfragment is geïsoleer en gekarakteriseer. Ensiemaktiwiteite vir TPS, TPP en trehalase, gemeet van jong stingel-proteïenekstrakte uit suikeriet variëteit N19, is 2.7 nmol.min^{-1}.mg^{-1}proteïen, 8.5 nmol.min^{-1}.mg^{-1} proteïen en 6.2 nmol.min^{-1}.mg^{-1} proteïen onderskeidelik. TPP ensiemaktiwiteit en transkripvlakke was hoër in *Saccharum spontaneum* as *Saccharum* interspesifieke hibriede.

TPP en trehalase aktiwiteite is kineties geanaliseer met die doel om data vir 'n *in silico* kinetiese model van trehalose en sukrose metabolisme te voorsien. Drie isovorme van TPP is geïdentifiseer en TPPAI, TPPAII en TPPB genoem. Beide TPPA-isovorme het pH-optima van 6.0 gehad, en TPPB ‘n optimum van pH 6.5. K_m waardes is bepaal as 0.447 ± 0.007 mM vir TPPAI, 13.82 ± 1.98 mM vir TPPAII en 1.387 ± 0.18 mM vir TPPB. Gedeeltelike suiwering en karakterisering van trehalase het 'n tweevoudige pH-optimum van 3.5 en 6.0 getoon, met K_m waardes tussen 0.345 en 0.375 mM. Hierdie data is gebruik as die basis vir 'n kinetiese model van trehalose metabolisme.

'n Voorheen beskrewe kinetiese model van sitosoliese sukrose metabolisme is aldus uitgebrei (met TPS, TPP en trehalase) om die trehalose-pad in te sluit. Die doel was om die inligting wat tans beskikbaar is oor sitosoliese sukrose-metabolisme in bergingsweefsel van suikerriet aan te vul,

punte van beheer tussen sukrose en trehalose-metabolisme te identifiseer, en om 'n platform te skep waarvandaan verdere eksperimentele werk en *in silico* modellering geloods kan word. Die model het trehalose vlakke van dieselfde grootte-orde voorspel as dié wat eksperimenteel bepaal is. Die grootste beheer van fluksie deur die trehalose-pad is gesetel in die TPS stap, met 'n fluksie-kontrole-koëffisiënt > 70% van die totale pad. Inkorporering van die trehalose-tak in die oorspronklike sukrose-model het getoon dat die reaksies van die oorspronklike model 'n beduidende effek op die bestendige-toestand-eienskappe van die trehalose-pad uitoefen.

As gevolg van die relatiewe lae fluksie deur die trehalose-tak van die uitgebreide model, die totale herwinning van glukose na trehalose degradasie, en die gebrek aan allosteriese regulering deur trehalose-6-fosfaat of trehalose op enige van die reaksies in die oorspronklike model, het inkorporering van die trehalose-tak geen beduidende effek op die bestendige-toestand van òf sitosoliese sukrosekonsentrasie òf die fluksie van sukrose na die vakuool gehad nie. Die uitgebreide model skep 'n basis vir verdere navorsing oor trehalose-metabolisme in die konteks van plant sukrose-akkumulering.

FOR MY FATHER

ACKNOWLEDGEMENTS

I would like to thank both Dr. F.C. Botha and Prof J.M. Rohwer for supervision of this study.

Frikkie, your input in both my life and this project will never be forgotten. I have learnt so much from you and feel privileged to have been your student.

Johann, your guidance has opened a whole new side of biology to me- thank you for your infectious enthusiasm, patience and hard work.

Thank you to Hennie Groenewald, Jens Kossmann and James Lloyd for consistently having an open-office-door policy for my many questions.

To Mauritz Venter and Wolfgang Schäfer, thank you both for your willingness to help at all times and for the many useful discussions.

Gratitude is expressed to Johan Thevelein and Patrick van Dijck who afforded me the opportunity of researching at the laboratory for Molecular Cell Biology, Katolieke Universiteit, Leuven, Belgium.

Special thanks to Jean Mackenzie of the Central Analytical NMR facility (University of Stellenbosch) for help with both experiments and data processing from the NMR work.

Financial support is gratefully acknowledged from the National Research Foundation, the South African Sugar Research Institute, the Bilateral SA-Belgian assoc., the Harry Crossley Trust, and the University of Stellenbosch.

The support and love of my husband made the last years in the lab possible. Morné, I cannot express my appreciation enough. I love you more every day.

Thanks to my parents. Dad, thanks for always believing in me and impelling me to success with encouraging words. Mum, you will always remain an inspiration of love and patience for me, thanks for just being my mom. I love you both.

The grace and love of the Father, and the wisdom of the Holy Spirit spurred me on each day.

TABLE OF CONTENTS

CHAPTER 7 : GENERAL DISCUSSION

LIST OF FIGURES, TABLES AND APPENDICES

FIGURES

TABLES

APPENDICES

LIST OF ABBREVIATIONS

°C	degrees centigrade
ATP	adenosine 5'-triphosphate
AtTPS	*Arabidopsis thaliana* TPS gene
Bq	Bequerel
bp	nucleic acid base pair
C	control coefficient
cDNA	complementary deoxyribonucleic acid
^{14}C	radio-labeled carbon
Da	Dalton
ddH_2O	double distilled water
DNA	deoxyribo nucleic acid
DTT	1,4-dithiothreitol
ε	elasticity coefficient
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
edn.	edition
EST	expressed sequence tag
F6P	fructose-6-phosphate
FRC	fructose
FW	fresh weight
G	gram
*x*g	gravitational force
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
GCMS	Gas Chromatography-Mass Spectrometry
gDNA	genomic DNA
GLC	glucose
H	hour
HCA	hierarchical cluster analysis
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1)
HPI	hexose phosphate isomerase
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
IID	inline isotope detector
J	steady-state flux
K_m	substrate concentration producing half maximal velocity
K_i	kinetic inhibition constant
L	litre
m	meter
M	molar
MCA	metabolic control analysis
MEP	methylphosphonate
MES	2-[N-morpholino] ethanesulfonic acid
min	minute
NAD^+	oxidised nicotinamide adenine dinucleotide
NADP	reduced nicotinamide-adenine phosphate dinucleotide
NMR	nuclear magnetic resonance
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
Pa	Pascal
PAD	pulsed amperometric detection
PCA	principal component analysis

PCR	polymer chain reaction
PEP	phosphoenolpyruvate
PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependant phosphofructokinase (pyrophosphate:D-fructose-6-phophate 1-phosphotransferase, EC 2.7.1.90)
PGI	phosphoglucoisomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
PVPP	polyvinylpolypyrrolidine
PySCeS	Python Simulator for Cellular Systems
R	response coefficient
RNA	ribonucleic acid
RNaseA	ribonuclease A
rpm	revolutions per minute
s	steady-state concentration
S6P	sucrose-6-phosphate
SDS	sodium dodecyl sulphate
se	standard error
SPS	sucrose phosphate synthase (UDP-glucose:D-fructose-6-P 2-α-D-glucotransferase, EC 2.4.1.14)
SUC	sucrose
SuSy	sucrose synthase (UDP-glucose:D-fructose 2-α-D-glucosyl-transferase, EC 2.4.1.13)
T6P	trehalose-6-phosphate
TBE	tris-borate/EDTA electrophoresis buffer
TBST	tris-buffered saline containing Tween
TCA	tricarboxylic
TE	tris/EDTA buffer
TEP	tri-ethyl phosphate
TPS	trehalose-6-phosphate synthase (EC 2.4.1.15)
TPP	trehalose-6-phosphate phosphatase (EC 3.1.3.12)
TRE	trehalose
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate
UDPG	UDP-glucose
UV	ultra violet
V	reaction rate
V	volume
V	Volt
V_{max}	maximum catakytic activity
w	weight

CHAPTER 1
GENERAL INTRODUCTION

Trehalose and carbon partitioning in sugarcane internodal tissues

1.1. Motivation

The predicted global refined sucrose consumption for 2004/05 is approximately 147 million tons. This translates to a market size of close to US$40 billion, calculated from the average spot price from the first quarter of 2005 (www.sugaronline.com). The predicted two million ton global sucrose production deficit for 2004/05 presents the challenge of improving sucrose yield that could meet this demand, and could boost foreign exchange earnings for a country like South Africa if it could capture this market. Currently, the South African sugar industry annually generates approximately US$1 billion of revenue, of which only 40% contributes to international trade and the world demand for sucrose. This makes it one of the smaller players in the world's sugar trade, leaving room for development. Sucrose is produced by two crops: sugarbeet and sugarcane that account for 30% and 70% of global sucrose production respectively (www.nuca.ie.ufrj.br). All of South Africa's sugar is produced from sugarcane that is propagated in regions with subtropical climatic conditions. Sugarcane is able to produce up to 14% of its fresh weight in sucrose (Moore 1995), and even though this is a remarkable attribute, increasing the productivity of such sucrose production is still the focus of much fundamental biochemical, breeding and agronomical research with a definite monetary incentive.

Commercial sugarcane varieties are interspecific hybrids of the genus *Saccharum* (Barnes 1974). Successful breeding of these hybrids to incorporate important traits from their ancestors, with particular emphasis on high sucrose yield and vigorous growth, has been successfully achieved using traditional crossing and screening methods. However, it seems that the natural genetic potential for sucrose production has been exploited, and novel breeding approaches need to be implemented to further increase sucrose yield (Grof & Campbell 2001). Such approaches embrace biotechnological advances, such as marker assisted breeding and genetic transformation. Despite the fact that this is a multibillion dollar industry with a significant investment in research and development, our understanding of the regulation of the primary carbohydrate metabolism, which produces and accumulates sucrose in this plant, is incomplete (Moore 1995; Moore et al. 1998; Moore 2005; Rae et al. 2005).

Sucrose is accumulated in the culm tissues of sugarcane plants, with sucrose content increasing with internodal maturity (Rose & Botha 2000). Until recently, research of sugarcane culm metabolism has had major emphasis on the metabolites and enzymes directly involved in the synthesis and degradation of sucrose, and branches of metabolism that compete with it for carbon. These include levels of the abundant sugars (sucrose, glucose and fructose) in different internodes (reviewed in Moore & Maretzki 1997), and the allocation of carbon to other water soluble and insoluble fractions (Bindon & Botha 2002, Whittaker & Botha 1997). There is only one report of levels of some of the phosphorylated intermediates of glycolysis in the sugarcane culm (Whittaker & Botha 1997), leaving a large number of unquantified metabolites such as organic and amino acids, and less abundant sugars and sugar-phosphates. Studies of sugarcane enzymes are limited to a few that are directly linked to sucrose, including sucrose phosphate synthase (EC 2.4.1.14) (Zhu et al. 1997; Botha & Black 2000); sucrose synthase (EC 2.4.1.13) (Buczynski et al. 1993; Botha & Black 2000; Schäfer et al. 2004a; Schäfer et al. 2004b); the invertases (EC 3.2.1.26) (Zhu et al. 1997; Vorster & Botha 1998; Vorster & Botha 1999; Rose & Botha 2000; Ma et al. 2000; Bosch et al. 2004); and two enzymes from glycolysis: fructokinase (EC 2.7.1.4) (Hoepfner & Botha 2003; Hoepfner & Botha 2004) and PFP(EC 2.7.1.90) (Whittaker & Botha 1999). The application of transcriptomic approaches to the analysis of sugarcane metabolism led to a more intricate picture than had previously been constructed from specifically focussed biochemical investigations (Carson et al. 2002; Casu et al. 2003), and the realisation that the current understanding is very simplistic in light of the exceptionally complex metabolism that supports sucrose accumulation.

Transcript analysis of young and maturing internodes revealed that only 2.1 to 2.4 % of transcribed genes had putative identities with known genes, and of these approximately 4% with carbohydrate metabolising enzymes (Casu et al. 2003). These transcripts with putative identities as carbohydrate metabolising gene sequences included enzymes of sucrose synthesis and catalysis; central hexose, pentose and triose phosphate metabolism; sugar transport; polysaccharide synthesis and catalysis; and other carbohydrate metabolism pathways. These findings were intriguing as the sampled tissues are characterised by high sucrose levels, yet comparatively few transcribed genes coded for enzymes directly involved in reactions with the disaccharide. Among these transcripts seemingly "unrelated" to sucrose, were two coding for the trehalose synthesising enzymes trehalose-6-phosphate synthase (EC 2.4.1.15, TPS), and trehalose-6-phosphate phosphatase (EC 3.2.1.28, TPP). This was surprising, as to date there are no literature references reporting the presence of

their biosynthetic product, trehalose, in sugarcane. Information available about sugarcane trehalose metabolism is limited to the above mentioned transcriptomics studies, and the characterisation of trehalase (EC 3.2.1.28), the enzyme responsible for the degradation of trehalose (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980).

The plant kingdom can be divided into two categories with regard to trehalose metabolism. The first are those plants that preferentially store trehalose (Adams et al. 1990; Drennan et al. 1993), and these are the exception. The second category includes plants whose trehalose levels are typically several orders of magnitude lower than other abundant sugars (Muller et al. 1995; Muller et al. 2001). It is assumed that sugarcane falls into the second category as its most abundant sugars are sucrose, glucose and fructose (Moore 1995). *Arabidopsis thaliana* is another plant that is a part of the second category. *A. thaliana* is a model organism for molecular plant physiology, and more information regarding trehalose metabolism is available for this species than any other in the plant kingdom. Striking observations from *A. thaliana* research have included the presence of 11 genomic copies of TPS, and 8 of TPP (Leyman et al. 2001); an absolute necessity of one of the isoforms of TPS for embryo development, normal vegetative growth and transition to flowering (Eastmond et al. 2002; Schluepmann et al. 2003; Schluepmann et al. 2004); and alterations in carbon partitioning due to increased *in vivo* trehalose-6-phosphate levels (Schluepmann et al. 2004).

These findings, in addition to the well defined roles of trehalose in growth, stress metabolism and carbon partitioning in yeast and bacteria renewed interest in the potential role of trehalose metabolism in sugarcane.

The aims of this thesis are fourfold:

- Firstly, the identification and quantification of trehalose in sugarcane, and its context in the internodal storage parenchyma metabolome.
- Secondly, identification of the machinery (transcripts and enzymes) required for trehalose synthesis and degradation, and investigation as to whether they are differentially expressed in sugarcane genotypes with different sucrose accumulation phenotypes.
- Thirdly, the description of the kinetic parameters governing the enzymes that catalyse the reactions that both produce and consume trehalose in sugarcane.
- And finally, the integration of the trehalose pathway into cytosolic sucrose metabolism with the purpose of determining control on steady-state flux and metabolite concentrations, with particular reference to sucrose accumulation.

1.2. Layout and aims of the following chapters

CHAPTER 2 : LITERATURE REVIEW

TREHALOSE: Carbon source, stress protectant or the missing link in the regulation of carbon partitioning?

Aims: Firstly, the literature available on trehalose metabolism in yeast and bacteria will be reviewed as a background to what has been reported in plant systems. Secondly, a discussion platform from reported observations of plant trehalose metabolism will be created from which sugarcane trehalose metabolism will be explored in the experimental chapters. Finally, developments in the approaches to describing and quantifying biological systems and their components in plant metabolism will be reviewed. These include the use of high-throughput technologies, bioinformatic statistical analyses and *in silico* modelling, which form an integral part of this thesis.

CHAPTER 3

The Metabolic Context of Trehalose in sugarcane

Aims: Trehalose will be identified and quantified from internodal tissues of five sugarcane varieties, and expressed in the context of the complete metabolome of two of these genotypes. The steady-state trehalose levels determined in this Chapter will be used for the validation of the kinetic model in Chapter 6. Potential roles for the intermediates of trehalose metabolism are suggested.

CHAPTER 4

Expression of transcripts and enzyme activities involved in sugarcane trehalose metabolism

Aim: Transcripts for TPS, TPP and trehalase are to be isolated, sequenced and their levels profiled in internodal tissues of different maturities and genotypes. Enzyme activities for all three trehalose metabolising enzymes will be determined. Reasons for the potential differential expression of transcript and enzyme activity levels will be discussed. Enzyme activities from this Chapter will be used in the construction of the *in silico* kinetic model in Chapter 6.

CHAPTER 5

Partial purification and kinetic characterisation of sugarcane TPP and trehalase

Aim: Complexity of the sugarcane trehalose pathway components will be investigated with regard to isoform abundance of TPP and trehalase. Kinetic characterisation of sugarcane TPP and trehalase will form part of the parameter complement required for the construction of the kinetic model in Chapter 6.

CHAPTER 6

Metabolic control analysis of sugarcane sucrose and trehalose metabolism

Aim: Data from all previous experimental chapters will be synthesised with the use of mathematical kinetic modelling. Two models are envisaged: one describing the linear trehalose pathway, and the other its integration into the current cytosolic sucrose metabolism model. Metabolic control analysis of the expanded model will be used to determine the control of the trehalose branch on sucrose metabolism.

CHAPTER 7 : GENERAL DISCUSSION

Aim: Observations and discussions from the previous six chapters will be examined in context with one another. Potential roles for trehalose metabolism in the sugarcane culm will be suggested, and future research on the topic proposed.

1.3. References

Adams RP, Kendal E, Kartha KK (1990) Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. Food and Chemical Toxicology 40: 871-898

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

Barnes AC (1974) The sugarcane. Leonard Hill Books, London, UK

Bindon K, Botha FC (2002) Carbon allocation to the insoluble fraction, respiration and triose-phosphate cycling in the sugarcane culm. Physiologia Plantarum 116: 12-19

Bosch S, Grof CPL, Botha FC (2004) Expression of neutral invertase in sugarcane. Plant Science 166: 1125-1133

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Australian Journal of Plant Physiology 27: 81-85

Buczynski SR, thom M, Chourey P, Maretzki A (1993) Tissue Distribution and Characterization of Sucrose Synthase Isozymes in Sugarcane. Journal of Plant Physiology 142: 641-646

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Drennan PM, Smith MT, Goldsworthy D, Van Staden J (1993) The Occurence of Trehalose in the Leaves of the Desiccation-Tolerant Angiosperm *Myrothamus flabelliofolius* Wele. Journal of Plant Physiology 142: 493-496

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Grof CPL, Campbell JA (2001) Sugarcane sucrose metabolism: scope for molecular manipulation. Australian Journal of Agricultural Research 28: 1-12

Hoepfner SW, Botha FC (2003) Expression of fructokinase isoforms in the sugarcane culm. Plant Physiology and Biochemistry 41: 741-747

Hoepfner SW, Botha FC (2004) Purification and characterisation of fructokinase from the culm of sugarcane. Plant Science 167: 645-654

Leyman B, Van Dijk P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends in Plant Science 6: 510-513

Ma H, Albert HH, Paull R, Moore PH (2000) Metabolic engineering of invertase activities in different subcellular compartments affects sucrose accumulation in sugarcane cells. Australian Journal of Plant Physiology 27: 1021-1030

Moore PH (1995) Temporal and Spatial Regulation of Sucrose Accumulation in the Sugarcane Stem. Australian Journal of Plant Physiology 22: 661-679

Moore PH (2005) Integration of sucrose accumulation processes across hierarchical scales: towards developing an understanding of the gene-to-crop continuum. Field Crops Research 92: 119-135

Moore PH, Botha FC, Furbank RT, Grof CPL (1998) Potential for overcoming physio-biochemical limits of sucrose accumulation. In BA Keating, JR Wilson, eds Intensive

sugarcane production: meeting ths challenges beyond 2000. CAB International, New York, pp 141-155

Moore PH, Maretzki A (1997) Sugarcane. In E Zamski, AA Schaffer, eds Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships, Ed. 2. Marcel Dekker Inc., New York, pp 643-669

Muller J, Aeschbacher RA, Wingler A, Boller T, Wiemkien A (2001) Trehalose and Trehalase in Arabidopsis. Plant Physiology 125: 1086-1093

Muller J, Boller T, Wiemkien A (1995) Trehalose and trehalase in plants. Plant Science 112: 1-9

Rae AL, Grof CPL, Casu RE, Bonnet GD (2005) Sucrose accumulation in the sugarcane stem: pathways and control points for transport and compartmentation. Field Crops Research 168

Rose S, Botha FC (2000) Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. Plant Physiology and Biochemistry 38: 819-824

Schäfer WE, Rohwer JM, Botha FC (2004a) A kinetic study of sugarcane sucrose synthase. European Journal of Biochemistry 271: 3971-3977

Schäfer WE, Rohwer JM, Botha FC (2004b) Partial purification and characterization of sucrose synthase in sugarcane. Journal of Plant Physiology

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Schluepmann H, van Dijken AJH, Aghdasi M, Wobbes B, Paul MJ, Smeekens SC (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate accumulation. Plant Physiology 135: 879-890

Vorster DJ, Botha FC (1998) Partial Purification and Characterisation of Sugarcane Neutral Invertase. Phytochemistry 49: 651-655

Vorster DJ, Botha FC (1999) Sugarcane Internodal Invertases and Tissue Maturity. Plant Physiology 155: 470-477

Whittaker A, Botha FC (1997) Carbon Partitioning during Sucrose Accumulation in Sugarcane Internodal Tissue. Plant Physiology 115 : 1651-1659

Whittaker A, Botha FC (1999) Pryophosphate:D-frucotse-6-phosphate 1-phosphotransferase activity patterns in relation to sucrose storage across sugarcane varieties. Physiologia Plantarum 107: 379-386

Zhu YJ, Komor E, Moore PH (1997) Sucrose Accumulation in the Sugarcane Stem Is regulated by the Difference between the Activities of Soluble Acid Invertase and Sucrose Phosphate Synthase. Plant Physiology 115: 609-616

CHAPTER 2
LITERATURE REVIEW

TREHALOSE:
Carbon source, stress protectant or the missing link in the regulation of carbon partitioning?

2.1. Introduction

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a non reducing sugar consisting of two glucose units bound by an α:1→1 linkage. It is heat and acid stable and its high hydrophilicity makes it an ideal substance increasing the hardiness of biological systems. Since trehalose was first identified in a living system, it has been isolated from at least 80 species including plants, algae, fungi, yeasts, bacteria, insects and other invertebrates (Elbein 1974). In addition to its proposed roles as carbon source and stress protectant, the 6-phosphate derivative (trehalose-6-phosphate) has been implicated in the regulation of carbon partitioning in yeast. This regulation is achieved through the control of carbon influx into glycolysis, by the inhibition of hexokinases and other yet undefined mechanisms (Gancedo & Flores 2004).

The two-step synthesis of trehalose, via the enzymes trehalose-6-phosphate synthase (EC 2.4.1.15 TPS) and trehalose-6-phosphate phosphatase (EC 3.1.3.12 TPP), is ubiquitous to most living systems and regarded as the major route of trehalose production. Current knowledge of plant trehalose metabolism limits the synthesis of trehalose to these two enzymes, and its hydrolysis by the enzyme trehalase (EC 3.2.1.28).

As a background to discussing the potential role(s) of trehalose metabolism in plants, and specifically sugarcane, we will first cover relevant aspects in yeast and bacteria. Technical and analytical challenges facing the study of trehalose metabolism in plants are also presented.

2.2. Trehalose metabolism in prokaryotes and simple eukaryotes

In prokaryotic organisms trehalose can serve as an external carbon source, compatible solute, structural component, metabolic intermediate and regulator via its route of synthesis and degradation (Arguelles 2000 and references there in).

Many species of bacteria and yeast have the ability to utilise trehalose as their sole carbon source. The system of trehalose uptake and metabolism has been well studied in many organisms including the "model" prokaryote *Escherichia coli*, and eukaryote *Saccharomyces cerevisiae*. Genes coding for enzymes that externally hydrolyse, transport, and regulate trehalose synthesis have been identified in both organisms. Much of the system has been characterised on enzyme level too, and this will be discussed here.

2.2.1. Function of trehalose in bacteria and yeast

Trehalose acts as an osmosticum in the cyanobacterium, *Desulfovibirio halophilus* and several purple sulfur and nonsulfur bacteria (Arguelles 2000). Apart from this the V_{max} of trehalose synthetic enzymes in these organisms are salt-dependent at an optimum of 100-300 mM NaCl, indicating that under stress conditions trehalose accumulation is stimulated (reaching levels up to 400 mM) (Howells et al. 2002). Increasing the concentration of trehalose in response to stress conditions in both *E. coli* and *S. cerevisiae* could serve to maintain protein and membrane stability under adverse conditions (Strom & Kaasen 1993). In unfavourable growth conditions the synthesis of trehalose has been shown to be essential for the survival of the pathogenic bacterium *Salmonella enterica* serovar *typhimurium*, allowing its survival in environments like soil and partially cooked foods (Howells et al. 2002).

Apart from being a carbon source and compatible solute, trehalose is accumulated as a reserve compound in yeast (Arguelles 2000).

The function of the trehalose molecule (which should be distinguished from flux through its synthetic pathway, or levels of this pathway's intermediates) as a carbon source or stress protectant in yeast and bacteria appears to be linked to significantly its high steady-state levels. With the exception of a few isolated cases, plants cannot be so dependent on trehalose for these functions for at least two reasons, both involving distinct metabolic pathways. Firstly, plants are photoautotrophic, a fact that excludes any other molecule except carbon dioxide from being the major carbon source (unless the plants are grown *in*

vitro, but this is then not an historical biological function for trehalose, but rather a biotechnological one). Secondly, the overwhelming majority of plants accumulate either sucrose or starch as reserve carbohydrates and are geared to mobilising either one when the need arises, making the use of trehalose as a storage reserve obsolete.

2.2.2. Routes of trehalose synthesis and degradation in bacteria and yeast

In both bacteria and yeast trehalose-6-phosphate synthase (EC 2.4.1.15 TPS) and trehalose-6-phosphate phosphatase (EC 3.1.3.12 TPP) are responsible for the two step synthesis of trehalose from UDP-glucose and glucose-6-phosphate (figure 2.1., shaded route). This route of trehalose formation is the only one that has been identified in the plant kingdom to date. Although this is the most well studied route of trehalose synthesis across species and kingdom borders, many others have been identified in bacteria and these will be discussed below.

As mentioned above trehalose metabolism plays a central role in the life of bacteria. In pathogenic bacteria such as *Mycobaceterium tuberculosis* the occurrence of multi-drug resistant strains has prompted research looking for suitable targets for pharmaceutical treatments. In this particular organism trehalose is an abundant cytosolic sugar as well as a significant component of its antigenic cord factors. As a result the routes of trehalose metabolism have been investigated in the search for effective pharmaceutical targets (De Smet et al. 2000). To this aim three pathways of trehalose synthesis have been identified (figure 2.1.). In most bacteria only one of the three routes described below have been identified on both gene and protein level, making *M. tuberculosis* unique in the regard that it expresses all three. The first is the classic TPS-TPP pathway, in bacteria named after the genes OtsA-OtsB first isolated from *E. coli*, and uses glucose-6-phosphate and UDP-glucose as substrates, as mentioned above. The second synthetic route is termed the TreY-TreZ pathway and uses the enzymes maltooligosyltrehalose synthase (EC 5.4.99.15 MOTs), and maltooligosyltrehalohydrolase (EC 3.2.1.141 MOTh) to produce trehalose units from glycogen. The third pathway utilises a mutase activity called trehalose synthase (EC 5.4.99.16 TS) that makes trehalose from maltose. In a separate study of *M. tuberculosis* TPS revealed that the highest activity was achieved using ADP-glucose, with decreasing rates using GDP-glucose, UDP-glucose, CDP-glucose and TDP-glucose under standard assay conditions (Pan et al. 2005). The enzyme was specific for glucose-6-phosphate as the second substrate and no activity was observed using mannose-6-phosphate, fructose-6-

phosphate or glucosamine-6-phosphate. This shows that the *M. tuberculosis* TPS is specific for its hexose-phosphate donor, and non-specific for the nucleoside diphosphate glucose donor. Such studies of the specificity of plant TPSs have not been carried out to date. In fact, no studies of the specificity of sucrose-phosphate-synthase (SPS EC 2.4.1.14) have been reported showing specificity for its hexose-phosphate substrate, thus not excluding the possibility that in plants this enzyme could partially fulfil the role of TPS.

A further route of synthesis has been identified in the mushroom *Agaricus bisporus.* The enzyme trehalose phosphorylase (EC 2.4.1.64 TP) converts trehalose and phosphate to glucose and glucose-1-phosphate in a reversible reaction (Wannet et al. 1998). TPS is a homotetramer with a total molecular weight of 240 kDa. Experimental evidence shows that the enzyme can operate in both directions *in vivo*, and that it could maintain the appropriate concentrations of its substrates and products in the cell.

Trehalose degradation is facilitated by various enzymes in bacteria. These include phosphotrehalase (EC 3.2.1.93), cytoplasmic trehalase (EC 3.2.1.28) and cytoplasmic hydrolase (Arguelles 2000).

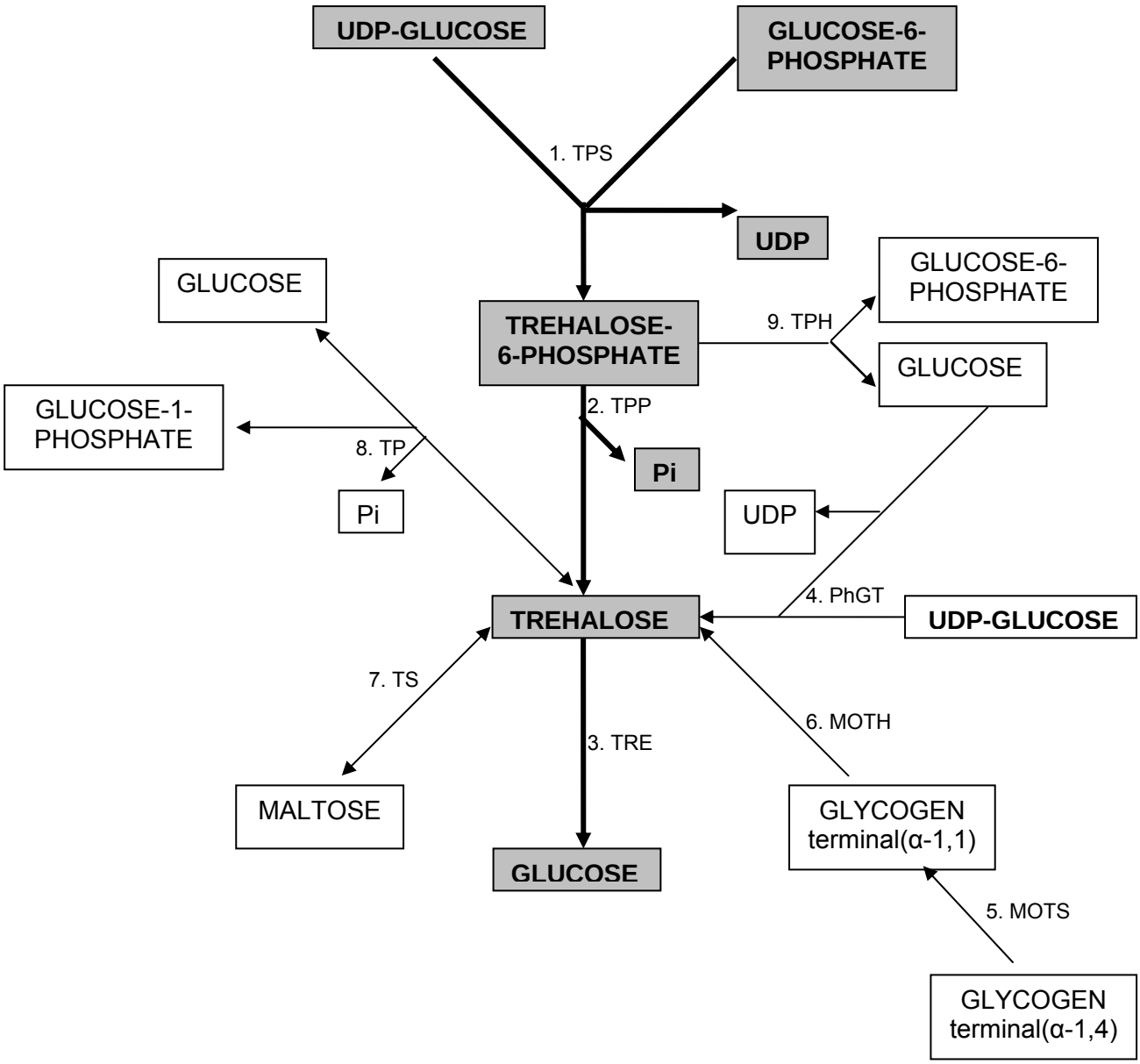


Figure 2.1. Routes of trehalose synthesis and breakdown identified in bacteria and yeast. The metabolic pathway through 1.TPS (trehalose-6-phosphate synthase), 2. TPP (trehalose-6-phosphate phosphatase) and 3. TRE (trehalase) is common to bacteria and yeast. 4. PhGT (*Pyrococcus horikoshii* glucosyltransferase) (Ryu et al. 2005). 5. MOTs (maltooligosyltrehalose synthase), and 6. MOTh (maltooligosyltrehalose hydrolase) only identified in bacteria (De Smet et al. 2000). 7. TS (trehalose synthase) isolated from bacteria (De Smet et al. 2000). 8. TP (trehalose phosphorylase) isolated from *Agaricus bisporus* (Wannet et al. 1998). 9. TPH (trehalose-6-phosphate hydrolase) characterised in bacteria (Rimmele & Boos 1994).

2.2.3. Trehalose metabolism and carbon partitioning in yeast

The biosynthesis of trehalose has been well characterized in yeast. Trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2), as well as two regulatory proteins (Tsl1 and Tsl3) have been cloned and described (Arguelles 2000). The enzymes form a multi-subunit aggregation that can be partially purified together (Londesborough & Vuorio 1993). The regulation of the complex is complicated because the catalytic subunits are also independently and differentially regulated by both phosphate and fructose-6-phosphate at physiological concentrations (see table 2.1.). Experimental evidence shows that the glucose-6-phosphate and fructose-6-phosphate pools are maintained at equilibrium with the isomerase reaction having an *in vitro* K_{eq} of 0.509 in the direction of fructose-6-phosphate formation (Kruger 1997). This means that there is always a supply of at least the glucose-6-phosphate as one of the substrates required for trehalose-6-phosphate synthesis, as well as the presence of the activator, fructose-6-phosphate.

Tps1 in yeast is activated by fructose-6-phosphate (F6P) and inhibited by free inorganic phosphate (Pi) (table 2.1.). Deletion of both regulatory proteins Tsl1 and Tps3 caused a 50% reduction in activity and relieved the phosphate inhibition, actually causing the opposite effect of activation (Bell et al. 1998). It has been suggested that Tps1 may actually operate in the cell separately from the complex. In this way it can be directly and differentially regulated by changing intra-cellular conditions.

At least four Tps1 mutants have added to our understanding of trehalose metabolism in yeast: *fdp1, cif1, byp1* and *glc6* (Van de poll et al. 1974):(Navon et al. 1979; Breitenbach-Schmitt et al. 1984; Cannon et al. 1994; Gancedo & Flores 2004) review that synthesised the data). These mutants were independently produced and later shown to have alterations in the same gene. Many of them did however, show similar metabolic permutations including the inability to grow on glucose and sporulate less efficiently than wild-type *Saccharomyces cerevisiae*. The first two mutants (*fdp1* and *cif*) (Van de poll et al. 1974; Navon et al. 1979) both demonstrated a significant increase in hexose-phosphate and a decrease in ATP levels in response to addition of glucose to a non-fermentable carbon source medium. This led to the conclusion that the product of this mutated gene was somehow involved in regulating the amount of carbon flux into glycolysis, or between the energy consuming and producing reactions of glycolysis.

By decreasing the amount of hexoses imported into the cell (by *CAT3* mutation) (Blazquez & Gancedo 1995), the *tps1* mutant was once again able to grow on glucose as its sole carbon source. The next committed step in the metabolism of glucose is the ATP dependent step catalysed by hexokinases. By deleting the most abundant form of this enzyme, *HXK2*, in the *tps1* mutant the lack of regulation was once again restored allowing the *tps1* mutant to grow on glucose (Hohmann et al. 1993). Both the *CAT3* and *HXK2* mutations in *tps1* show that restricting the amount of carbon flux into the cell restored the glycolytic balance to an extent that it could function normally. By making *tps1* mutants that also had the ability to stimulate the energy producing reactions of glycolysis by providing Pi and reductant through overexpression of the glycerol production and export pathways, growth was restored on glucose.

All of the above experiments contributed to a cohesive story: that the *tps1* mutant in yeast could not regulate flux through glycolysis when grown on glucose, by relieving the effect of a yet unknown glycolytic suppressor.

Because the product of TPS is trehalose-6-phosphate and the next metabolic step leads to trehalose it was hypothesised that either one or both could be responsible for regulating a key step in glucose uptake, signalling or glycolysis (Blazquez et al. 1993; Gancedo & Flores 2004). Because *tps1* mutants can grow on galactose, it was thought that the regulation could therefore only be at the level of glucose uptake, signalling or phosphorylation, as carbon from galactose enters glycolysis as glucose-6-phosphate. Trehalose-6-phosphate at micromolar levels competitively inhibits hexokinase II (Blazquez et al. 1993). Apparently the *tps1* mutant which lacked its product trehalose-6-phosphate, had no regulation on hexokinase and the ATP used by this run-away activity out-stripped its production in the energy-producing reactions of glycolysis causing a “burn-out”, lethal phenotype. However, there is still one as yet inexplicable phenomenon of the *tps1* mutant: the mutant accumulates high levels of hexose phosphates when supplied with glucose (Thevelein & Hohmann 1995), suggesting that the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase is not sufficiently activated to complete glycolytic fermentation. Although this is an attractive theory there is no experimental evidence to link Tps1 or its product trehalose-6-phosphate to the function of activation of G3P dehydrogenase.

Across yeast and fungal species there is no consistent trend in phenotypic characteristics of *tps1* mutants. Such mutants have been generated in *Kluyveromyces lactis*, *Yarrowia lypitica, Hansenula polymorpha, Aspergillus niger Aspergillus nidulans, Schizosaccharomyces*

pombe, Candida albicans, Cryptococcus neoformans and Magnaporthe grisae (Gancedo & Flores 2004). Although these mutants have not been fully investigated it would appear that those that relied on a high flux through glycolysis had hexokinase isoforms sensitive to trehalose-6-phosphate (*K. lactis, Y. lipitica, A. niger* and *A. nidulans*). However, this sensitivity does not always lead to the same phenotype observed in the *S. cerevisiae tps1* mutants.

It is evident that our understanding of the function of Tps1 and its product trehalose-6-phosphate is incomplete, and that it would appear that we are missing a crucial point of control other than hexokinase in the flow of carbon in yeast cells.

In times of slow growth caused by stress conditions, *S. cerevisiae* accumulates trehalose (Hottiger et al. 1987b). Under normal growth conditions trehalose does not function as a carbon source in yeast as the amount of glucose resulting from trehalase activity is not enough to account for the catabolic activities. Tps1 is activated by dephosphorylation which may occur under stress conditions when protein phosphatases are activated (Shin et al. 1987; Hottiger et al. 1987b). This reaction to stress phenomena allows the production of varying amounts of trehalose-6-phosphate which in turn are able to the regulate carbon flux accordingly. Not only this, but increasing TPS activity increases a cycle of carbon from the hexose phosphate pool to the glucose pool via the degradative activity of trehalase. It has been suggested that the trehalose pathway maintains cytosolic glucose levels in times of stress (Hottiger et al. 1987b).

Further insight into properties of trehalose biosynthesis was gained from a few studies that have kinetically characterized TPS and TPP enzymes in yeast and bacteria (table 2.1.). The inconsistency of *S. cerevisiae* TPS kinetic parameters between studies is attributed to the assay methods (Vandercammen et al. 1989). A comparison of TPS assay methods showed that the most accurate results were generated from using UDP-D-[U-^{14}C]-glucose and detecting label in trehalose-6-phosphate and trehalose over time (Vandercammen et al. 1989). Kinetic parameters for TPS from the latter study will be incorporated into the kinetic model in Chapter 6.

Table 2.1. Kinetic parameters of trehalose synthesising enzymes

		K_m (mM)				
	Organism	***TPS_{G6P}***	***$TPS_{UDP\text{-}glc}$***	***TPP_{T6P}***	***Comments***	***Ref[§]***
1	*S. cerevisiae*	28	1.3		TPS activity activated by F6P TPP activity inhibited by Pi High levels of F6P can overcome Pi inhibition. Tps1 subunit 56 kDa Tps2 (TPP) subunit 102 kDa	A
2	*S. cerevisiae*	3.5	0.5	0.5	Pi inhibition of TPS activity 50% inhibition of TPS by >1 mM UDP or UTP	B
3	*Mycobacterium tuberculosis*	7	18		Activity of recombinant TPS used nucleoside diphosphate donors ADP-glc, CDP-glc, GDP-glc, TDP-glc and UDP-glc TPS activity activated by the addition of heparin	C
4	*E.coli*	3.7	8.6	2.5	Recombinant TPS from OtsA	D

[§] A : (Londesborough & Vuorio 1993), B (Vandercammen et al. 1989), C (Pan et al. 2005), D (Seo et al. 2000)

In order for trehalose to be assimilated from storage into catabolic metabolism it needs to be mobilised into a fermentable sugar. In baker's yeast trehalose is cleaved to its two glucose moieties by the enzyme trehalase (Londesborough & Varimo 1984). Two isoforms have been identified operating optimally *in vitro* at either neutral or acid pH conditions. Similar properties of plant trehalases have been characterised and will be discussed in Chapter 5.

Having reviewed the function and regulation of trehalose and its metabolism in yeast and bacteria, we now discuss current knowledge about this disaccharide in plants.

2.3. Trehalose in plants

2.3.1. Exploring the functions of trehalose in plants

Historically it was believed that trehalose was not present in most plant species. However, with more sensitive detection methods developed in the recent times the disaccharide has been identified in many species of plants investigated (Roessner et al. 2000; Vogel et al. 2001; Muller et al. 2001a; Muller et al. 2001b; Eastmond et al. 2002; Roessner-Tunali et al. 2003), including sugarcane (Chapter 3). Along with metabolomics advancements, the presence and role of trehalose in plants came under the spotlight after many EST programs identified partial cDNAs with high homology to the enzymes of trehalose metabolism from yeast and bacteria. Such ESTs were isolated from sugarcane genomics projects and are discussed in Chapter 4. Because of the apparent association of trehalose metabolism especially the ability to improve desiccation tolerance and act as a stabilising agent in yeast (Arguelles 2000), trehalose metabolism in plants became a topic of interest in plant biotechnology. The manipulation of trehalose metabolism could potentially increase drought tolerance in crops, or increase stability of other alternate commercial products produced in plants when dried or frozen. Apart from its potential direct commercial importance the possibility exists that flux through the trehalose pathway could have an effect on the regulation of carbon metabolism, as it has been shown in yeast that the disaccharide controls the influx of sugars into glycolysis (Goddijn & van Dunn 1999).

The background of trehalose metabolism in yeast and bacteria has been established in the discussion above. We will now explore the likelihood that trehalose metabolism is involved as carbon source, stress protectant or a regulator of carbon partitioning in plants, with particular interest in its potential role in sugarcane.

2.3.1.1. Carbon source

As discussed in section 2.2.1., it is unlikely that trehalose could act as a carbon source in plants because of their photoautotrophic nature, and preferential storage of carbon as sucrose or starch. *In vitro* culture of plants on trehalose as the sole carbon source has led to several phenotypic deviations that make such an application of this disaccharide unsuitable. Initial experiments aimed at elucidating the role of trehalose in plant metabolism examined the effects of growing plants on media either containing trehalose or the potent trehalase inhibitor validamycin A (Wingler et al. 2000; Fritzius et al. 2001; Muller et al. 2001a). Growth

of *A. thaliana* seedlings on trehalose inhibited root elongation and seedling growth (Wingler et al. 2000). These phenotypic effects were reversed by transferring the plants to media containing sucrose and/or glucose even in the presence of trehalose. In a separate experiment *A. thaliana* plants were grown in the presence of 30 mM trehalose and a significant increase in non-structural carbohydrates (sucrose and starch being the most noticeable) was observed (Bae et al. 2005). The increase in starch has been attributed to posttranslational redox activation of ADP-glucose pyrophosphorylase (Kolbe et al. 2005). Proteins extracted from the same tissues were subjected to 2D gel electrophoresis. Those that showed differential expression from the control treatment were digested and sequenced, revealing upregulation of proteins involved in stress response and detoxification. So rather than acting as a carbon source, increasing exogenous trehalose levels increased endogenous trehalose levels and appeared to stress the plants. The presence of exogenous trehalose, whether from pathogen attack or some other source, and its seemingly toxic effects on plants could explain why large amounts of trehalase were found in the media of soybean cultures (Muller et al. 1992).

Another method of increasing intracellular trehalose levels was the introduction of bacterial genes responsible for trehalose synthesis. Trehalose levels were increased in potato and tobacco transformed with bacterial trehalose synthetic genes (Goddijn et al. 1997). A correlation between the presence of the bacterial cytosolic trehalose synthetic genes, the accumulation of trehalose and stunted growth was observed in these plants. Trehalose was not detected in the control plants but was detectable between 0.02 and 0.11 $mg.g^{-1}FW$ (53 to 290 $nmol.g^{-1}FW$) in transgenic lines containing only the TPS transgene. In tobacco lines harbouring both the TPS and TPP bacterial genes trehalose levels were increased. Some of the potato plants with the dual transformation produced no tubers. It is not known how specific TPP is to its substrate and it could be possible that tuber development could have been inhibited by a pleiotropic effect not directly linked to trehalose itself. It would appear that trehalose has detrimental effects on normal growth and development. The ubiquitous presence of trehalase in plant tissues may be a protection against pathogenic introduction of trehalose, or *in planta* accumulation by endogenous genes.

With the observation that trehalose has an effect on normal root development and induces shoot starch accumulation, it has been suggested that endogenous plant trehalose could act as a signal molecule in the control of plant metabolism and growth (Vogel et al. 2001), and not as a storage carbohydrate or source of carbon. Both trehalose and trehalose-6-phosphate have been hypothesised as agents of carbon signalling and regulation.

2.3.1.2. Trehalose and stress protection in plants: drought resistance

The presence of trehalose has been found to correlate with an organism or tissue's dessiccation tolerance across kingdom and species boundaries (Hottiger et al. 1987a; Adams et al. 1990; Drennan et al. 1993; Garg et al. 2002; Chen & Murata 2002; Avonce et al. 2004; El-Bashiti et al. 2005; Cortina & Culianez-Macia 2005). There appear to be at least two distinct characteristics of these systems. The first is observed in those organisms that accumulate trehalose as a compatible solute to concentrations that bring about physical protection of membranes and cellular components. This group includes yeasts like *S. cerevisiae* (Hottiger et al. 1987a), and the resurrection plants *Myrothamnus flabellifolius* (Drennan et al. 1993) and *Selaginella lepidophylla* (Adams et al. 1990). The second group acquires drought tolerance by some other pleiotropic effect of upregulation of flux through the trehalose synthesis pathway. This trait has been exploited in agricultural research in an effort to produce plants with the ability to grow in conditions of suboptimal water supply.

Transgenic rice plants expressing a fusion gene of *E.coli TPS* and *TPP* (*OtsA-OtsB*) under control of either tissue-specific or stress-responsive promoters, accumulated trehalose to levels well below 1 $mg.g^{-1}$ FW, yet recovered completely after two cycles of 100h drought stress and subsequent watering for 3 weeks (Garg et al. 2002), while control plants did not. Even though these trehalose levels were relatively low in comparison with other abundant solutes, they were still significantly higher than wild type plants indicating that the flux through the trehalose pathway was greatly increased. A concurrent increase in soluble carbohydrates was observed in these drought tolerant plants, potentially enhancing the compatible solute theory. Apart from this an increased photosynthetic ability under normal and adverse conditions supports the theory that some component of trehalose metabolism is involved in regulatory and signalling aspects of carbon metabolism.

Besides drought tolerance, introduction of the OtsA-OtsB gene construct conferred resistance to salt stress. Similar effects were observed when an OtsA-OtsB construct fused to the maize (*Zea mays)* ubiquitin promoter was expressed in rice (Jang et al. 2003). The observations were again reflected in a study in tomato *Lycopersicon esculentum* transformed with the *S. cerevisiae TPS1* gene under control of the CaMV35S promoter (Cortina & Culianez-Macia 2005). The use of this promoter meant that the gene was constitutively expressed, causing a severe growth phenotype. However, the plants did display improved tolerance to drought, salt and oxidative stress. As observed in *A. thaliana* plants with increased trehalose levels (Wingler et al. 2000), these tomato plants had increased starch

levels. Introduction of genes to alter trehalose metabolism in tobacco resulted in a higher threshold of stress tolerance, but in most cases this was accompanied by severe phenotypic changes (Holstrom et al. 1996; Pilon-Smits et al. 1998; Zhao et al. 2000). The reason for the increased tolerance observed in all these transgenic lines is still unclear.

Analysis of stress tolerant and sensitive varieties of *Triticum aestivum* demonstrated the presence of trehalose in all three varieties investigated (El-Bashiti et al. 2005). However, under drought conditions, stress tolerant varieties accumulated at least 200% more trehalose in their roots, and 50% more in their leaves than the stress sensitive control. Although the authors claim to have measured higher TPS activity in the stress tolerant variety, their TPS assay procedure is not able to distinguish between TPS and sucrose-6-phosphate synthase (SPS). No reference is made to levels of sucrose or its synthesizing enzyme SPS and therefore the data are questionable. They do however report a reduction in trehalase activity under stress conditions in tolerant varieties. The extent of the reduction (a decrease of approximately 0.2 $\mu mol.min^{-1}.g^{-1}FW$, assuming the protein content is close to 3 $mg.g^{-1}FW$), equates to a decreased turnover of over 8000 µmol trehalose per gram FW in 10 days. The absolute change in trehalose is recorded around 5000 $\mu mol.g^{-1}FW$ over the same time period, and it is therefore evident that the change in trehalase activity alone could account for the increase in trehalose content. However, the highest recorded level of trehalose in this study was approximately 13 $nmol.g^{-1}$ FW (5000 $\mu g.g^{-1}FW$) which is insufficient to act as a compatible solute. In light of this, and the transgenic studies discussed in this section, it is clear that in plants dessiccation tolerance (stress protection) is not conferred by accumulating high levels of trehalose.

2.3.1.3. Trehalose metabolism in a signalling capacity

All the evidence discussed above points to the role of the trehalose pathway in plants (including its metabolites and proteins) as involved in regulation of metabolism in a signalling capacity. This is confirmed by the low steady-state levels of trehalose. Such levels have been measured at approximately 250 $nmol.g^{-1}DW$ in flowers, stems and root of *A. thaliana* plants grown under sterile conditions (Vogel et al. 2001; Muller et al. 2001a). These levels (between 10 and 100 times lower than the reported sucrose levels) suggest that the endogenous function of trehalose (or its precursor trehalose-6-phosphate) in most plants is probably not that of carbon source or stress protectant, but rather a signal molecule involved in metabolic regulation, causing alterations in carbon partitioning and dessiccation tolerance.

Taking the inhibition of hexokinase observed in *S. cerevisiae* into account, the first logical experiment was to determine trehalose-6-phosphate mediated inhibition on plant hexokinases. No inhibition was observed when *A. thaliana* hexokinase was assayed with glucose and up to 5 mM trehalose-6-phosphate (Eastmond et al. 2002). The possible implications of such an inhibition sugarcane hexokinase (including glucokinase and both forms of fructokinases) will be explored in Chapter 6.

Further investigation of the role of trehalose-6-phosphate levels in *A. thaliana* was achieved by introduction of trehalose metabolism genes from *E. coli*. *OtsA, OtsB* and *treC* encode for *E. coli* enzymes TPS, TPP and TPH (trehalose-6-phosphate hydrolase: producing glucose-6-phosphate and glucose from trehalose-6-phosphate, see figure 2.1.) respectively. Introduction of the first gene into *A. thaliana* increased trehalose-6-phosphate levels, and the latter two resulted in a decrease (Schluepmann et al. 2003). Accumulation of trehalose-6-phosphate was coincident with smaller, darker leaves: a reverse of the pale-leaf phenotype of plants with lower trehalose-6-phosphate levels. In addition to this, because the phenotypes of the plants transformed with *OtsB* and *treC* were the same and that TPP and TPH reactions have different products, it was thought that the effect is directly linked to the level of their substrate: trehalose-6-phosphate. Expression of transgenic trehalase resulted in no change in trehalose-6-phosphate levels, and these transgenics were phenotypically indistinguishable from the wild-type. Increasing sugar supply to plants with lower than normal trehalose-6-phosphate levels resulted in an accumulation of respiratory intermediates (including glucose-6-phosphate, fructose-6-phosphate and citrate). This reflects the same phenotype observed in yeast *tps1* mutants that have a fatal reaction to supply of glucose in the growth medium (Bonini et al. 2003). In plants whose trehalose-6-phosphate levels were higher than wild-types, addition of excessive levels of sugar supply improved growth (Schluepmann et al. 2003). As mentioned above hexokinases in plants have not been shown to be sensitive to trehalose-6-phosphate, leaving us with the possibility that the changes observed in visual phenotype and changes in carbon partitioning are caused by trehalose-6-phosphate mediated regulation on glycolytic respiration distinct from that observed in yeast.

With the most obvious regulatory effects of trehalose metabolism in plants explored, characterisation of the metabolism (including transcript and enzyme levels) was needed, and this is discussed below.

2.3.2. Enzymes of trehalose metabolism in plants: identification and their role in regulating plant growth and carbon partitioning

The biosynthesis of trehalose in plants has been suggested to occur in two steps. Trehalose-6-phosphate synthase (TPS) catalyses the synthesis of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate. Trehalose-6-phosphate is dephosphorylated by trehalose-6-phosphate phosphatase (TPP) rendering trehalose. Although bacteria have three enzymes of trehalose degradation, only one has been identified in plants. Trehalase hydrolyses trehalose to two glucose units. Trehalase is a glycoprotein with a predicted molecular weight of approximately 56 kDa (Aeschbacher et al. 1999), and has dual pH optima at both acid and neutral regions (Muller et al. 2001a). Significant trehalose activity in many plants whose levels of trehalose are undetectable (Glasziou & Gayler 1969; Muller et al. 2001a).

2.3.2.1. Trehalose-6-phosphate synthase (TPS)

With the sequencing of the *Arabidopsis thaliana* genome, the trehalose pathway has become the topic of much interest in this model plant's metabolism (Vogel et al. 1998; Blazquez et al. 1998; Vogel et al. 2001; Muller et al. 2001a). A putative full-length cDNA coding for TPS (*AtTPS1*, accession number Y08568) was isolated from *A. thaliana*, and its function verified by complementation of a yeast *tps1* mutant (Blazquez et al. 1998) and measurement of its *in vitro* activity in these yeast cells. Eleven potential isoforms of TPS in the *A. thaliana* genome were identified by mining the vast amount of available genomic data in this organism (Leyman et al. 2001). *AtTPS1* to *AtTPS4* appear to be authentic *TPS* genes when compared with other known sequences of *TPS* (class I TPS genes). The other 7 contain conserved TPP domains, and it has been postulated that they would be able to facilitate the dual activities of both TPS and TPP activities (class II TPS genes). However to date, this has not been proven experimentally.

Very little is known about the biochemical characteristics of plant TPS enzymes. Characterisation of the functional *A. thaliana* and *S. lepidophylla* TPS1 (accession number SLU96736) cDNAs resulted in proteins with molecular weights of 106 and 109 kDa respectively (Blazquez et al. 1998; Zentella et al. 1999). The *S. lepidophylla* TPS protein was partially purified with a native molecular weight of 440 kDa, suggesting its *in vivo* occurrence as a homotetramer (Valenzuela-Soto et al. 2004). It is not a glycosylated protein, and its *in vitro* pH optimum was determined as pH 7.0. *S. lepidophylla* TPS maximum

catalytic activity was observed at 50 mM K^+ and 5 mM $MgCl_2$. The enzyme preparation was active at low levels (<30% of activity with glucose-6-phosphate) with fructose-6-phosphate, glucose, sucrose, galactose or lactose in reaction with UDP-glucose, suggesting that the enzyme had low affinity for these substrates, or that the preparation was not fully purified. Activity with glucose-6-phosphate and UDP-glucose as substrates was doubled in the presence of 100 mM fructose-6-phosphate, 400 mM glucose or 400 mM fructose (Valenzuela-Soto et al. 2004). This could again indicate an activation of the enzyme or contamination with other enzymes, for example SPS.

Presumably because extractable TPS activity was below detection limit, or that sample sizes were too small from *A. thaliana,* further investigations centred around the only *AtTPS* whose functionality had been conclusively proven experimentally: *AtTPS1*. Truncation of *TPS1* cDNAs from *A. thaliana* and *Selaginella lepidophylla* caused 10 to 20 fold increase in activity, when expressed in the yeast *Δtps1* deletion mutant (Van Dijck et al. 2002). It is therefore evident that the functional cDNAs have regulatory regions that affect the catalytic activity of the enzyme. The transcript expression of *AtTPS1* is not organ specific and displays highest expression in leaf tissues when compared with stems, roots and flowers (Vogel et al. 2001). *AtTPS1* was expressed in levels 4 to 6-fold higher in developing seeds when compared with leaves and roots (Girke et al. 2000), perhaps indicating a function in reproduction and germination as opposed to normal growth metabolism.

Production of *A. thaliana tps1* mutants that have either disruption in the gene (Eastmond et al. 2002; van Dijken et al. 2004) or overexpression of the gene (Avonce et al. 2004), have provided a tool to investigate the importance of *AtTPS1* activity in the development and metabolism of this model plant.

Disruption of the *AtTPS1* gene in *A. thaliana* led to a loss in the ability of the embryo to store carbon reserves during initial development (Eastmond et al. 2002). At this stage of embryo development sucrose levels were significantly higher than in wild type plants, and it appears as if this gene perturbation has affected sucrose signalling. The mutation also caused embryo lethality. The phenotype was partially rescued by growth on media with low sucrose levels. Although this phenomenon is mirrored in the phenotype of *Saccharomyces cerevisiae tps1* deletion mutants, down regulation of the predominant hexokinase activity in these *AtTPS1* mutants did not rescue the embryo lethality. This was not surprising in that trehalose-6-phosphate that regulates hexokinase activity in yeast has no effect on its plant equivalents (Wiese et al. 1999; Eastmond et al. 2002). It is therefore apparent that the effect

of disruption of the *TPS1* gene in *A. thaliana* is not mediated in the same way that TPS regulates carbon flux into glycolysis in yeast. Rather, it is evident that either the *AtTPS1* protein itself, or its reaction product trehalose-6-phosphate, affects points of metabolic control other than the entry of carbon into glycolysis via hexokinase. Other potential enzymes whose activity affects the entry of carbon into glycolysis are the gluco- and fructokinases, invertase, sucrose synthase and UDP-glucose pyrophosphorylase. However, none of their activities are affected by trehalose-6-phosphate (Eastmond et al. 2002), ruling out the possibility that *AtTPS1*'s enzymatic product regulates carbon metabolism at these points.

In order to determine whether *AtTPS1* is necessary beyond embryo development the *tps1* mutant describe above was transformed with a chemically inducible *AtTPS1* construct (van Dijken et al. 2004). This allowed embryo development to occur normally under chemical stimulus. Vegetative growth of the mutant on inducer-free media led to reduced root growth, slower plantlet growth rate, and a lack of transition to flowering, proving that the gene is necessary beyond the embryo stage. Overexpression of *AtTPS1* in *A. thaliana* resulted in faster germination rates, once again confirming its role in embryo development (Avonce et al. 2004). These plants also displayed glucose and ABA insensitivity, implicating the gene once again in a sugar- or hormone-sensing role.

It is clear that TPS is essential for plant growth and development, the mechanisms that it uses are however not so evident. Evidence for the presence of *TPS* transcript and TPS activity from sugarcane will be presented in Chapter 4. The control of TPS on the flux through trehalose synthesis, and indeed that of the sugarcane sucrose metabolism will be discussed in Chapter 6.

2.3.2.2. Trehalose-6-phosphate phosphatase (TPP)

The dephosphorylation of trehalose-6-phosphate by TPP renders free trehalose. Much less research emphasis has been placed on this enzyme, presumably because of the assumption that TPS enzymes have a more specific character, and are potentially the rate-limiting step in trehalose-6-phosphate turnover *in planta.* Two TPP cDNAs from *Arabidopsis* have been characterised on a molecular level and their proteins expressed in yeast complementation studies (Vogel et al. 1998). Two transcripts were designated the names *AtTPPA* (accession number AA000926) and *AtTPPB* (accession number AA022625). Both of these genes are expressed in single copies in the *A. thaliana* genome. Highest transcript expression was

observed in young developing seedlings and developing flowers; mirroring a similar expression pattern of *AtTPS1* discussed above. In all plant tissues tested *AtTPPA* had higher transcript expression than AtTPPB. Both complemented the heat sensitive yeast *tps2* deletion mutant, producing trehalose, and were able to dephosphorylate both sucrose-6-phosphate and glucose-6-phosphate. A further *TPP* cDNA was isolated from tobacco (accession number AY570725) and also complemented the yeast *tps2* deletion mutant phenotype (Wang et al. 2004), although its activity was not determined in an enzyme assay.

In sugarcane a putative *TPP* EST has been isolated (Carson et al. 2002), and its sequence will be used to determine TPP transcript expression in sugarcane in Chapter 4. TPP activity has never been measured from sugarcane and will be discussed in the context of its transcript expression in Chapter 4. Further characterisation of the enzyme will be presented in Chapter 5.

2.3.2.3. Trehalase

Trehalase, an α-glucosidase that hydrolyses trehalose into two glucose units, has been described on molecular and activity distribution levels in *A. thaliana* (Muller et al. 2001a). Highest transcript expression and trehalase activity have been determined in flowering parts of mature plants and in green siliques. Inhibition of trehalase (using validamycin A) causes an increase in endogenous trehalose levels. In flowers, leaves and stems with increased trehalose levels, sucrose and starch concentrations were significantly higher than in control plants. These plants also showed impaired fruiting patterns with no seed production. Although the authors discuss the possibility that trehalose may be either a sensor or signal in regulating carbon allocation, they also mention the possibility that the validamycin A could have pleiotropic effects on metabolism that could cause the observed changes. Although trehalose was quantified and potentially implicated as an important metabolite, its precursor trehalose-6-phosphate cannot be ignored.

The soybean trehalase protein was isolated from nodule tissues and used to identify a cDNA coding for the activity (Aeschbacher et al. 1999). The protein has an approximate molecular weight of 66 kDa, and its cDNA has an ORF of 1674 bp. The gene was present in a single copy in the soybean genome, and its transcript most highly expressed in roots and nodules compared with leaves and flowers. *Phaseolus vulgaris* trehalase has a molecular weight of 45 kDa (Garcia et al. 2005), and a broad pH range between pH 3.6 and pH 6.6, and *in vitro* temperature optimum of 60°C. Using sucrose as substrate the enzyme displayed 70% of the

observed activity with trehalose, showing that it is not substrate specific. The enzyme had a K_m of 0.109 mM and a V_{max} of 5.11 nmol.min^{-1}.mg^{-1} protein.

Sugarcane trehalases were of the first plant trehalases described in the literature (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980). The enzyme has pH optimum of 6.2 and was therefore thought to be located in the cytosol (Glasziou & Gayler 1969). Activity was highest in immature stalk tissues and its function initially assigned to the assistance of carbohydrate transfer across the tonoplast. However, trehalose has never been detected in sugarcane tissues and the hypothesis that trehalase is linked to transport of soluble sugars could never be substantiated. Experimentally determined K_m values vary between 0.1 and 0.63 mM (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980). Due to variations in the kinetic data available, the partial purification and characterisation of sugarcane trehalase will be revisited in Chapter 5.

2.4. Practical challenges and limitations when investigating trehalose metabolism in sugarcane

2.4.1. Sugarcane: a dedicated sucrose storer

Central to the ability of plants to produce reduced carbon from CO_2 and to transport and store these carbohydrates is the synthesis of sucrose. This study focused on sugarcane, which is renowned for its ability to produce and store high levels of sucrose in the culm. SPS is the main sucrose synthesising activity in the sugarcane culm (Botha & Black 2000). Substrates for this enzyme are fructose-6-phosphate and UDP-glucose. UDP-glucose is the common substrate between SPS and TPS (figure 2.2.). Sugarcane also has significant levels of phosphoglucoisomerase (PGI). So when using UDP-glucose and glucose-6-phosphate as substrates for TPS in a crude enzyme preparation, interference by high levels of both PGI and SPS would outcompete TPS. In fact using the first technique described below (UDP couple) can give false results. In some studies no compensation or explanation has been given for this interference, calling into question the validity of the results (Valenzuela-Soto et al. 2004; El-Bashiti et al. 2005). Development of a technique for measuring TPS in plant extracts is central to the characterisation of trehalose metabolism in plants.

Accurately measuring the activity of TPS and TPP has presented many practical challenges. In plants this is further confounded by potentially low levels of TPS and TPP, coupled with high levels of PGI. The predominant assay for TPS activity (UDP-glucose + glucose-6-phosphate → trehalose-6-phosphate + UDP), is by coupling the production of UDP to the utilisation of NADH (via pyruvate kinase and lactate dehydrogenase, with the addition of phosphoenolpyruvate and NADH as substrates) and its consequent decrease in spectrophotometric absorbance at 340 nm. In crude extracts this method is unreliable (Pan et al. 2005). The second technique includes dephosphorylation of trehalose-6-phosphate and the assay of trehalose either spectrophotometrically (using trehalase and a glucose detecting couple) or colorimetrically (using the anthrone method- this method does not require dephosphorylation because it detects both trehalose and trehalose-6-phosphate). Both these methods' detection systems cannot distinguish between TPS and SPS activity, and are therefore not suitable for accurate assaying in crude samples. A third technique is quantification of the incorporation of radiolabel over time into the product pool from a 14C-UDP glucose donor substrate. Coupled with the competition for substrate are the very low levels of TPS expression in plant systems, making it impossible to measure this enzyme's activity without sensitive detection methods such as Gas Chromatography-Mass Spectrometry (GCMS), high pressure liquid chromatography-pulsed amperometric detection

(HPLC-PAD) or high pressure liquid chromatography-inline isotope detection (HPLC-IID). In Chapter 4 we show the use of TPS product analysis using both GCMS and HPLC-IID in determining sugarcane TPS activity.

In plants, the low levels of trehalose and specifically trehalose-6-phosphate have been a limitation in the study of this disaccharide's presence, let alone it's *in planta* metabolism. With the development of GC-MS and HPLC-PAD, measuring trehalose has become quite routine. However, trehalose-6-phosphate levels are also crucial to completing the picture. Methods used included dephosphorylation of sugar-phosphate extracts and measurement by HPLC or enzyme-linked assays. Although this works, the process is laborious and therefore does not lend itself to large sample groups. Recoveries have not been reported with this method. Another method quantifies the scale of inhibition of a trehalose-6-phosphate sensitive hexokinase as an indication of the amount of T6P in a crude metabolite extraction. Although this method is more sensitive than the former, it cannot account for other metabolites in the extract that could affect the HK activity. Another accurate and sensitive enzyme linked method uses *B. subtilis* phosphotrehalase (Van Vaeck et al. 2001). This enzyme breaks trehalose-6-phosphate down into glucose and glucose-6-phosphate. Both these substrates are sensitively measured by enzyme coupled reactions. However the limitation with this technique is the availability of purified phosphotrehalase.

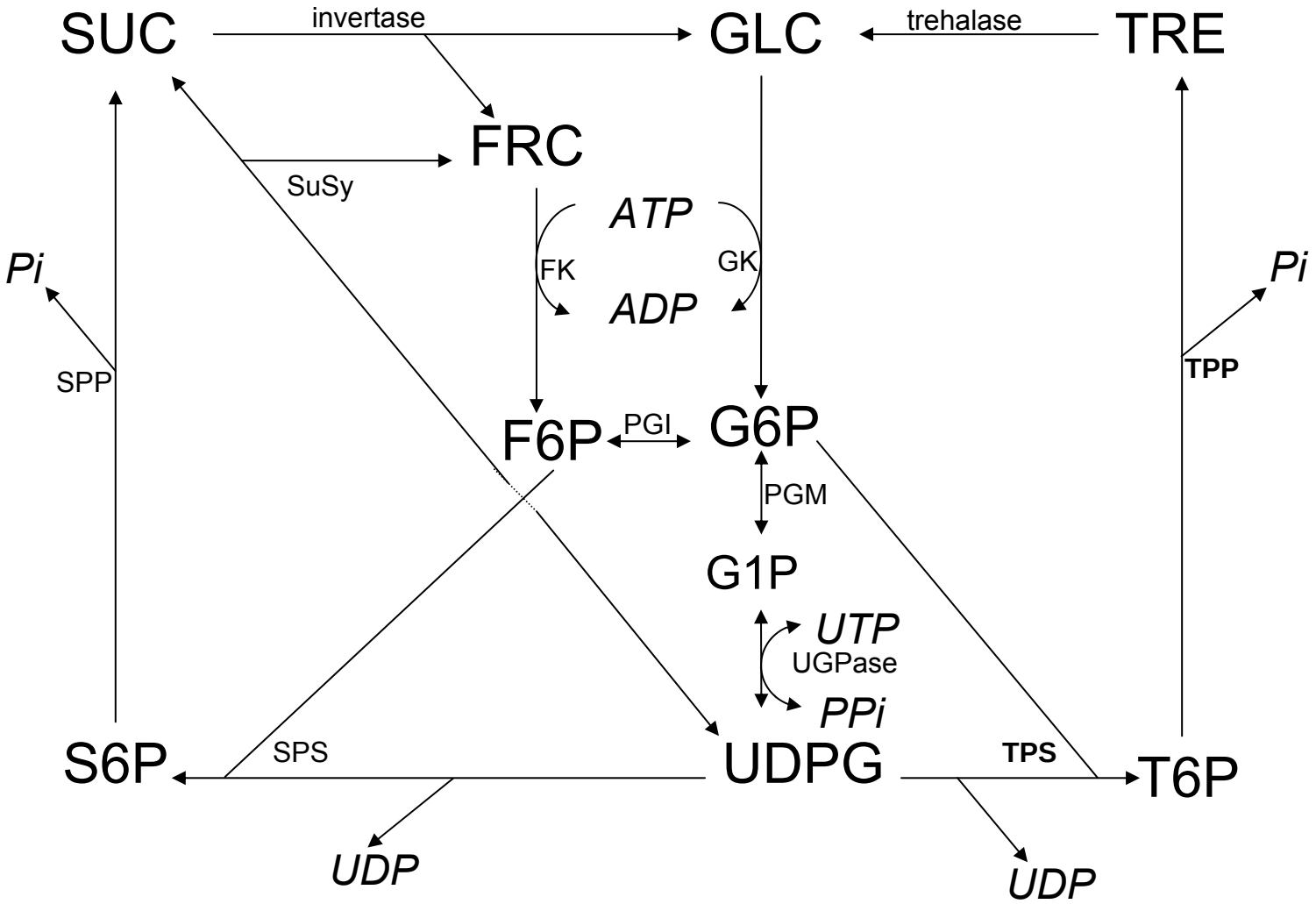


Figure 2.2. Sucrose and trehalose metabolism share substrates for both synthesis and degradation. Abbreviations: SUC: sucrose, GLC: glucose, FRC: fructose, TRE: trehalose, T6P: trehalose-6-phosphate, S6P: sucrose-6-phosphate, UDPG: UDP-glucose, G1P: glucose-1-phosphate, G6P: glucose-6-phosphate, F6P: fructose-6-phosphate, FK: fructokinase, GK: glucokinase, PGM: phosphoglucomutase, PGI: phosphoglucoisomerase, UGPase: UDP-glucose pyrophosphorylase, SuSy: sucrose synthase; SPS: sucrose phosphate synthase, SPP: sucrose phosphate phosphatase, TPS: trehalose-6-phosphate synthase, TPP: trehalose-6-phosphate synthase

2.4.2. Sugarcane and trehalose

To date the only literature referring to trehalose metabolism and sugarcane are two reports of ESTs putatively involved in trehalose biosynthesis (Carson et al. 2002), and documentation of trehalase activity as mentioned above (Alexander 1973; Fleischmacher et al. 1980; Gibon et al. 2002; Casu et al. 2003). This has delineated a very large gap that leaves room for the definition of most of the components of trehalose metabolism in sugarcane and how it is integrated into the sugarcane metabolic system as a whole.

In this thesis a wealth of information about trehalose metabolism in sugarcane has been generated: trehalose levels in sugarcane are quantified for the first time (Chapter 3); these trehalose levels are placed in the context of the metabolome of different sugarcane genotypes (Chapter 3); transcript levels and enzyme activities for sugarcane TPS, TPP and trehalase are measured in the developing culm (Chapter 4); and kinetic characterisation of partially purified TPP and trehalase are presented (Chapter 5). However, synthesis of this data is required to understanding the potential role(s) of trehalose in sugarcane. Metabolic control analysis of the trehalose pathway, and its relationship to the current sugarcane sucrose model (Rohwer & Botha 2001), is presented in Chapter 6. Below we discuss the use of bioinformatics tools to make sense of the large amounts of data produced in projects like this one.

2.4.3. Metabolomics and kinetic modelling as tools to understand metabolism

Elucidation of the regulation of plant metabolism has moved beyond investigating a single enzyme and changes in only its substrate and product pools. In the post-genomic era, tools that enable us to examine the proteome and metabolome are becoming more accessible and user friendly. Identifying changes in steady state levels of particular metabolites could indicate points of metabolic control in a specific pathway. Using technology such as GCMS (Roessner et al. 2000), HPLC-PAD and NMR metabolite levels can be accurately monitored, including those occurring in both high and low abundance. Many studies have successfully used metabolomics to describe changes observed across metabolism when comparing genotypes (Roessner et al. 2001a; Roessner et al. 2001b; Roessner-Tunali et al. 2003; Fernie 2003; Obiadalla-Ali et al. 2004).

Application of metabolomics has been particularly useful in studying differences in transgenic lines. Not only can one profile a large variety of metabolites with these non-specific techniques, but the potential to detect effects not directly related to the altered gene is far greater than using specific metabolite assays. The nature of the data sets produced from

such metabolomics studies demand the use of sophisticated methods of analysis. There is no way that meaningful conclusions can be drawn from data sets of over one hundred metabolites and many genotypes presented in tables or 2D correlations. Several methods of data analysis have been applied to, or developed for metabolomics. Statistical multivariate analyses were the first used. These include both hierarchical cluster analysis (HCA) and principle component analysis (PCA) (Roessner et al. 2001a). HCA uses successive pairwise comparison of variables (e.g. metabolites) to quantify similarity between samples (e.g. genotypes). In this way a dendrogram can be constructed depicting the "relatedness" of samples by linkage distances. PCA also correlates all possible combinations of variables, but uses the variance in each sample set. The multiple variances are reduced to two dominant vectors which are used as the axes for a 2D-plot. Samples are then plotted according to their variance to the vector. The closer the sample is to 0 of a particular vector (or principal component), the closer it (the sample) adheres to the definition of that principal component. Analysis of a large number of samples using a PCA and producing the 2D principal component plot, allows the researcher to identify samples that relate differently (i.e. have a greater variance) to the principal components when related to the rest of the data set.

An elegant example that illustrates the strength of integrating metabolite profiling and multivariate analysis is the metabolite profiling of developing tomato fruits that overexpress hexokinase. PCA shows that at the green fruit stage transgenics and wild types are distinctly different from each other with regard to the relationship of steady-state metabolite levels (Roessner-Tunali et al. 2003). Red fruit metabolite levels of transgenics and wild types clustered similarly with regard to the two principal components, i.e. the variance between samples diminishes with ripening. From this two clear conclusions were drawn. Firstly, altering hexokinase activity has the greatest effect on early fruit development. Secondly (a point that is relevant to our current study), PCA analysis of the metabolome can be used to distinguish between developmental stages and genotypes. In a complementary way HCA has also been used to show how "similar" transgenic lines are to each other and to the wild type (Roessner et al. 2001a).

Apart from these authentic statistical analyses (PCA and HCA), other methods and internet databases have been customised to the specific analysis of metabolite levels. These tools make for more meaningful analysis of plant metabolomic data and have been discussed at length (Sumner et al. 2003). One of these methods has been used in Chapter 3's analysis of the sugarcane genome: Metabocliques (Kose et al. 2001). This bioinformatics tool analyses a dataset for all significant ($R>0.90$) linear relationships between variables, and then creates a visual output where one can see how each metabolite is related to another, and also the

interaction between groups of metabolites. The authors of this method have made it accessible to any researcher in that it is a free download (www.mpimp-golm.mpg.de) and uses MS Excel as the interface. Another program that is freely available for academic and non-commercial use is MSFACTs (Duran et al. 2003) developed in L.W. Sumner's group whose research includes *Medicago truncatula* metabolomics.

Metabolomics should not be viewed in isolation of the rest of a biological system. Steady-state metabolite levels are the result of enzyme activities. These enzymes were transcribed from genes. Thus it is clear to see that regulation on the level of gene transcription and enzyme activity could cause signification change in the metabolome. And although metabolomics can identify those metabolites that are affected by such regulation, it cannot describe regulation on enzyme activity or transcript expression. In the same way an isolated study of transcriptomics or proteomics cannot *accurately* be used to predict changes in metabolite levels. This is where the strength of *in silico* modelling of metabolic systems comes into its own.

2.4.3.1. Integration of the metabolome with metabolism

Kinetic modelling of metabolism can meaningfully supplement metabolomics data. Comparing changes in mass action ratios between control and experimental samples can indicate points of divergence in the control of metabolism. These changes are the result of a difference in enzyme activity caused by a variance in the amount of the specific protein or a change in its regulation. In light of this, kinetic characterisation of an enzyme should include determining its maximum catalytic activity (V_{max}) and substrate affinity (K_m). Although these parameters describe the enzyme in question, on their own they contribute little to the understanding of the integration of that enzyme in the context of position in a metabolic pathway, or indeed its role in a greater portion of metabolism. Enzyme kinetic parameters are in effect a mathematical description of biological regulation, and combining these mathematical descriptions of all the enzymes in a pathway results in a mathematical model of that particular part of metabolism. This model can then be used to predict levels of intermediate metabolites and observe which enzymes exercise the greatest control on the pathway by application of steady-state and metabolic control analysis (MCA).

MCA is a quantitative method of analysis of the control of metabolism as described by Kacser & Burns (Kacser & Burns 1973) and Heinrich and Rapoport (Heinrich & Rapoport 1974). This approach defines the principles of flux and concentration control, and illustrates the concept of shared control in a metabolic pathway, i.e. all the enzymes in the pathway

contribute to the control of flux (and therefore steady-state metabolite levels) for each step as opposed to the "classic" hypothesis that regulation lies with "rate-limiting" enzymes. MCA requires the quantification of changes in system variables (steady-state metabolite and flux levels) in response to small changes in system parameters (enzyme V_{max} and K_m values). In principle this can be experimentally achieved, however in practice there are many limitations. These practical challenges, with particular reference to the application of experimental MCA in plants, have been thoroughly discussed (ap Rees 1994). A far more achievable starting point to MCA is its combination with *in silico* kinetic modelling. In this way only the kinetic parameters of the individual enzymes in a pathway need to be experimentally determined, without the need for perturbing each enzyme's activity individually and quantifying the consequent changes in steady-state metabolite and flux levels. *In silico* modelling provides a platform from which to effectively design experiments with specific outcomes.

MCA makes use of mathematically defined coefficients calculated from both parameter and variable changes when a system is perturbed. These coefficients fall into two categories: local and global. Elasticity coefficients describe the '"local" sensitivity of a specific enzyme activity (v) to fractional changes in metabolites (s) directly connected to the reaction (either substrate, product, inhibitor or activator), described by equation 2.1:

$$\varepsilon_s^v = \frac{\delta v/v}{\delta s/s} \qquad (2.1)$$

The second category of coefficients are those that describe global or systemic control. This implies that parameters of one enzyme are able to affect the variables of another that is not necessarily connected by a shared substrate or product. Response coefficients quantify the sensitivity of the steady-state flux through a specific reaction (J) due to a fractional change in a reaction parameter (x) of another reaction:

$$R_x^J = \frac{\delta J/J}{\delta x/x} \qquad (2.2)$$

Perhaps the most useful coefficients are the control coefficients. These fall in the global category along with response coefficients and quantitatively describe the change in a steady-state variable caused by a change in the rate of any one reaction within a described metabolic pathway. Concentration-control coefficients quantify the change in a particular metabolite's steady-state level (s) in response to a change in reaction rate (v) (equation 2.3).

Similarly, flux-control coefficients quantify the sensitivity of flux (J) through a specific reaction in relation to any single reaction rate (v) in a pathway (equation 2.4).

$$C_v^s = \frac{\delta s/s}{\delta v/v} \qquad (2.3)$$

$$C_v^J = \frac{\delta J/J}{\delta v/v} \qquad (2.4)$$

The greater the numerical value of a control coefficient the greater the control that step's reaction rate exercises over the variable in question. All flux-control coefficients for a specific parameter will sum to one, and concentration-control coefficients to zero, because MCA is performed at steady-state conditions and because of the mathematical nature of the definition of the coefficients. These relationships are aloes known as the summation theorems of MCA. A positive control coefficient indicates that an increase in the parameter will cause an increase in the variable. In the same way a negative control coefficient indicates a negative effect on variable due to an increase of the parameter. Consequently control coefficients should be viewed for the entire system and not in isolation to determine which reactions exercise the greatest control over each individual steady-state variable. Therefore, the control on a system, and its individual components, can be quantitatively described with the combined attributes of the elasticity, response and control coefficients.

These modelling techniques and analyses have been successfully applied to add to the understanding of many metabolic pathways. A useful database of models (JWS Online) is available online at http://jjj.biochem.sun.ac.za (Olivier & Snoep 2004). This website provides an interface to both familiarise the user with the concepts and construction of kinetic models, as well as to provide the opportunity to run 'ready-made' models, change parameters and observe the effects. It also offers a secure service to journals for the review of models before publication. Applications of these models include identification of suitable drug targets in the pathogen *Trypanosoma brucei* (Bakker et al. 1999), prediction of metabolic manipulation for increased production of diacetyl in *Lactococcus lactis* (Hoefnagel et al. 2002), and identification of specific points of control in the accumulation of sucrose in sugarcane (Rohwer & Botha 2001). A great advantage of JWS Online is that it allows biological scientists with little computer programming background to use peer-reviewed models as a tool to aid their research. However, the user cannot add reactions to any model online as

this is not the purpose of the site. This should not deter the user from further exploration of kinetic modelling as there are a number of software packages available to this aim (reviewed in (Rohwer 2005).

Two widely used Windows based programming tools for modelling biochemical pathways are Gepasi (Mendes 1997) and Scamp (Sauro 1993). Another recently developed application is PySCeS, the Python Simulator for Cellular Systems (Olivier et al. 2005). PySCeS is an open-source tool, available online at http://pysces.sourceforge.net , and runs on any computer or operating system that is able to support Python as a programming language. PySCeS can be run either interactively or from a Python script and the output of data can be recorded in text, HTML or graphical format. Because of its suitability for our study of the interaction of sucrose and trehalose metabolism in sugarcane internodes, PySCeS was chosen as the modelling software tool in Chapter 6.

As mentioned above, a kinetic model of sugarcane sucrose metabolism is available on the JWS Online site (Rohwer & Botha 2001). This model uses kinetic parameters of all the enzymes involved in sucrose metabolism to predict steady-state metabolite levels. The power of the model is twofold. Firstly, it places all enzymes in relationship with each other in sucrose metabolism. Secondly, it allows the user to make *in silico* changes in the amount or regulation of an enzyme activity and observe changes in metabolite levels. This approach allows one to rationally choose points of metabolism for experimental manipulation in order to make a desired change. In this way the modelling of sugarcane trehalose metabolism (Chapter 6) will provide insight into the role of TPS, TPP, trehalase as well as trehalose-6-phosphate and trehalose in the accumulation of sucrose. The model itself requires data of steady-state metabolite levels plants in order to validate its predictive accuracy, and this is where metabolomics (Chapter 3) interfaces with the enzyme parameters of TPS, TPP and trehalase (Chapters 4 and 5).

2.5. Conclusion

The lack of understanding in regard to the control of carbon partitioning in higher plants, with specific reference to sugarcane, has formed the starting point for this study. The trehalose pathway will be investigated in sugarcane internodal tissues because of its potential role in the regulation of carbohydrate metabolism. In the majority of plants, it would appear that trehalose metabolism's main function is in a signalling capacity, and not directly as a carbon source or stress protectant. In this study we examine trehalose metabolism and employ the tools of metabolomic analysis and kinetic modelling to begin to place this disaccharide in its metabolic context in the genus *Saccharum*.

2.6. References

Adams RP, Kendal E, Kartha KK (1990) Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. Food and Chemical Toxicology 40: 871-898

Aeschbacher RA, Muller J, Boller T, Wiemkien A (1999) Purification of Trehalase GMTRE1 from Soybean Nodules and Cloning of Its cDNA. *GMTRE1* Is Expressed at a Low Level in Multiple Tissues. Plant Physiology 119: 489-495

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

ap Rees T (1994) Metabolic control analysis of plant metabolism. Plant, Cell and Environment 17: 587-599

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signalling. Plant Physiology 136: 3649-3659

Bae H, Herman E, Sicher R (2005) Exogenous trehalose promtes non-structural carbohydrate accumulation and induces chemical detoxification stress response proteins in *Arabidopsis thaliana* grown in liquid media. Plant Science 168: 1293-1301

Bakker BM, Michels PAM, Opperdoes FR, Westerhoff HV (1999) What Controls Glycolysis in the Bloodstream form *Trypanosoma brucei*? Journal of Biological Chemistry 274: 14551-14559

Bell W, Sun W, Hohmann S, Wera S, Reinders A, De Virgilio C, Wiemkien A, Thevelein JM (1998) Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. Journal of Biotechnology 273: 33311-33319

Blazquez MA, Gancedo C (1995) Mode of action of the *qcr9* and *cat3* mutations in restoring the ability of *Saccharomces cerevisiae tps1* mutants to grow on glucose. Molecular genes and genetics 249: 655-664

Blazquez MA, Lagunas R, Gancedo C, Gancedo JM (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Letters 329: 51-54

Blazquez MA, Santos E, Flores C-L, Martinez-Zapater J-M, Salinas J, Gancedo C (1998) Isolation and molecular characterization of the *Arabidopsis TPS1* gene, encoding trehalose-6-phosphate synthase. The Plant Journal 13: 685-689

Bonini BM, Van Dijck P, Thevelein JM (2003) Uncoupling the glucose growth defect and the deregulation of glycolysis in *Saccharomyces cerevisiae teps1* mutants expressing trehalose-6-phosphate-insensitive hexokinase from *Schizosaccharomyces pombe*. Biochimica et Biophysica Acta 1606: 83-93

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Australian Journal of Plant Physiology 27: 81-85

Breitenbach-Schmitt I, Schmitt HD, Heinisch J, Zimmerman FK (1984) Genetic and physiological evidence for the existance of a second pathway in yeast parallel to the phosphofructokinase-aldolase reaction sequence . Molecular genes and genetics 195: 536-540

Cannon JF, Pringle JR, Fietcher A, Khalil M (1994) Characterisation of glycogen-deficient glc mutants of *Saccharomyces cerevisiae*. Genetics 136: 485-503

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Current Opinion in Plant Biology 5: 250-257

Cortina C, Culianez-Macia FA (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Science in press:

De Smet KAL, Weston A, Brown IN, Young DB, Roberston BD (2000) Three pathways for trehalose biosynthesis in mycobacteria. Microbiology 146: 199-208

Drennan PM, Smith MT, Goldsworthy D, Van Staden J (1993) The Occurence of Trehalose in the Leaves of the Desiccation-Tolerant Angiosperm *Myrothamus flabelliofolius* Wele. Journal of Plant Physiology 142: 493-496

Duran AL, Yang J, Wang L, Sumner L (2003) Metabolomics spectral formatiing, alignment and conversion tools (MSFACTs). Bioinformatics 19: 2283-2293

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

El-Bashiti T, Hamamci H, Oktem HA, Yucel M (2005) Biochemical analysis of trehalose and its metabolizing enzymes in wheat under abiotic stress conditions. Plant Science in press:

Elbein AD (1974) The metabolism of α–α trehalose. In RS Tipson, D Horton, eds Advances in Carbohydrate Chemistry and Biochemistry Vol 30. Academic Press, New York, pp 227-256

Fernie AR (2003) Metabolome characterisation in plant system analysis. Functional Plant Biology 30: 111-120

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Fritzius T, Aeschbacher RA, Wiemkien A, Wingler A (2001) Induction of *ApL3* Expression by Trehalose Complements the Starch-Deficient Arabidopsis Mutant *adg2-1* Lacking ApL1, the Large Subunit of ADP-Glucose Pyrophosphorylase. Plant Physiology 126: 883-889

Gancedo C, Flores C-L (2004) The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Research 4: 351-359

Garcia NAT, Iribarne C, Lopez M, Herrera-Cervera JA, Lluch C (2005) Physiological implications of trehalase from *Phaseolus vulgaris* root nodules: partial purification and characterization. Plant Physiology and Biochemistry in press:

Garg AK, Kim J-K, Owens TG, Ranwala AP, Choi YD, Kochlan LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. PNAS 99: 15898-15903

Gibon Y, Vigeoleas H, Tiessen A, Geigenberger P, Stitt M (2002) Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates and glycolytic intermediates based on a novel enzymic cycling system. The Plant Journal 30: 221-235

Girke T, Todd J, Ruuska S, White J, Benning C, Ohlrogge J (2000) Microarray analysis of developing Arabidopsis seeds. Plant Physiology 124: 1570-1581

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Goddijn OJM, van Dunn K (1999) Trehalose metabolism in plants. Trends in Plant Science 4: 315-319

Goddijn OJM, Verwoerd TC, Voogd E, Krutwagen RWHH, de Graaf PTHM, Poels J, van Dunn K, Ponstein AS, Damm B, Pen J (1997) Inhibition of Trehalse Activity Enhances Trehalose Accumulation in Transgenic Plants. Plant Physiology 113: 181-190

Heinrich R, Rapoport TA (1974) A linear steady-state treatment of enzymatic chains. European Journal of Biochemistry 42: 89-95

Hoefnagel MH, Starrenburg MJ, Martens DE, Hugenholtz J, Kleerebezem M, Van Swam II, Bongers R, Westerhoff HV, Snoep JL (2002) Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modelling, metabolic control and experimental analysis. Microbiology 148: 1003-1013

Hohmann S, Neves MJ, de Koning W, Alijo R, Ramos J, Thevelein JM (1993) The growth and signalling defects of the *ggs1* (*fdp1/byp1*) deletion mutant on glucose are supressed by a deletion of the gene coding for hexokinase PII. Current Genetics 25: 89-94

Holstrom K-O, Mantyla E, Welin B, Mandal A, Palva ET , Tunnela OE, Londesborough J (1996) Drought tolerance in tobacco. Nature 379: 683-684

Hottiger T, Boller T, Wiemkien A (1987a) rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccaromyces cerevisiae* cells subjected to temperature shifts. FEBS Letters 220: 113-115

Hottiger T, Schmutz P, Wiemkien A (1987b) Heat-Induced Accumulation and Futile Cycling of Trehalose in *Saccharomyces cerevisiae*. Journal of Bacteriology 169: 5518-5522

Howells AM, Bullifent HL, Dhaliwal K, Griffin K, de Castro AG, Frith G, Tunnacliffe A, Titball RW (2002) Role of trehalose biosynthesis in environmetnal survival and virulence of *Salmonella enterica* serovar *typhimurium*. Research in Microbiology 153: 281-287

Jang I-C, Oh S-J, Seo J-S, Choi W-B, Song SI, Kim CH, Kim YS, Seo H-S, Choi YD, Nahm BH, Kim J-K (2003) Expression of Bifunctional Fusion of *Escherichia coli* Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. Plant Physiology 131: 516-524

Kacser H, Burns JA (1973) The control of flux. Symposia of the Society of Experimental Biology 28: 65-104

Kolbe A, Tiessen A, Schluepmann H, Paul MJ, Ulrich S , Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. PNAS 102: 11118-11123

Kose K, Weckwerth W, Linke T, Fiehn O (2001) Visualizing plant metabolomic correlation networks using clique-metabolite matrices. Bioinformatics 17: 1198-1208

Kruger N (1997) Carbohydrate synthesis and degradation. In DT Dennis, DH Turpin, DD Lefebvre, DB Layzell, eds Plant Metabolism. Longman, Essex, pp 83-104

Leyman B, Van Dijk P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends in Plant Science 6: 510-513

Londesborough J, Varimo K (1984) Characterisation of two trehalases in baker's yeast. Biochemical Journal 219: 511-518

Londesborough J, Vuorio OE (1993) Purification of trehalose synthase from baker's yeast. European Journal of Biochemistry 216: 841-848

Mendes P (1997) Biochemistry by numbers: simulation of biochemical pathways with Gepasi. Trends in Biochemical Sciences 22: 361-363

Muller J, Aeschbacher RA, Wingler A, Boller T, Wiemkien A (2001a) Trehalose and Trehalase in Arabidopsis. Plant Physiology 125: 1086-1093

Muller J, Boller T, Wiemkien A (2001b) Trehalose becomes the most abundant non-structural carbohydrate during senescence of soybean nodules. Journal of Experimental Botany 52: 943-947

Muller J, Staehelin R, Mellor T, Wiemkien A (1992) Partial purification and characterization of trehalase from soybean nodules. Journal of Plant Physiology 140: 8-13

Navon G, Shulman RG, Yamane T, Eccleshal TR, Lam KB (1979) Phosphorus-31 nuclear magnetic resonance studied of wild-type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. Biochemistry 18: 4487-4499

Obiadalla-Ali H, Fernie AR, Kossmann J, Lloyd JR (2004) Developmental analysis of carbohydrate metabolism in tomato (*Lycopersicon esculentum* cv. Micro-Tom) fruits. Physiologia Plantarum 120: 196-204

Olivier BG, Rohwer JM, Hofmeyr J-HS (2005) Modelling cellular systems with PySCeS. Bioinformatics 21: 560-561

Olivier BG, Snoep JL (2004) Web-based kinetic modelling using JWS Online. Bioinformatics 20: 2143-2144

Pan YT, Carroll JD, Elbein AD (2005) Treahlose-phosphate synthase of *Mycobacterium tuberculosis*. Cloning, expression and properties of the recombinant enzyme. European Journal of Biochemistry 269: 6091-6100

Pilon-Smits EA, Terry N, Sears T, Kim H, Zayed A, Hwang S, van Dunn K, Voogd E, Verwoerd TC, Krutwagen RWHH, Goddijn OJM (1998) Trehalose-producing transgenic tobacco plants show improved growth performance under drought stress. Journal of Plant Physiology 152: 525-532

Rimmele M, Boos W (1994) trehalose-6-Phosphate Hydrolase of *Escherichia coli*. Journal of Bacteriology 176: 5654-5664

Roessner-Tunali U, Hegemann G, Lytovechenko A, Carrari F, Bruedigam C, Granot D, Fernie AR (2003) Metabolic Profiling of Transgenic Tomato Plants Overexpressing Hexokinase Reveals That the Influence of Hexose Phosphorylation Diminishes during Fruit Development. Plant Physiology 133: 84-99

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001a) Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmetally Modified Plant Systems. The Plant Cell 13: 11-29

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. The Plant Journal 23: 131-142

Roessner U, Willmitzer L, Fernie AR (2001b) High-Resolution Metabolic Phenotyping of Genetically and Environmentally Diverse potato Tuber Systems. Identification of Phenocopies. Plant Physiology 127: 749-764

Rohwer JM (2005) Strategies and Tools for Computational Systems Biology. Screening Trends in Drug Discovery 6: 23-25

Rohwer JM, Botha FC (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochemical Journal 358: 437-445

Ryu S-I, Park C-S, Cha J, Woo E-J, Lee S-B (2005) A novel trehalose-synthesizing glucosyltransferase from *Pyrococcus horikoshii*: Molecular cloning and characterization. Biochemical and Biophysical Research Communications 329: 429-436

Sauro HM (1993) SCAMP: a general purpose simulator and metabolic control program. CABIOS 9: 441-450

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Seo H-S, Koo YJ, Lim JY, Song JT, Kim CH, Kim JK, Lee JS, Choi YD (2000) Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*. Applied and Environmental Microbiology 66: 2484-2490

Shin D-Y, Matsumoto K, Iida H, Uno I, Ishikawa N (1987) Heat shock response of *Saccharomyces cerevisiae* mutants altered in cyclic AMP-dependant protein phoshorylation. Molecular and Cellular Biology 7: 244-250

Strom A, Kaasen I (1993) Trehalose metabolism *Escherichia coli*: stress protection and stress regulation of gene expression. Molecular Microbiology 82: 204-210

Sumner LW, Mendes P, Dixon RA (2003) Plant Metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62: 817-836

Thevelein JM, Hohmann S (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? Trends in Biological Sciences 20: 3-10

Valenzuela-Soto EM, Marquez-Escalante JA, Iturriaga G, Figueroa-Soto CG (2004) Trehalose 6-phosphate synthase from *Selaginella lepidophylla*: purification and properties. Biochemical and Biophysical Research Communications 313: 314-319

Van de poll K, Kerkenaar A, Schamhart DHJ (1974) Isolation of a regulatory mutant of fructose-1,6-bisphosphatase in *Saccharomyces carlsbergenssis*. Journal of Bacteriology 965-970

Van Dijck P, Mascorro-Gallardo JO, De Bus M, Royackers K, Iturriaga G, Thevelein JM (2002) Truncation of *Arabidopsis thaliana* and *Selaginella lepidophylla* trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochemical Journal 366: 63-71

van Dijken AJH, Schluepmann H, Smeekens SC (2004) Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegitative Growth and Transition to Flowering. Plant Physiology 135: 9

Van Vaeck C, Wera S, Van Dijck P, Thevelein JM (2001) Analysis and modification of trehalose-6-phosphate levels in the yeast *Saccharomyces cerevisiae* with the use of *Bacillus subtilis* phosphotrehalase. Biochemical Journal 353: 157-162

Vandercammen A, Francois J, Hers H-G (1989) Characterization of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *Saccharomyces cerevisiae*. European Journal of Biochemistry 182: 613-620

Vogel G, Aeschbacher RA, Muller J, Boller T, Wiemkien A (1998) Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of yeast *tps2* mutant. The Plant Journal 13: 673-683

Vogel G, Fiehn O, Jean-Richard-dit-Brussels L, Boller T, Wiemkien A, Aeschbacher RA, Wingler A (2001) Trehalose metabolism in *Arabidopsis*: occurence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. Journal of Experimental Botany 52: 1817-1826

Wang Y-J, Hao Y-J, Zhang Z-G, Chen T, Zhang J-S, Chen S-Y (2004) Isolation of trehalose-6-phosphate phosphatase gene from tobacco and its functional analysis in yeast cells. Journal of Plant Physiology in press:

Wannet WJB, denCamp HJM, Wisselimk HW, van der Drift C, van Griensven LD, Vogel G (1998) Purification and characterization of trehalose phosphorylase from the commercial mushroom *Agaricus bisporus*. Biochimica et Biophysica Acta 1425: 177-188

Wiese A, Groner F, Sonnewald U, Deppnar H, Lerchl J, Hebbeker U, Flugge U, Weber A (1999) Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Letters 461: 13-18

Wingler A, Fritzius T, Wiemkien A, Boller T, Aeschbacher RA (2000) Trehalose induces the ADP-Glucose Pyrophosphorylase Gene, *ApL3*, and Starch Synthesis in Arabidopsis. Plant Physiology 124: 105-114

Zentella R, Mascorro-Gallardo JO, Van Dijck P, Folch-Mallol J, Bonini BM, Van Vaeck C, Gaxiola R, Covarrubias AA, Nieto-Sotelo J, Thevelein JM, Iturriaga G (1999) A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant. Plant Physiology 119: 1473-1482

Zhao HW, Chen YJ, Hu YL, Gao Y, Lin ZP (2000) Construction of a trehalose-6-phosphate synthase gene driven by drought-responsive promoter and expression of drought-resistance in transgenic tobacco. Acta Bot Sinica 42: 616-619

CHAPTER 3

THE METABOLIC CONTEXT OF TREHALOSE IN SUGARCANE

Abstract

The regulation of sucrose accumulation in higher plants has not been completely elucidated even though significant research effort has been invested in this subject. Due to the potential role of trehalose metabolism in the regulation of carbohydrate partitioning we have identified trehalose from five *Saccharum* varieties that differentially accumulate sucrose. Levels of trehalose varied between 3.91 ± 0.99 and 0.31 ± 0.09 $nmol.g^{-1}$ fresh weight (FW) in the five genotypes. Trehalose was linearly correlated with xylose, ribose and inositol, but not with any of its proposed synthetic substrates (UDP-glucose and glucose-6-phosphate), or its hydrolytic product, glucose. It would appear that trehalose does not directly affect sucrose accumulation although this does not preclude involvement of trehalose-6-phosphate in the regulation of carbon partitioning. Analysis of the metabolome of high and low sucrose storing genotypes (*Saccharum* interspecific hybrid; N19 and *Saccharum spontaneum (*US6656-15)), showed highest steady state levels of glycolytic, TCA and amino acid intermediates in the young internodes of the low sucrose storer. This could indicate that a differential regulation of partitioning in N19 compared to US6656-15, with a greater percentage of its soluble carbohydrate pool being accumulated as sucrose. The ability to measure metabolite levels in different cellular compartments, as opposed to the current total cellular determinations presented in this chapter, would greatly increase the accuracy and interpretability of such metabolomic data.

3.1. Introduction

The ability of commercial sugarcane varieties to accumulate up to 14 % of their fresh weight in sucrose (www.sugar.org.za) has made it one of the world's important crops. Breeding programs the world over have succeeded in increasing the sucrose content using traditional crossing methods, and screening progeny for high-sucrose phenotypes. However, the regulation of sucrose metabolism and accumulation is still poorly understood despite the significant research effort in this area (Moore 1995; Huber & Huber 1996; Whittaker & Botha 1997; Farrar et al. 2000; Rohwer & Botha 2001; Lunn & MacRae 2003).

In the last few years the study of the regulation of plant metabolism has expanded to include proteomics and metabolomics. In the same way that genomics studies the differential expression of genes and their potential interactions, proteomics and metabolomics examine expression profiles on the protein and metabolite levels. Changing steady state levels of metabolites could be an indication of points of control in a specific pathway. With the improvement of technology in metabolite identification and quantification it is now possible to identify up to 170 compounds from one extract in one assay (Roessner et al. 2000). These compounds include those occurring in both high and low abundance. The technology of GC-MS separates and identifies multiple metabolites. Multivariate analysis of the ensuing data, and integration into mathematical models allows us to compare their levels in an attempt to identify points of metabolic control. Using this technique coupled with HPLC and enzyme-linked spectrophotometric assays, a more complete picture of the sugarcane metabolome will be achieved. To date there is only one publication reporting metabolite levels other than the abundant sugars in sugarcane (Whittaker & Botha 1997). Although the glycolytic intermediates and some metabolites directly linked to sucrose metabolism are discussed, there is no reference to amino and organic acids, low abundance sugars, sugar alcohols or all the nucleotides. This additional information will not only add to our understanding of the entire context of sucrose metabolism, but also help validate kinetic models that attempt to predict this metabolism (Rohwer & Botha 2001).

In the genomics approach sugarcane EST programs across the world have produced over 300 000 partial transcripts. Among these are fragments showing homology to published sequences for trehalose-6-phophate synthase (EC 2.4.1.15 TPS) and trehalose-6-phosphate phosphatase (EC 3.1.3.12 TPP) (Carson et al. 2002; Casu et al. 2003). Trehalose is a sugar that acts as a storage carbohydrate, stress metabolite or signalling molecule in prokaryotes and simple eukaryotes (Arguelles 2000). In light of this the isolation and identification of

trehalose from plant material has caused much interest. In plants it is still unclear what the exact roles of trehalose are, although it has been suggested that it aids the desiccation tolerance of resurrection plants (Adams et al. 1990), could signal of pathogen attack or could act as a signal molecule in the regulation of carbon partitioning (Vogel et al. 2001). With this in mind the identification of these sugarcane ESTs coding for genes of trehalose synthesis sparked interest in the potential role of this disaccharide in sucrose metabolism.

In this Chapter we have analysed the sugarcane metabolome in the culm. This allowed us to place trehalose in its context of carbon partitioning in sugarcane. The data and its analyses serve as tools to better elucidating the poorly understood regulation of sucrose accumulation in this dedicated sucrose storer.

3.2. Materials and Methods

3.2.1. Plant material

Sugarcane was grown in the field at the South African Sugarcane Research Institute at Mount Edgecombe, KwaZulu Natal, South Africa. Interspecific *Saccharum* varieties N19, N27 and N31 were sampled representative of commercially cultivated sugarcane. Ancestral species *Saccharum spontaneum* and *Saccharum robustum* were represented by varieties US6656-15 and NG7794 respectively. Plants were harvested after growth periods of 12 and 18 months from initial planting. Immediately after harvest the tissues were sectioned and frozen in liquid nitrogen.

3.2.2. Metabolite extraction

Metabolites were extracted from sugarcane internodal tissues (Stitt et al. 1983). The method was scaled down to application in a microcentrifuge tube. Approximately 100 mg of tissue was mixed with 620 µl extraction buffer (200 mM Hepes, pH 8.5, 65% (v/v) methanol, 24% (v/v) chloroform and 10 µg ribitol per sample as internal standard for GCMS metabolite quantification). The mixture was mixed at room temperature for 10 minutes. Addition of 400µl water separated the water soluble and non-polar components after centrifugation at 13 000 rpm, for 15 minutes at 4°C. The aqueous phase was aliquoted into 240µl and 24µl fractions and dried under partial vacuum (Speed Vac ® Plus SC110A, Savant Instruments, Inc., Holbrook, NY, USA) for further derivitisation and analysis by GCMS. For other soluble metabolite determinations 300 µl of the aqueous phase was reduced to dryness and resuspended in 60 µl distilled water.

3.2.3. Metabolite analysis by GCMS

Metabolites were derivitised in a two step process of methoxymation and trimethylsylilation (Roessner et al. 2000). Analysis was performed using a Thermofinnigan TRACE GCMS fitted with an AS2000 autosampler. Separation was achieved using a Restek Rtx-5Sil analytical column (30m x 0.25 mm ID). One µl derivitised sample was injected into a splitless injector at 230°C. The flow rate of the carrier gas (Helium) was 1 $ml.min^{-1}$. The initial oven temperature was 70°C and ramped at 1 $°C.min^{-1}$ for 6 minutes. The second ramp was between 76 and 350°C at $6°C.min^{-1}$. A final hold of 3 minutes at 350°C completed the run, after which the oven was re-equilibrated to 70°C for the following sample injection. The TRACEMS is a quadropole mass spectrometer equipped with an Electron Impact (EI+)

source. The GC interface was set at 250°C, the ion source at 200°C. Emission current was set at 150 µA, and the detector at 350V. Data acquisition was delayed for 7.5 minutes to avoid solvent peaks. MS acquisition was set on Full scan mode between 43 and 579 atomic mass units (amus). All chromatograms (GC) and m/z fingerprints (MS) were analysed using the software package Xcalibur.

Known standards were made up in milliQ water in a cocktail with a final concentration of 1mM. Dilutions were made between 0.001 and 0.5mM for calculation of calibration curves and quantification from unknown samples.

3.2.4. Enzyme linked measurement of sugar and glycolytic intermediates

Sucrose, glucose and fructose were extracted as described above analysed as described in (Rose & Botha 2000). Glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate were quantified at dual wavelengths 340nm and 410nm (Schluepmann et al. 2003). Phosphoenolpyruvate and pyruvate were determined as previously described (Bergmeyer & Bernt 1974).

3.2.5. Statistical analyses and graphs

Principal Component Analysis (PCA) and cluster analyses were carried out using the software program Statistica 7 © Statsoft. Visualisation of metabolite correlations was achieved using Metabocliques (Kose et al. 2001). Graphs were drawn in either Statistica 7 ©, or SigmaPlot ©, Systat.

3.3. Results

3.3.1. Sugar levels in the culm

To determine to sugar levels, five sugarcane varieties were sampled after a 12 month growth period in the field. The five genotypes were selected to include commercial varieties and two ancestral species. The commercial varieties N19, N27 and N31 are interspecific *Saccharum crosses* and they accumulated sucrose up to 500 µmol.g^{-1} FW (figure 3.1A). *S. spontaneum (*US6656-15) and *S. robustum* (NG7794) are "wild" ancestral sugarcane species and their sucrose levels in the mature internodes (represented by internode 11, figure 3.1.) were at least 25% lower than the interspecific hybrids. In all the genotypes the sucrose content increased with culm maturity Glucose and fructose levels followed an opposite trend to that of sucrose with lowest levels in the mature internodes (figure 3.1B and C).

Trehalose was detected in all five genotypes. Its content was five orders of magnitude lower than sucrose in the same tissue (figure 3.2.). With the exception of variety N31, the trehalose content in the internodes decreased with increasing tissue maturity. No simple linear correlations between trehalose and sucrose, or trehalose and either of the abundant hexoses were evident. Comparison of genotypes revealed that trehalose levels were generally higher in the ancestral varieties than the commercial ones.

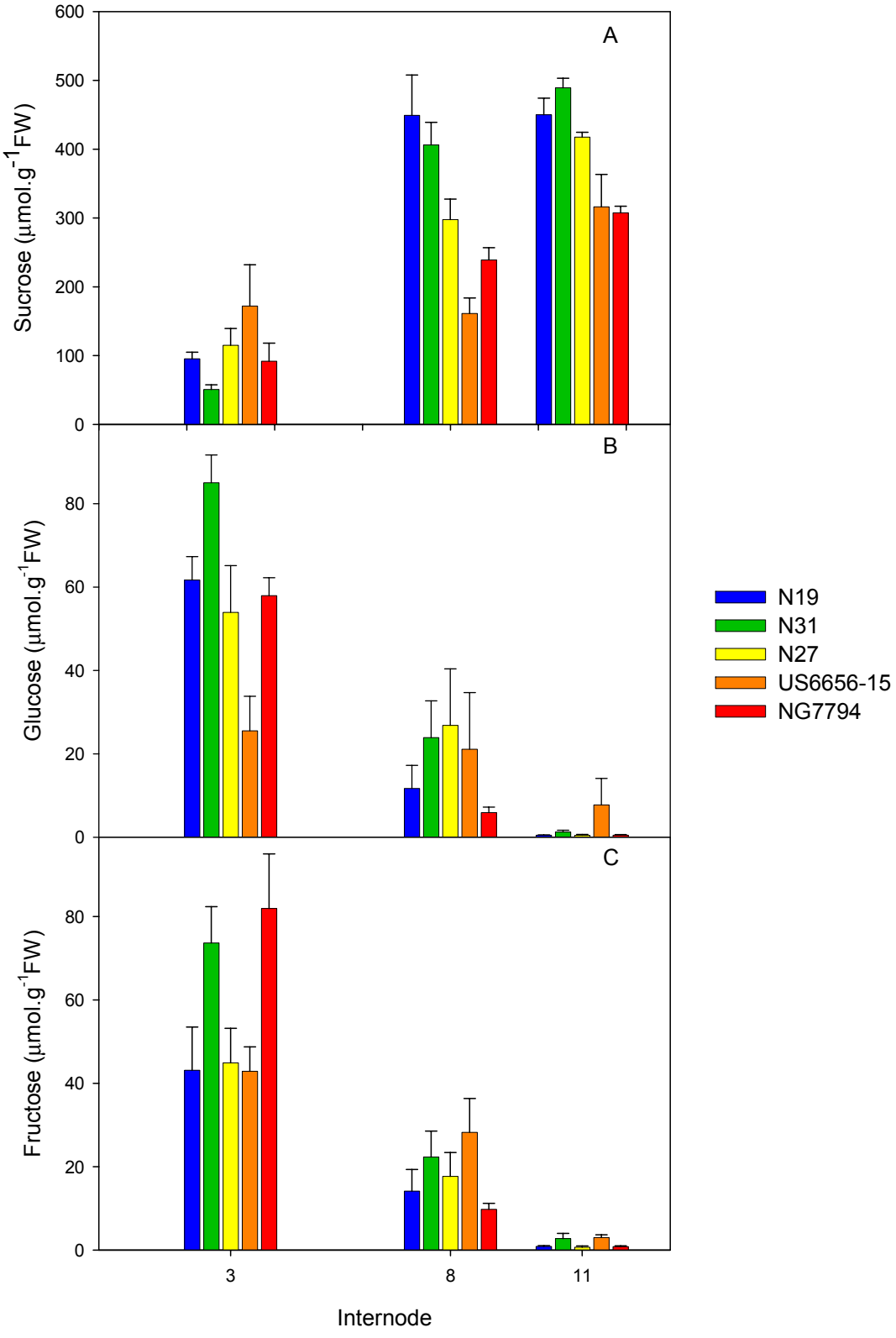


Figure 3.1. Sugar contents measured from extracts of five field-grown sugarcane genotypes. Panels A, B and C represent sucrose, glucose and fructose respectively. All values are the mean of at least three independent replicates ± standard error.

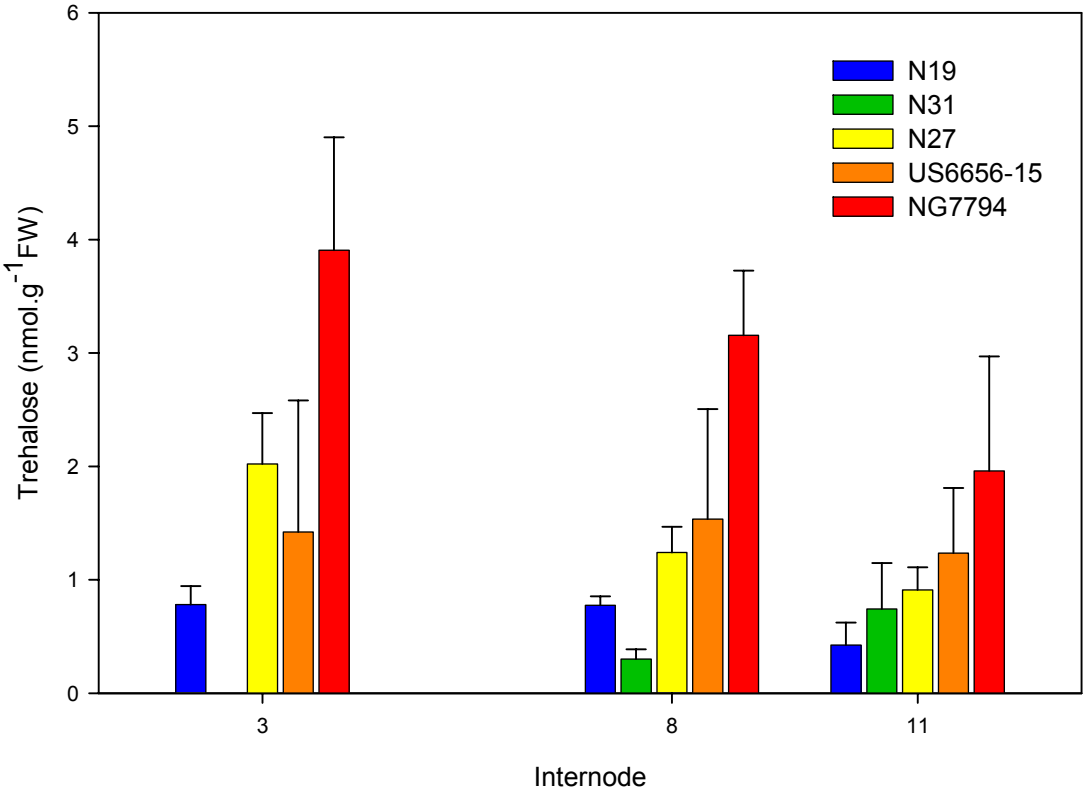


Figure 3.2. Trehalose content (nmol.g^{-1}FW) in internodal tissues of five sugarcane varieties. Data are the mean of at least three independent replicates ± standard error.

To determine the relationship between varieties with regards to their patterns of sugar accumulation, with specific reference to glucose, fructose, sucrose and trehalose, hierarchical cluster analysis (HCA) was performed. HCA is a bioinformatics analysis tool used to cluster samples (in this case genotypes) by similarities in their data sets (in this case levels of steady state sugars) (Roessner et al. 2001; Stitt & Fernie 2003; Sumner et al. 2003). An HCA methodically groups samples by progressive pairwise correlation of their data, and presents the results in as a dendrogram whose arm lengths correspond to similarity of the samples. It is a very useful visualisation tool when comparing multiple components of a data set in order to determine relationships between samples. By using HCA to analyse the patterns of sugar accumulation in the five genotypes sampled, we were able to perceive which genotypes displayed the most similar characteristics.

When comparing all four sugar levels (sucrose, glucose, fructose and trehalose) as a function of variety, the commercial varieties clearly segregated away from the *S. spontaneum*

and *S. robustum* genotypes (figure 3.3.). It could be expected that this was due to the lower sucrose and higher reducing sugars present in the two ancestral genotypes in comparison to the commercial varieties. Interestingly, even though trehalose levels were close to one hundred thousand times lower than sucrose levels in a specific tissue (e.g. N19 internode 3), trehalose still contributed significantly to the genotype relationship seen in figure 3.3. This is because HCA makes use of pairwise correlations and is not weighted according absolute amounts. Omitting trehalose from the analysis resulted in a close clustering of N19 and N31 (i.e. short linkage distance between the two), and a second clade clustering N27 to both US66656-15 and NG7794 with greater distance between them. From this it was evident that steady state trehalose accumulation patterns contributed to a sample segregation pattern (figure 3.3.) that was closest to that expected for the genotypes sampled.

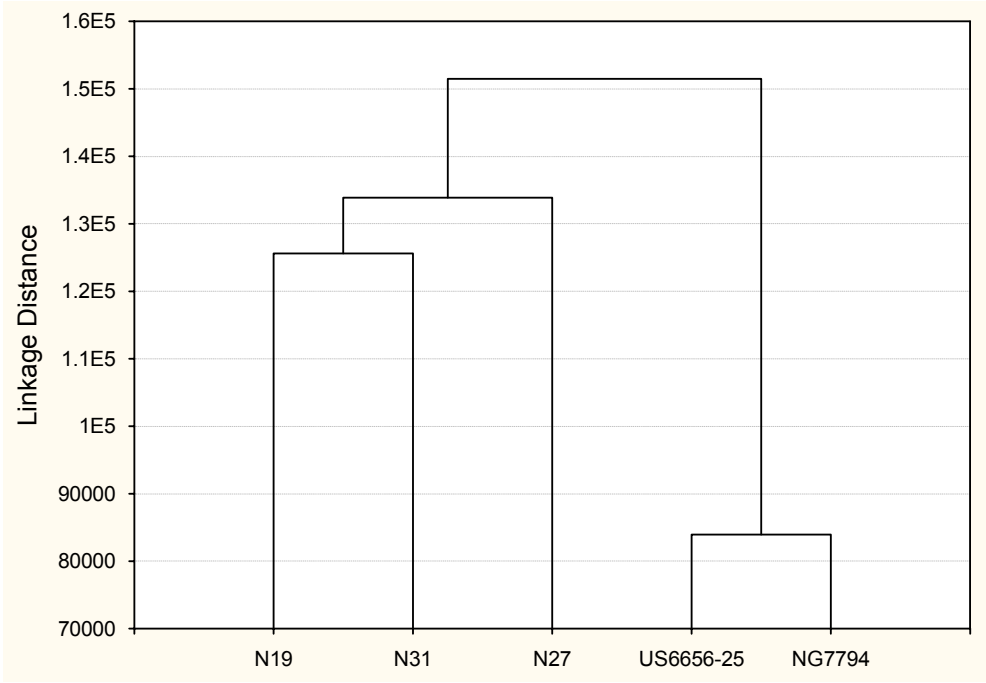


Figure 3.3. Cluster analysis of 5 sugarcane varieties with regard to distribution of sucrose, glucose, fructose and trehalose levels in internodes 3, 8 and 11.

3.3.2. Metabolite profile

At the end of the growing season (18 months) *Saccharum* N19 and US6656-15 were selected from the five genotypes discussed in section 3.3.1 and harvested to determine metabolite levels with the aim of creating metabolite profiles that could potentially highlight differences in the control of carbon partitioning between them. In this harvest season, internode 10 represented a mature internode, while internode 11 was used in the previous section. With the aid of GCMS and enzyme linked assays metabolites from sucrose, glycolytic, TCA and amino acid metabolism were determined from internodes 3,6 and 10 (table 3.1.). In both varieties the abundant sugars (sucrose, glucose and fructose) dominated the total soluble metabolite pool with concentrations ranging between 2.0 ± 0.3 and 293 ± 18 $\mu mol.g^{-1}FW$. The only other metabolites that were present at $\mu mol.g^{-1}FW$ levels were the organic acids aconitate, malate, shikimate, quinate and a single amino acid glutamate, and then only in young tissues (internode 3). Trehalose and the other less abundant sugars, glycolytic and TCA intermediates, amino acids were all in the $nmol.g^{-1}FW$ range.

It was evident that at the time of harvest there was no longer a significant difference between these varieties in sucrose levels in the mature internodes (internode 10, table 3.1.). Despite this, there were still variations between other metabolite steady state levels. In order to visualise these we compared all concentrations of each metabolite relative to its level determined in N19 internode 3 (figure 3.4.). Here, a few interesting trends emerged. When comparing only young (internode 3) and mature (internode 10) metabolite levels, only sucrose concentrations (and maltose in US6656-15) increased. The majority of differences in metabolite levels between the two varieties were evident in internode 3. Glucose-6-phosphate, fructose-6-phosphate, triose-phosphates, phosphoenolpyrvate (PEP) and pyruvate were all significantly higher in US6656-15 than in N19. Except for methionine, amino acid levels were higher in US6656-15 internode 3 than N19. Again in US6656-15 internode 3 aconitate and 2-oxo-glutarate levels were over six and four times higher, respectively, than in N19.

Table 3.1. Metabolite levels from sugarcane internodes 3, 6 and 10. Concentrations are given in nmol.g^{-1} FW, except for the high abundance sugars sucrose, glucose and fructose whose concentrations are in µmol.g^{-1}FW. Data are the mean of at least three replicates ± standard errors.

		Saccharum hybrid (N19)						*S. spontaneum (US6656-15)*					
		Internode 3		***Internode 6***		***Internode 11***		***Internode 3***		***Internode 6***		***Internode 11***	
high abundance sugars													
(µmol.g^{-1}FW)	**Glucose**	74.8	±6.6	24.4	±7.3	1.99	±0.35	62.5	±8.3	31.1	±8.7	21.3	±7.0
	Fructose	68.7	±5.3	24.4	±6.1	3.34	±0.99	67.2	±8.5	26.7	±8.1	10.3	±1.7
	Sucrose	132	±7	212	±7	293	±18	113	±14	219	±16	283	±9
low abundance sugars													
(nmol.g^{-1}FW)	**Trehalose**	0.78	±0.16	0.53	±0.15	0.42	±0.20	1.42	±1.16	1.53	±0.97	0.573	±0.573
	Xylose	2.90	±0.57	3.92	±1.05	nd		11.3	±5.5	12.1	±2.5	3.93	±0.83
	Ribose	7.52	±1.71	7.42	±1.25	3.07	±0.81	10.2	±3.3	11.1	±2.7	3.80	±0.69
	Arabinose	7.77	±4.25	1.80	±0.86	0.20	±0.05	4.98	±0.88	2.13	±0.73	0.53	±0.15
	Inositol	11.4	±3.2	17.3	±7.4	4.59	±0.61	24.3	±8.3	36.8	±18.6	5.2	±0.2
	Maltose	2.51	±0.49	20.9	±3.2	0.00	±0.00	12.8	±4.7	10.8	±5.9	23.0	±3.4
sugar phosphates													
(nmol.g^{-1}FW)	**glucose-6-phosphate**	27.1	±3.8	14.0	±1.7	nd		66.3	±6.8	8.68	±2.35	19.7	±1.7
	fructose-6-phosphate	33.4	±9.2	12.4	±3.5	nd		62.7	±16.9	5.98	±3.02	3.30	±1.68
	glucose-1-phosphate	28.2	±5.4	7.62	±4.91	nd		nd		nd		nd	
	udp-glucose	18.6	±4.2	6.81	±3.41	4.5	±2.4	nd		nd		nd	
	fructose-1,6-bisphosphate	68.2	±13.1	54.0	±7.4	35.3	±11.7	116	±25	33.2	±6.6	32.4	±3.4
	phosphoenolpyruvate	50.8	±21.8	82.8	±29.6	23.5	±11.3	475	±76	150	±23	115	±10
	triose phosphates	217	±32	141	±16	119	±2	610	±124	110	±23	123	±12
Organic acids													
(nmol.g^{-1}FW)	**Pyruvate**	150	±11	113	±20	103	±14	693	±120	135	±14	91.5	±14.2
	Succinate	6.85	±1.01	10.6	±6.5	nd		22.8	±12.9	6.25	±1.19	nd	
	Malate	5600	±703	265	±49	14.1	±7.1	981	±150	339	±94	39.6	±5.0
	2-oxo-glutarate	8.96	±1.90	9.6	±2.3	nd		57.9	±23.9	nd		nd	
	shikimic acid	1510	±164	371	±116	2.02	±0.64	1200	±96	646	±221	126	±51
	citric acid	126	±48	161	±13	48.7	±34.2	162	±66	311	±175	212	±63
	quinic acid	1570	±90	1410	±221	8.60	±3.23	4490	±301	2590	±698	350	±94
	aconitic acid	3330	±631	177	±58	nd		55010	±8365	2410	±1501	721	±474

Table 3.1. cont….

		Saccharum hybrid (N19)						*S. spontaneum (US6656-15)*					
		Internode 3		***Internode 6***		***Internode 11***		***Internode 3***		***Internode 6***		***Internode 11***	
amino acids ($nmol.g^{-1}FW$)	**Alanine**	27.4	±10.8	19.0	±3.2	0.220	±0.060	66.9	±30.1	3.94	±2.05	1.25	±0.55
	Valine	135	±38	46.4	±11.3	nd		214	±61	15.7	±8.0	nd	
	Serine	277	±61	37.8	±10.5	0.623	±0.143	321	±141	1.65	±0.36	1.73	±0.52
	Isoleucine	131	±47	45.3	±11.1	nd		273	±67	nd		nd	
	Glycine	53.9	±15.7	14.5	±7.5	nd		55.0	±15.5	nd		nd	
	Threonine	1680	±165	262	±53	nd		2000	±684	nd		nd	
	Methionine	67.2	±44.1	37.8	±20.0	nd		nd		2.53	±1.37	1.11	±0.69
	aspartic acid	409	±66	1060	±387	64.1	±41.4	722	±167	260	±83	nd	
	trans-4-hydroxy proline	1620	±930	303	±84	24.2	±14.6	1030	±441	177	±90	7.04	±7.04
	glutamic acid	10900	±3890	2500	±775	nd		13000	±6056	9190	±824	nd	

nd : levels too low to detect

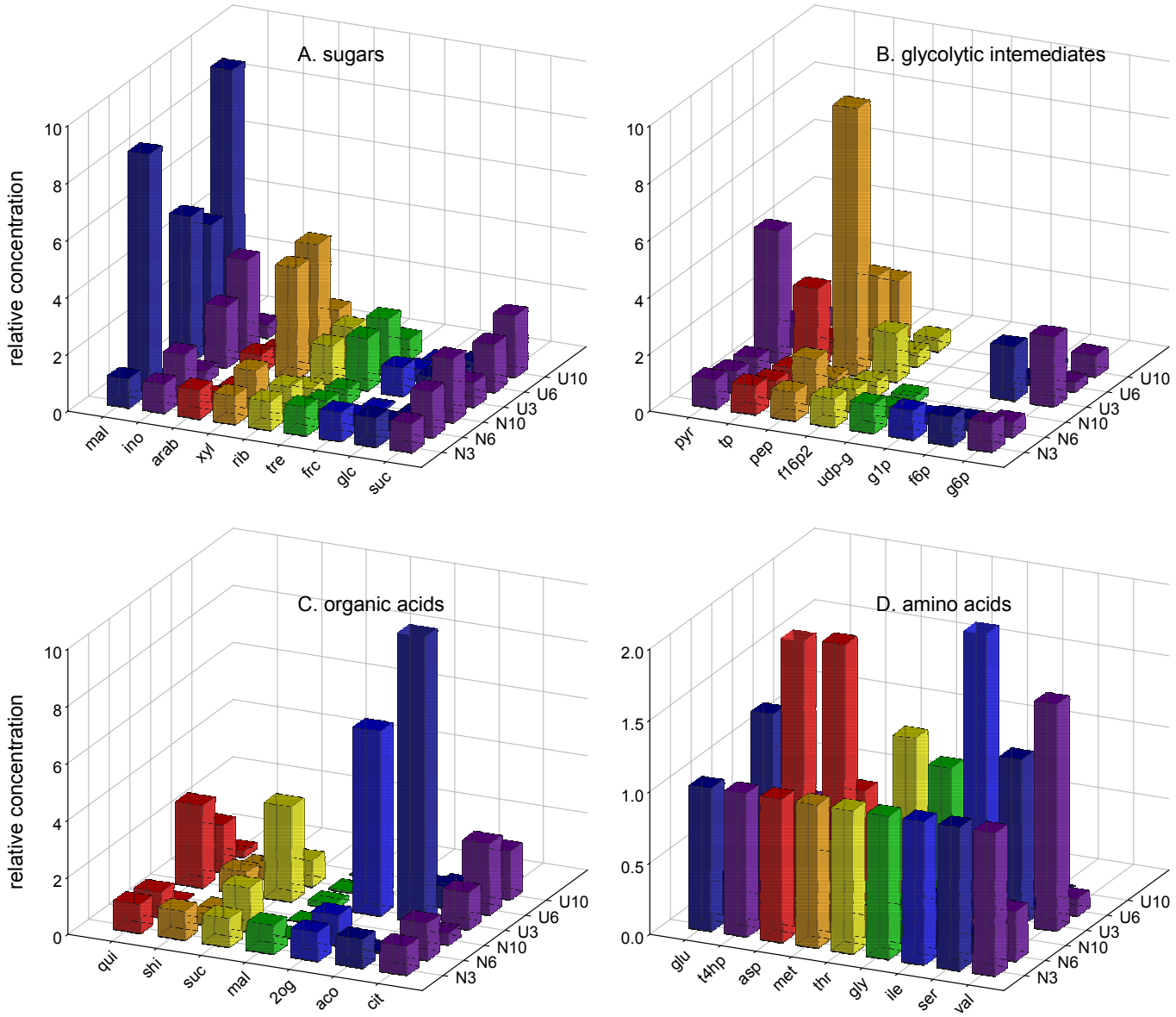


Figure 3.4. Metabolite levels from *Saccharum* interspecific hybrid N19 (N), and *S. spontaneum* US6656-15 (U). Internodes 3, 6 and 10 were sampled in triplicate from culms of separate plants. Concentrations are represented relative to N19 internode 3. Panels A, B, C, and D represent metabolites from sugar, glycolytic, organic acid and amino acid pools respectively. Abbreviations: Panel A: malate (mal), inositol (ino), arabinose (arab), xylose (xyl), ribose (rib), trehalose (tre), fructose (frc), glucose (glc), sucrose (suc); Panel B: pyruvate (pyr), triose-phosphates (tp), phosphoenolpyruvate (pep), fructose-1,6-bisphosphate (f16P2), UDP-glucose (udp-g), glucose-1-phosphate (g1p), fructose-6-phosphate (f6p), glucose-6-phosphate (g6p); Panel C: quinic acid (qui), shikimic acid (shi), succinate (suc), malate (mal), 2-oxo-glutarate (2og), aconitate (aco), citrate (cit); Panel D: glutamate (glu), trans-4-hydroxy-proline (t4hp), aspartate (asp), methionine (met), threonine (thr), glycine (gly), isoleucine (ile), serine (ser), valine (val).

3.3.3. The metabolome in context

There are many challenges in exploring the plant metabolome. Extracting and quantifying large groups of metabolites, whose chemistry and stability vary greatly, is the first step to overcome. Metabolomic studies invariably produce very large data sets, as the aim is often to describe a particular metabolite, or genotype, in the context of the full complement of detectable metabolites. Such data sets cannot be adequately analysed without the use of computer aided bioinformatics tools.

Two of the most frequently used analyses are hierarchical clustering analysis (HCA) and principal component analysis (PCA). Both methods systematically compare all possible metabolite pairs in a data set in order to identify correlations between them. PCA illustrates variance between individuals in terms of principle components derived from a multi-dimensional vector analysis. All principal components identified contribute to the total variance of a data set. Often more than 90% of the variance can be accounted for by as few as two principal components. These components are then used as the axes for a 2D plot that shows the relationship of a specific individual to that principal component. The closer the individual lies to the origin of that principal component the more closely it adheres to the pattern it defines. In other words, application of PCA to plant metabolomics allows us to group individuals (whether they be genotypes, tissue types or metabolites) by adherence to patterns derived from a multivariate analysis. In this way, those individuals that cluster together share a potentially stronger regulation or relationship, than those that are outliers.

The strength of using both HCA and PCA lies in the principles behind each method. HCA is established on the *similarity* of samples, whereas PCA illustrates *variance* between the samples. Because of this, the two analyses complement one another when used to describe metabolomics data.

3.3.3.1. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) of metabolite interactions

Comparison of all metabolites levels versus tissue type using a PCA, showed that with regard to the first factor internodes 6 and 10 of both N19 and US6656-15 clustered closer together than internode 3 (figure 3.5.), suggesting a greater variance of metabolite interaction between genotypes in actively growing tissues. This would imply that there was less of a variation in the pattern of steady state level metabolites in the more mature internodes.

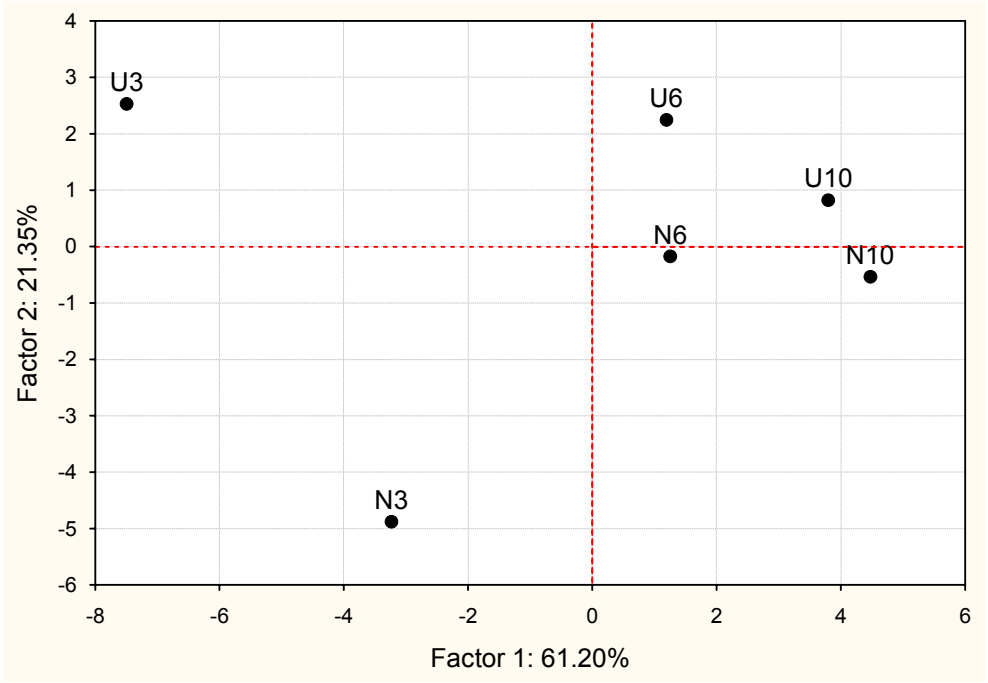


Figure 3.5. Principal component analysis of all measured metabolites as a function of tissue type. Interspecific *Saccharum* hybrid N19 (N) and *S. spontaneum* clone US66-56-15 (U) were sampled. Internodes 3, 6 an 10 were selected for analysis.

Using the complete set of metabolite data, a dendrogram of varietal tissue relationship was constructed (figure 3.6.). Confirming the PCA result, clustering of samples was according to tissue type and not genotype, suggesting that the greatest similarities in metabolite interaction was observed between internodes and not variety.

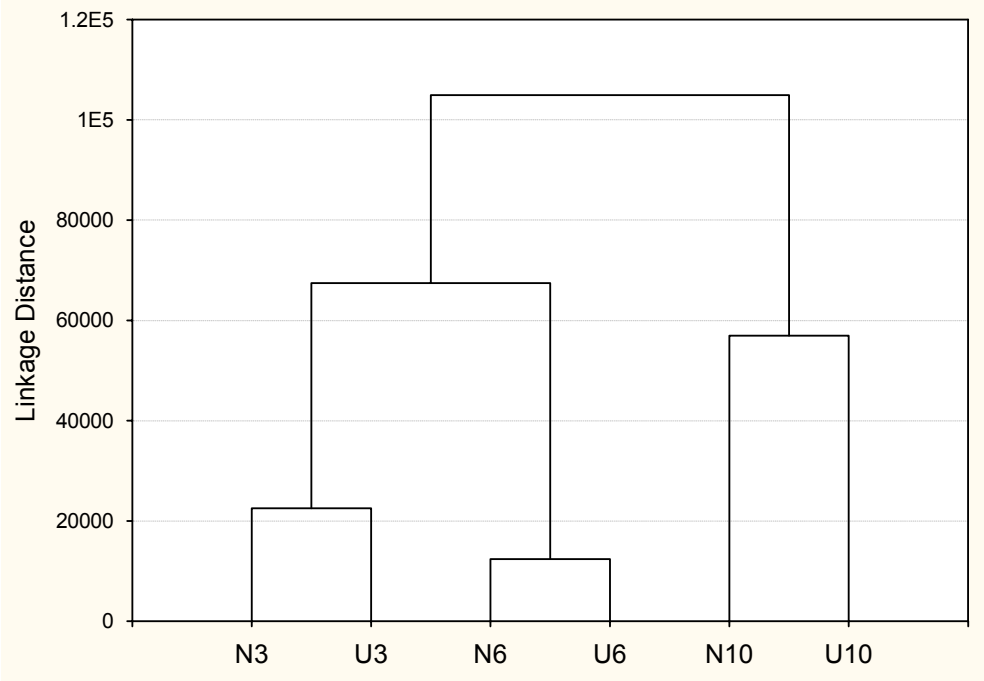


Figure 3.6. Cluster analysis of all metabolite levels determined as a function of tissue type. Sugarcane varieties N19 (N) (*Saccharum* interspecific hybrid) and US6656-15 (*S. spontaneum*) (U) were sampled. Tissues analysed were internodes 3, 6 and 10.

When comparing internodes as the variable between varieties the majority of metabolites clustered together (figure 3.7.). In all three internodes sampled (3, 6 and 10) sucrose seemed not to relate in the same way to the two factors of the PCA. This is not a surprising result from the PCA as it is designed to recognise those metabolites that do not conform to its principal components. With internode maturity, and a decrease in hexose levels it was observed that in internode 10 (figure 3.7C) only sucrose was not closely related to the first factor of the PCA that accounted for 99.87 % of the total variance between varieties in this tissue. This PCA serves to confirm the phenotypic difference between the two genotypes- the observed differences in steady state levels of sucrose (table 3.1.).

One of the features of this type of metabolomics analysis is the identification of other phenotypic variances that would not have been seen, due to such a large, diverse data set. Aconitate was one such variance. Noting such an outlier in the PCA, and returning to table

3.1., it was clear to see the 10 fold difference between N19 and US6656-15, and that significantly more carbon been partitioned aconitate in the latter sugarcane variety. Although it is imaginable that this difference could have been noticed from a few simple graphical representations (as in figure 3.4.), metabolomic experiments are tending towards much larger data sets whose visual identification of variance could be virtually impossible. This is the strength of such statistical analyses.

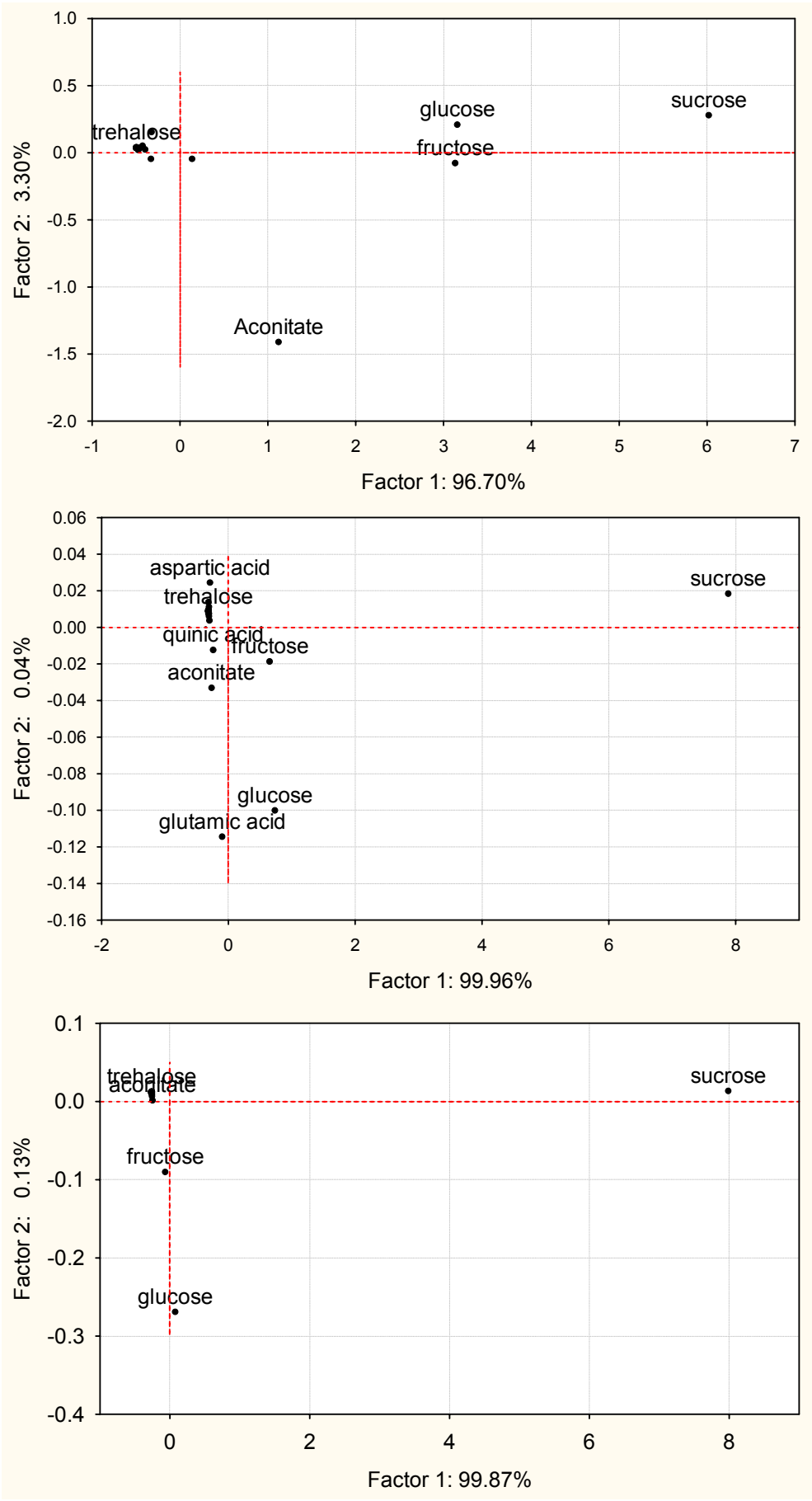


Figure 3.7. PCA of all metabolites measured using N19 and US6656-15 as the variables. Only outliers are annotated, other metabolites have clustered together. Analysis of data collected from internodes 3, 6 and 10 are shown in panels A, B and C respectively.

3.3.3.2. Metabolome analysis using linear correlations

PCA and cluster analysis give a reduced comparison of metabolite distribution between samples. However, such analyses lack the correlation of specific metabolites with one another in a linear regression (both on the individual and the inter-metabolite basis). Performing manual linear correlations on a set of data as large as the one presented in table 3.1. would be laborious in its interpretation. The program Metabocliques was written with this kind of analysis in mind (Kose et al. 2001). The program correlates each individual metabolite against every possible other metabolite in the data set. The user defines the linear regression correlation threshold between 0 and 1 (the closer the value is to one, the higher the statistical validity of the calculated relationship). In this study we have clamped the threshold at 0.9 (the authors of the model suggest 0.8 as the lower limit for significant results). Once all correlations below the threshold have been discarded, the program employs a branch-and-bound algorithm to find all maximum cliques in the correlation set. Thereafter, the cliques (groups of correlating metabolites) are ordered in a visual output that uses colour to link metabolites that are directly correlated to one another.

Figure 3.8. represents correlations of metabolites in all tissues of both *Saccharum* interspecific hybrid N19, and *S. spontaneum,* US6656-15 in the Metabocliques output. The data was analysed as a complete set to see which metabolites were universally correlated to one other. Cells indicated in yellow are those metabolites and cliques that correlate above the 0.90 threshold. Numbers in each cell indicate the number of cliques that a particular metabolite is involved in. The clique with the highest number of metabolites in it is ranked at the top of the matrix. In this case, clique 22 has eight metabolites that have strong linear correlations with each other. These metabolites represent glycolytic and amino acid metabolism intermediates.

The entire clique matrix is complex, but can be broken down into regions (for example figure 3.8A, B and C). Taking a closer look at sucrose, we see that it is involved in 6 cliques, and metabolites that are both directly and indirectly linked to its metabolising enzymes. Those metabolites that are directly involved include fructose, glucose and fructose-6-phosphate. Arabinose and the amino acids, glycine, serine, valine and threonine, as well as the organic acid shikimate all respond significantly in a linear correlation with sucrose, although they are not substrates or products of any reactions involving sucrose. Sucrose is negatively correlated with all metabolites that rendered significant linear regressions against this disaccharide. The nature of the linear correlation is not evident from the Metabocliques' output and therefore, in order to look closer at some of these interactions sucrose was plotted against fructose-6-phosphate, UDP-glucose, glucose, shikimate, fructose and trehalose (figure 3.9.). It was evident that the substrates for sucrose synthesis were differentially

related to their product. Fructose-6-phosphate had a significant negative correlation with sucrose content (R^2 = 0.83, p = 0.0117), however UDP-glucose showed no linear relationship at all (R^2 = 0.14, p = 0.4619) (figure 3.9A and B). The products of the invertase reaction: glucose and fructose both had significant negative correlations with sucrose content (figure 3.9C and E). Trehalose and sucrose were evidently not significantly correlated in a linear manner (figure 3.9F).

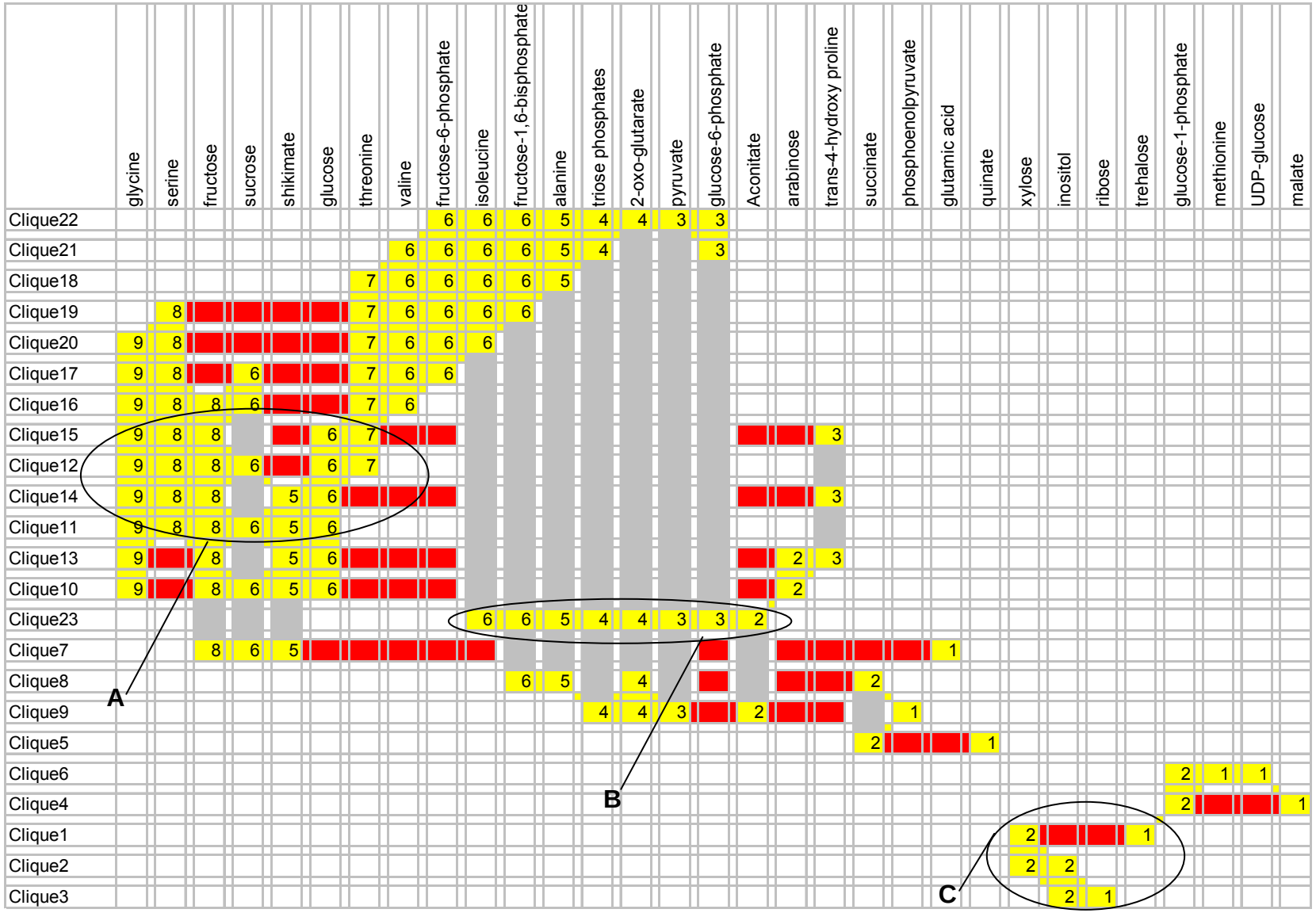


Figure 3.8. Analysis of linear interactions of metabolites in all tissues sampled from sugarcane varieties N19 (*Saccharum* interspecific hycrid) and US6656-15 (*S. spontaneum)*. Analysis was done using the program Metabocliques. The threshold was set at R = 0.90. Three clusters of metabolite interactions (A,B and C) are discussed in the text.

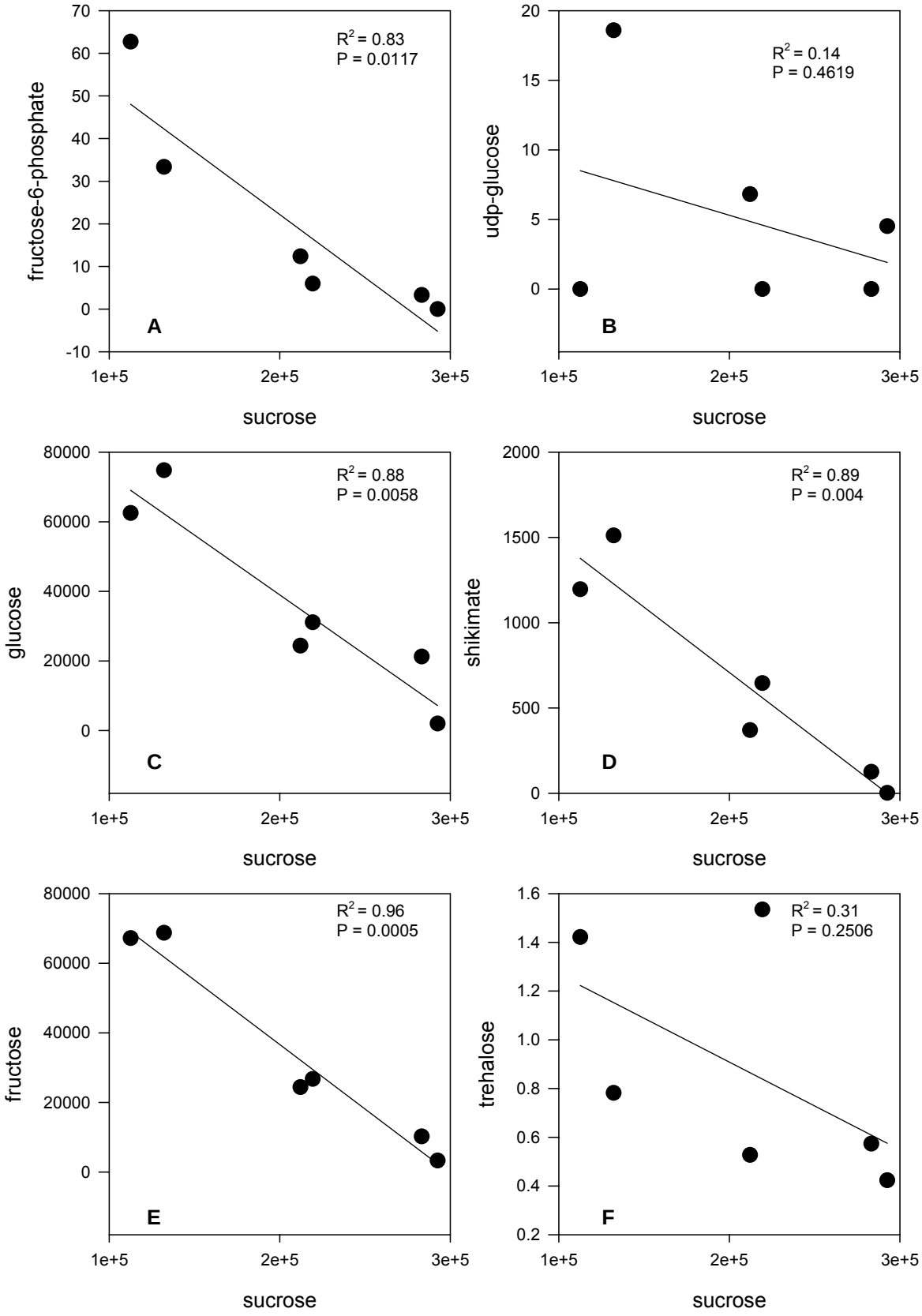


Figure 3.9. Linear correlations of metabolites involved in sucrose metabolism as indicated in figure 3.8A. All metabolite levels are given in nmol.g^{-1}FW, (A) fructose-6-phosphate, (B) UDP-glucose, (C) glucose, (D) shikimate, (E) fructose, (F) trehalose. Data are the mean of at least 3 replicates from three different tissue types and two sugarcane varieties.

The clique involving trehalose in figure 3.8C revealed a smaller set of correlations than the sucrose clique (figure 3.8A). Here we see trehalose only involved with three other metabolites: xylose, ribose and inositol. Figure 3.10. shows the linear correlations between all possible combinations of these metabolites. This validated the Metabocliques method for correlation analysis by individually analysing the linear correlations used to construct the output.

Another important use of this correlation matrix is to observe relationships between well studied metabolites and those of which very little is known. For instance, aconitate had high levels in the young internodes of sugarcane tissues (table 3.1.). In *S. spontaneum* these levels were comparable to the amount of glucose in the same tissue. Assessment of the clique in which aconitate interacted showed that it significantly correlated with other organic acids such as pyruvate and 2-oxo-glutarate, as well as intermediates in the top section of glycolysis. However, plotting these interactions on 2D graphs (figure 3.11.), showed how careful one should be in interpreting data produced from programs like Metabocliques. Although the output of this program presents true correlations that hold above a 0.90 threshold, observation of the 2D linear correlations showed that they were dominated by one point- the aconitate level from US6656-15 internode 3 (table 3.1.). Without this point the relationships would be quite different- some of them would still be linear, but some of them not. This is further discussed in section 5, below.

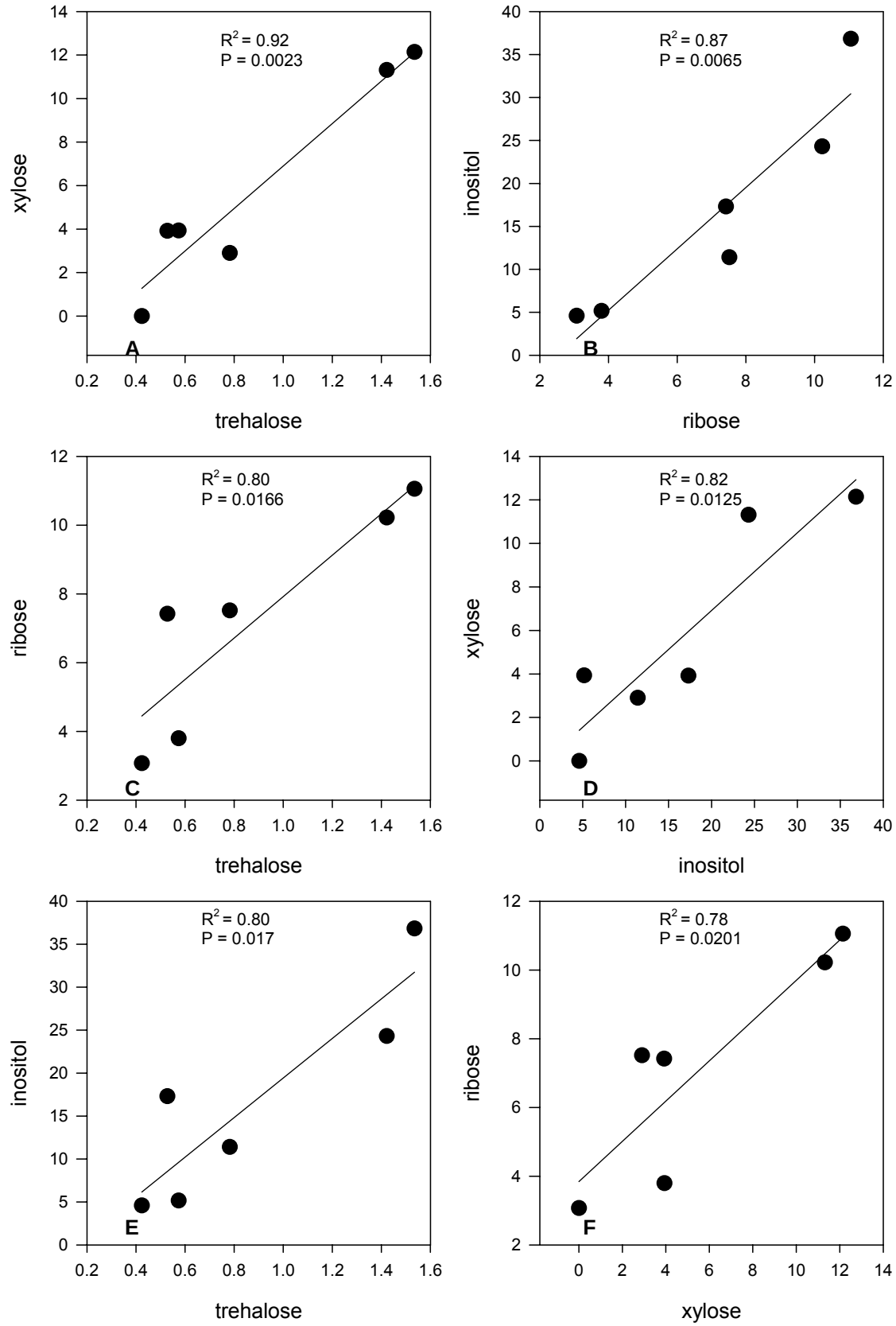


Figure 3.10. Linear correlations of metabolites involved in the trehalose clique as indicated in Figure 3.8C. All metabolite levels are given in nmol.g^{-1}FW, (A) trehalose vs. xylose, (B) ribose vs. inositol, (C) trehalose vs. ribose, (D) inositol vs. xylose, (E) trehalose vs. inositol, (F) xylose vs. ribose. Data are the mean of at least 3 replicates from three different tissue types and two sugarcane varieties.

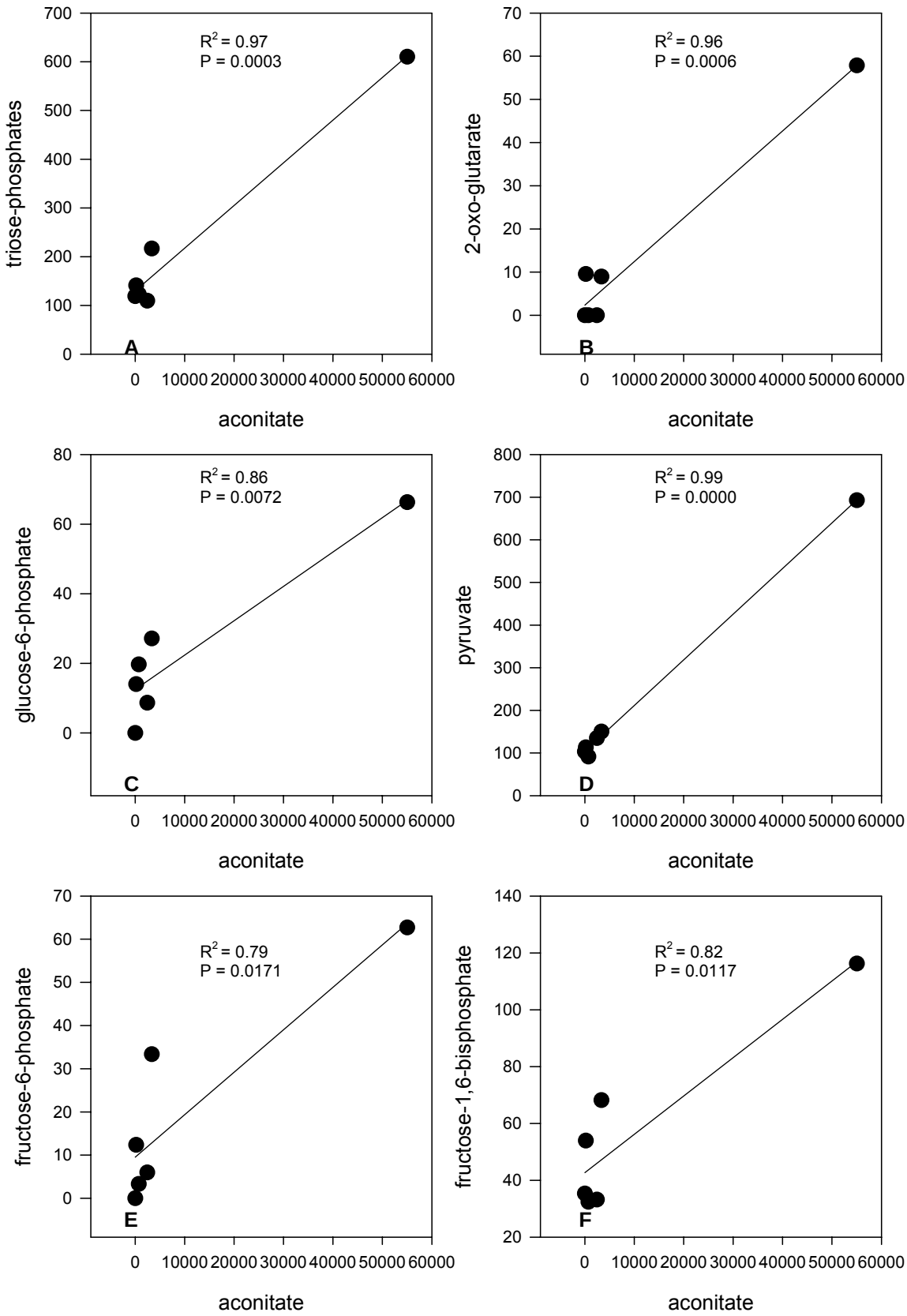


Figure 3.11. Linear correlations of aconitate vs. metabolites as indicated in Figure 3.8B. All metabolite levels are given in nmol.g^{-1}FW, (A) triose-phosphates, (B) 2-oxo-glutarate, (C) glucose-6-phosphate, (D) pyruvate, (E) fructose-6-phosphate, (F) fructose-1,6-bisphosphate. Data are the mean of at least 3 replicates from three different tissue types and two sugarcane varieties.

3.4. Discussion

3.4.1. Trehalose in sugarcane internodal tissues

Enzymatic activity responsible for the degradation of trehalose was first identified in plants from sugarcane protein extracts (Glasziou & Gayler 1969). Despite this there have been no publications of levels of the disaccharide itself in sugarcane tissue. Here we have unquestionably identified trehalose extracted from the culm tissue of five different sugarcane genotypes. This was achieved by using the tandem capabilities of gas chromatography and mass spectrometry (GCMS).

In all five sugarcane varieties sampled, sucrose levels increased with tissue maturity (figure 3.1A). N19 and N31 displayed the greatest gradient of sucrose accumulation between developing and sucrose storing internodes, 3 and 11 respectively. US6656-15 and NG7794 both had significantly lower purity (ratio of sucrose to sucrose plus hexoses) in mature internodes. These results gave us a good platform from which to investigate differences in carbon partitioning between genotypes whose phenotypes were so obviously different with regards their most important commercial attribute, i.e. sucrose accumulation.

Identification of ESTs coding for putative trehalose synthetic genes (trehalose-6-phosphate synthase: TPS, and trehalose-6-phosphate phosphatase: TPP) in sugarcane (Carson et al. 2002; Casu et al. 2003) meant there was a possibility that sugarcane could produce its own endogenous trehalose. Coupled with this, we know that sugarcane has the substrates for the trehalose-6-phosphate synthase reaction: UDP-glucose and glucose-6-phosphate (Whittaker & Botha 1997). In this study we have measured trehalose, and know therefore that the plant can produce it endogenously. The route of this synthesis is assumed to be through TPS and TPP, and is shown for the first time in sugarcane in chapter 4. Because of the effects of trehalose metabolism on the regulation of carbon flux in yeast, characterisation of the sugar and its derivatives in sugarcane could indicate whether such a role is possible in the plant kingdom too.

Using the five genotypes described above we measured trehalose levels in culm tissues of different maturities. The general trend that trehalose decreased with culm maturity held in four varieties, with commercial interspecific *Saccharum* hybrid N31 the exception to the rule (figure 3.2.). Although there was a trend that trehalose levels decreased with a concurrent increase in sucrose content, the relationship could not be described as significant by a linear

regression (figure 3.9F). Using the sugar levels to describe the five varieties in a cluster analysis, the interspecific (N19, N31, N27) and specific genotypes (US6656-15, NG7794) separated in two distinct clades (figure 3.3.). This served to confirm the difference between the varieties in their capacity to accumulate sucrose and metabolise abundant sugars.

Although these data are useful, determining what role trehalose plays in carbon partitioning was greatly aided by a more thorough investigation of the sugarcane metabolome. At the end of the growth season, varieties N19 and US6656-15 were selected for this task. Because the cane had ripened, there was no significant difference in sucrose in the mature internodes between the varieties (table 3.1.), however there was variation in other metabolite levels.

3.4.*2. Analysis of the sugarcane metabolome*

A bird's eye perspective

Measurement of the sugarcane metabolite complement (table 3.1.) has greatly increased our knowledge of steady state levels in the sugarcane culm. The most evident result is that the bulk of soluble metabolites decreased with tissue maturity as sucrose increased (figure 3.4.). This is not surprising as the enzymes for sucrose synthesis are present in higher amounts in these tissues than the immature internodes (Botha & Black 2000), and those that degrade sucrose are down regulated (Vorster & Botha 1999; Rose & Botha 2000). Comparison of N19 and US6656-15 metabolite levels (figure 3.4.) brought a few interesting things to light. Higher levels of maltose were observed in the mature internode of US6656-15. This could be indicative of starch turnover that was not as active in the commercially bred *Saccharum* interspecific hybrid. Levels of glycolytic intermediates, some organic acids and amino acids were higher in the ancestral variety. This could have been as a result of a higher allocation of carbon to respiration as an alternate sink to sucrose. With all these observations in mind it is clear that the mature sugarcane internode is unequivocally a dedicated sucrose storer. Nevertheless, we still have many gaps in our understanding of the regulation of sucrose accumulation in higher plants.

Observations from a bioinformatics perspective

Analysing interactions of steady state levels of the complete metabolome may give us an indication as to the points of metabolic control. PCA of all metabolites in both varieties showed that the older internodes (6 and 10) clustered together (figure 3.5.). This was confirmed in the HCA in figure 3.6. From these descriptive statistics it would appear that,

between these two varieties, tissues of the same age have similar metabolic functions based on metabolite interactions.

In internode 10, sucrose was the component that least conformed to the principal components (figure 3.7C). When comparing the varieties per internode as the variable in the PCA, the three internodes again had different metabolite distributions (figure 3.7.). The fact that sucrose, glucose and fructose did not cluster with other metabolites was not so surprising, as the system is geared towards producing large amounts of these metabolites, while the others remain in relatively low abundance. An exception to this is aconitate (figure 3.7A). Aconitate is an intermediate in the TCA cycle and accumulated to high levels in young internodes (table 3.1.). In comparison with other metabolites, using the linear matrix analysis represented in figure 3.8., aconitate appeared to be co-regulated with other glycolytic and TCA cycle intermediates (figure 3.11.). However, as mentioned in the results section, these correlations were forced by a single data point (from *S. spontaneum* young internodes), that was so much higher than other tissues that the linearity of the relationship was made significant. This observation would never have been made from the Metabocliques analysis alone, and serves to show that blind application of analysis tools can be dangerous. Had this not been the case, and aconitate levels decreased more gradually with tissue maturity, still giving linear correlations to other metabolites, the Metabocliques analysis would have provided a useful platform from which to analyse other components and interactions of aconitate metabolism, such as gene and protein expression and regulation.

Trehalose displayed linear correlations to the sugars xylose and ribose and the sugar alcohol, inositol (figure 3.10.). None of these metabolites serve as substrates or products for any reaction involving the other. The reason(s) for their linear correlations were not plainly obvious. Trehalose and inositol share the substrate glucose-6-phosphate in their biosynthetic pathway. Neither trehalose, nor inositol showed significant correlations to glucose-6-phosphate, indicating either that the analysis is confounded by interpreting the extractions as non-compartmentalised, or otherwise that they are regulated at a level other than substrate availability. The fact that there was no significant correlation between trehalose and sucrose was not surprising as the degradative rate through trehalase is high (10 $nmol.min^{-1}.g^{-1}$ FW) (Fleischmacher et al. 1980) when compared to the average low levels of its substrate (2 $nmol.g^{-1}$FW) determined in this study, implying rapid turnover. The product of the reaction is glucose which is rapidly assimilated into metabolism. So unless trehalose or one of its derivatives acts as a signalling molecule, from the correlations it does not appear to directly affect sucrose metabolism in the sugarcane culm.

The insider's perspective

At this stage is in important to recall a couple of aspects of plant anatomy. Firstly, any one tissue consists of numerous cell types, whose functions vary. This variation in function anticipates a variation in measured metabolite levels. Extracting metabolites from such a collection of cells, and analysing ensuing metabolite data would allow us to interpret the data in light of the nature of tissue sampling. Secondly, the plant cell does not exist as a single compartment. If one assumes that in these tissues the vacuole is 90% of the cellular volume (Komor 1994), and the metabolites measured are localised to various compartments, including the cytosol, plastid and mitochondrion, it is clear that data from complete cell extracts can only be analysed and correlated to a certain point, beyond which we tread on too many assumptions. Techniques are being developed to determine sub-cellular metabolite levels (Stitt & Fernie 2003). Even though such data would give greater insight to a study like the one presented in this Chapter, current techniques are not able to produce data of so many individual metabolites in as short a period of time as those optimised for invasive, whole tissue metabolomics.

Apart from the nature of plant tissues, the value of metabolomics can only be realised when the correct analyses are applied to the data. For example, the researcher needs to rationally explain the many linear correlations that can result from studies such as this. Some are easily elucidated, for example the linear correlations between fructose-6-phosphate and glucose-6-phosphate, or glucose-1-phosphate and UDP-glucose (figure 3.8.). Both reactions are separated by a single enzyme (phosphoglucomutase and UDP-glucose pyrophosphorylase, respectively) that operates close to equilibrium. Thus it would be strange if these correlations were not significantly linear. In contrast, rationalising the linear relationship between fructose-6-phosphate and 2-oxo-glutarate (figure 3.8.) is more difficult. These metabolites are present in the cytosol and mitochondrion respectively and involved in completely different metabolic pathways. Drawing any conclusion of the control over each other's regulation from the observed relationship would be presumptuous.

A realist's perspective

The purest form of biological science is the description of a living system in mathematical terms, and this with the purpose of creating a platform from which to create new hypotheses. In the quest for describing the intricacies of living systems around us, we are faced with many technical challenges and practical limitations. One has to do the best with what is

available to endeavour to disprove the hypothesis that one started out with. The work that is presented in this Chapter should be regarded from such a perspective.

The contribution of describing the sugarcane metabolome, even with its restrictions of mixed cell type and sub-cellular compartments, has contributed greatly to our knowledge of the genus *Saccharum*. Leaving the data here would be careless. Metabolomics is not a study that should be regarded on its own, rather it should be seen in the context of both the transcriptome and the proteome, and should be used to further define a holistic understanding of metabolism. The data should be incorporated into the description of a living system. In Chapter 6 we present such a description in the form of a kinetic model that analyses metabolic control. Although the model is built on kinetic parameters of the enzyme reactions mapped, the steady-state levels of these metabolites will contribute to the validation of the model. In this way the sugarcane metabolomics data collected here will be incorporated into a mathematical description of sugarcane sucrose metabolism with the purpose of identifying points of metabolic control, and creating a place from which further hypotheses can be created.

3.5. Conclusion

Trehalose has been identified and quantified from five different sugarcane varieties for the first time. Its concentration displayed no obvious direct relationship to sucrose levels, and it remained at levels so low that it contributed less than 0.0004% of the total sugar pool. Perhaps the only mechanism by which trehalose metabolism could affect sucrose levels would be in a signalling capacity. In this study we have not measured cellular levels of trehalose-6-phosphate, which is a possible contributor to carbon regulation, if one were to extrapolate its function from yeast (Thevelein & Hohmann 1995). The current data will be used to validate the metabolic control analysis presented in Chapter 6.

3.6. References

Adams RP, Kendal E, Kartha KK (1990) Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. Food and Chemical Toxicology 40: 871-898

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Bergmeyer HU, Bernt E (1974) Sucrose. In HU Bergmeyer, ed Methods of Enzymatic Analysis, Ed. 2. Verlag Chemie Weinheim, Academic press, New York, pp 1176-1179

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Australian Journal of Plant Physiology 27: 81-85

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Farrar J, Pollock C, Gallagher JA (2000) Sucrose and the integration of metabolism in vascular plants. Plant Science 154: 1-11

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Huber SC, Huber JL (1996) Role and Regulation of Sucrose-Phosphate Synthase in Higher PLants. Annual Review of Plant Physiology and Plant Molecular Biology 47: 431-444

Komor E (1994) Regulation of futile cycles: the transport of carbon and nitrogen in plants. In E-D Schulze, ed Flux Control in Biological Systems. Academic Press, San Diego, CA, pp 153-201

Kose K, Weckwerth W, Linke T, Fiehn O (2001) Visualizing plant metabolomic correlation networks using clique-metabolite matrices. Bioinformatics 17: 1198-1208

Lunn JE, MacRae E (2003) New complexities in the synthesis of sucrose. Current Opinion in Plant Biology 6: 208-214

Moore PH (1995) Temporal and Spatial Regulation of Sucrose Accumulation in the Sugarcane Stem. Australian Journal of Plant Physiology 22: 661-679

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmetally Modified Plant Systems. The Plant Cell 13: 11-29

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. The Plant Journal 23: 131-142

Rohwer JM, Botha FC (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochemical Journal 358: 437-445

Rose S, Botha FC (2000) Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. Plant Physiology and Biochemistry 38: 819-824

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Stitt M, Fernie AR (2003) From measurements of metabolites to metabolomics: an 'on the fly' perspective illustrated by recent studies of carbon-nitrogen interactions. Current Opinions in Biotechnology 14: 1-9

Stitt M, Gerhardt R, Kurzel B, Heldt HW (1983) A role for Fructose 2,6-Bisphosphate in the Regulation of Sucrose Synthesis in Spinach Leaves. Plant Physiology 72: 1139-1141

Sumner LW, Mendes P, Dixon RA (2003) Plant Metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62: 817-836

Thevelein JM, Hohmann S (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? Trends in Biological Sciences 20: 3-10

Vogel G, Fiehn O, Jean-Richard-dit-Brussels L, Boller T, Wiemkien A, Aeschbacher RA, Wingler A (2001) Trehalose metabolism in *Arabidopsis*: occurence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. Journal of Experimental Botany 52: 1817-1826

Vorster DJ, Botha FC (1999) Sugarcane Internodal Invertases and Tissue Maturity. Plant Physiology 155: 470-477

Whittaker A, Botha FC (1997) Carbon Partitioning during Sucrose Accumulation in Sugarcane Internodal Tissue. Plant Physiology 115 : 1651-1659

CHAPTER 4

EXPRESSION OF TRANSCRIPTS AND ENZYME ACTIVITIES INVOLVED IN SUGARCANE TREHALOSE METABOLISM

Abstract

Although trehalose is present in sugarcane (figure 3.2.) little is known about its metabolism in the genus *Saccharum* (sugarcane). Until recently, only trehalase activity had been measured in sugarcane extracts (Alexander 1973), but nothing was known about the other enzymes or any of the genes involved in trehalose biosynthesis in sugarcane. Transcriptomics research provided partial transcripts that were putatively identified as trehalose-6-phosphate synthase (*TPS*) and trehalose-phosphate phosphatase (*TPP*) (Carson et al. 2002). The aim of this study was to identify the transcript and protein machinery necessary for the production of trehalose. In this study two novel sugarcane partial cDNAs that coded for trehalase (*tre*) and *actin* were isolated. The latter was required as a standard to normalise gene expression against. Transcript expression profiles were completed using RT-PCR analysis of RNA extracted from different *Saccharum* genotypes, and internodal tissues at different stages of maturity. A putative full-length *SugTPS* transcript was isolated and characterised by both *in silico* analysis, and *in vivo* complementation of the *Saccharomyces cerevisiae Δtps1* mutant. Enzyme activities for TPS, TPP and trehalase were measured from plant protein extracts. In *Saccharum* interspecific hybrid N19, 2.7 $nmol.min^{-1}.mg^{-1}$protein TPS, 8.5 $nmol.min^{-1}.mg^{-1}$protein TPP and 6.2 $nmol.min^{-1}.mg^{-1}$protein trehalase activities were measured from young internodal protein extracts. Trehalose-6-phosphate degradative enzyme activity and transcript levels were higher in *S. spontaneum* than *Saccharum* interspecific hybrids.

4.1. Introduction

Transcriptomics has added greatly to the investigation and understanding of metabolism, not only in plants but in all living organisms (Hegde et al. 2003; Meyers et al. 2004). Many questions about the regulation of gene expression and metabolism in different scenarios, such as changing environmental conditions, different developmental stages, diverse organs and their constituent tissue types have been answered (Buckhout & Thimm 2003; Gibson 2005). However, along with those sought after answers, many new questions have arisen. One such example is that presented in this Chapter. While searching for differentially expressed genes in the sugarcane internodal transcriptome, cDNAs coding for putative trehalose biosynthetic genes were sequenced (Carson et al. 2002; Casu et al. 2003). The presence of such genes that could potentially lead to the production of a disaccharide, that at the time had not been shown to be present in this dedicated sucrose storer, led to the inception of this project. In plants it is thought that the combined activities of trehalose-6-phosphate synthase (EC 2.4.1.15, TPS) and trehalose-6-phosphate phosphatase (EC 3.1.3.12, TPP) are responsible for the synthesis of trehalose (Goddijn & van Dunn 1999). Degradation of trehalose is accounted for by the enzyme trehalase (EC 3.2.1.28) (Goddijn & van Dunn 1999).

To date, the only published references to trehalose metabolism in sugarcane are the measurements of the hydrolytic activity of trehalase (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980). In fact, trehalase was first identified in plants in sugarcane, but no conclusion as to its role in metabolism could be agreed upon because of the apparent lack (or undetectable levels) of its substrate in sugarcane tissues. Interest in the metabolism was renewed upon sequencing of ESTs putatively involved in trehalose metabolism in sugarcane, owing to the importance of the trehalose metabolic pathway in the yeast *Saccharomyces cerevisiae*, and especially the intermediate trehalose-6-phosphate, whose presence at even µM levels has been shown regulate the flux of carbon entering glycolysis (Arguelles 2000).

Mining the sequence data of the completed *Arabidopsis thaliana* genome rendered many potential trehalose metabolism gene candidates (Leyman et al. 2001). In this plant 11 putative *TPS*s exist, of which *AtTPS1* has been the most extensively characterised of any of the genes involved in *A. thaliana* trehalose metabolism (Eastmond et al. 2002; Van Dijck et al. 2002; van Dijken et al. 2004; Avonce et al. 2004). On a transcript level this enzyme is most highly expressed in flowers and siliques. *In planta* mutations of this gene have shown it

to be vital to embryo maturation, vegetative growth, transition to flowering and involvement in various signalling pathways. Besides this one, none of the other isolated *AtTPS* genes have been successfully expressed in a heterologous organism, or their TPS function determined (Vogel et al. 2001; Van Dijck et al. 2002). This has led to the assumption that this multigene family has a more complicated interaction than initially expected. Despite all the investigations of the *AtTPS* genes, there has been no literature reference to TPS activity measured from *A. thaliana* plant extracts. This is perhaps due to competitive enzymes that complicate its assay, as well as the limited amount tissue available from this plant's organs. TPS activity has been described in the resurrection plant *Selaginella lepidophylla* whose distinguishing feature is its accumulation of trehalose preferentially to sucrose (Valenzuela-Soto et al. 2004). Because of the latter fact, this plant's metabolism is not suited for comparison to *A. thaliana,* and indeed *Saccharum* species that are geared towards sucrose accumulation and utilisation.

In this Chapter we report on two novel transcripts for *trehalase* and *actin* that together with those of *TPS* and *TPP* were profiled in different *Saccharum* genotypes and internodal tissues. Isolation of a putative full-length sugarcane *TPS* (*SugTPS)* is described along with its *in silico,* and *in vivo* characterisation in the yeast *Δtps1* mutant. In addition, enzyme activities of TPS, TPP and trehalase from sugarcane tissues are reported, and the latter two profiled in two distinct *Saccharum* species.

4.2. Materials and Methods

4.2.1. Plant material

Saccharum interspecific hybrids N19, N27 and N31 along with *S. spontaneum* US6656-15 and *S. robustum* NG7794 were grown in field plots at the South African Sugar Research Institute, Mount Edgecombe, KwaZulu Natal, South Africa. Plants were harvested at the end of an 18 month growth season.

4.2.2. Reagents for biochemical and molecular biological analyses

Substrates, cofactors and coupling enzymes for biochemical assays were purchased from Roche Diagnostics GmbH, Penzberg, Germany. Restriction enzymes and Taq DNA Polymerases were purchased from the Promega Corporation, Madison, USA. The *E. coli* DH5α-strain (Hanahan 1983) was used for all subcloning and construct assembly.

4.2.3. Isolation of partial cDNAs coding for trehalase and actin

RNA was isolated from *Saccharum* interspecific hybrid N19 (Bosch et al. 2004). cDNA was reverse transcribed from 4 µg total RNA using SuperScript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, California, USA) according to the manufacturer's instructions. Amplification of a cDNA putatively identified as a *trehalase (tre)* gene was achieved using 1 unit Taq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward primer Tre_for (5'-GGT CAG GTC CAT AAT TTG TCT CGG-3'), 0.2 µM reverse primer Tre_rev (5'-CCA GAT TGC TCT TCG GCG AGA CC-3') and 2 µl cDNA reaction template in a final volume of 20 µl. PCR conditions were as follows: 4 minute denaturation at 94°C; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for one minute; Final elongation step of 72°C for 7 minutes. The resulting amplicon of approximately 900 bp was separated on a 1% (w/v) agarose gel, excised and sequenced for identification. Sequencing was done using automated procedures with the ABI PRISM Dye Terminator kit (PerkinElmer Life Sciences, Boston, MA, USA).

A novel *actin* fragment was amplified from cDNA synthesised from *Saccharum* interspecific hybrid N27 as described above for *tre*. PCR amplification was as described for *tre* above with the annealing temperature at 50°C, and primers actin_forw1 (5'-TCACACTTTCTACAATGAGCT-3') and actin_rev1 (5'-GATATCCACATCACACTTCAT-3'). The resulting approximately 600 bp fragment was identified as a sugarcane *actin* by sequence analysis.

4.2.4. Primer design for transcript isolation and expression profiling of TPS and TPP

Primers used for the amplification of *TPS* and *TPP* partial cDNAs for expression profiling were designed against EST clones I2-245 and I7-154 respectively (Carson et al. 2002) (table 4.1.).

Table 4.1. Primers used in the amplification of *TPS* and *TPP* transcripts for expression profiling. Restriction sites were designed in the primer sequences to aid cloning, these are highlighted.

Target gene	***Primer name***	***Restriction site***	***Sequence (5'→3')***
TPS	TPS_for	*Bam*HI	CTAGGGATCCTACATCGAGCACAAG
	TPS_rev	*Eco*RI	GTCGGAATTCCACACGATGCTCTC
TPP	TPP_for	*Eco*RI	CGGGGAATTCGTTGCAAAGCGTTTC
	TPP_rev	*Sal*I	CGGGGTCGACACCTTCAATTTCACG

A putative full-length *SugTPS* was isolated from N19 cDNA reverse transcribed from internode 3 RNA. Primers for the amplification were designed against sugarcane ESTs from the NCBI database that had high homology to other known *TPS*s (figure 4.5.). Sequencing of the *SugTPS* gene was done by sequentially designing primers as represented in table 4.2.

Table 4.2. Primers used in the amplification and sequencing of *SugTPS*.

Primer name	***Position on SugTPS***	***Sequence (5'→3')***
TPS_ATG	1→23	ATGCCTTCGCTCTCCTCGCACAA
TPS_1	947→932	GCGTCGACAAACGTCATTCTGTTC
TPSi_R	1200→1178	GTAGTAGGCGGCCTTCTCCTGCG
TPSi_F	1207→1227	GCCGAGTGCTGTGTCGTGAGC
TPS_2	1759→1736	TCAACACAGAGATCACCTCACTGC
TPS_for	1985→2010	CTAGGGATCCTACATCGAGCACAAG
TPS_rev	2298→2275	GTCGGAATTCCACACGATGCTCTC
TPS_3	2597→2573	AACCTGTCGATCCTGTAACTGCTG

4.2.5. Complementation of the Saccharomyces cerevisiae Δtps1 mutant

4.2.5.1. Strains and media

Yeast strains were derived from wild-type W303-1A (*Mat***a** *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2*); *Δtps1,* YSH290 (W303-1A, *Δtps1*: :*TRP1*); *Δtps2,* YSH450 (W303-1A, *Δtps2*: :*LEU2*); *Δtps1Δtps2,* YSH290 (W303-1A, *Δtps1*: :*TRP1, Δtps2*: :*LEU2*). Rich media (YPgal) contained 20% (w/v) peptone, 10% (w/v) yeast extract and 2% (w/v) galactose. Minimal media (SD) contained 1.7% (w/v) Yeast Nitrogen Base (Difco, Detroit, USA), 5% (w/v) ammonium sulphate, 2% (w/v) glucose or galactose as carbon source, and amino acids without uracil (0.04% (w/v) adenine, 0.05% (w/v) arginine, 0.08% (w/v) aspartate, 0.02% (w/v) histidine, 0.05% (w/v) isoleucine, 0.1% (w/v) leucine, 0.05% (w/v) lysine, 0.02% (w/v) methionine, 0.05% (w/v) phenylalanine, 0.1% (w/v) threonine, 0.05% (w/v) tryptophan, 0.05% (w/v) tyrosine and 0.14 % (w/v) valine). For induction of gene expression 100 µM $CuSO_4$ was added to the media.

4.2.5.2. Cloning the SugTPS into a yeast expression vector

The sugarcane transcript (*SugTPS*) containing the 2487 bp ORF was amplified with primers containing *Xho*I and *Kpn*I restriction sites on the 5' and 3' ends respectively. The amplicon was ligated into the pGEM®-T Easy vector (Promega Corporation). The S*ugTPS* fragment was restricted from the pGEM®-T Easy- S*ugTPS* construct using *Xho*I and *Kpn*I and ligated into the pSAL4 yeast expression vector (Appendix 4.C.). This vector placed the gene under control of the Cu^{2+} inducible CUPI promoter.

4.2.5.3. Yeast transformation

Three ml liquid YPgal were inoculated with the mutant under investigation and incubated overnight at 30°C, 220 rpm. One and a half ml of this was used to inoculate 50 ml YPgal the next morning. The culture was incubated at 30°C, 220 rpm until an OD_{600} of ± 1 was reached. Cells were collected by centrifugation at 800 *xg* for 5 minutes. The supernatant was discarded and cells resuspended in one ml 100 mM lithium acetate and transferred to a 1.5 ml Eppendorf tube. The suspension was centrifuged at 2000 *xg* for two minutes and the supernatant discarded. The pellet was resuspended in a maximum volume of 500 µl 100 mM lithium acetate (enough for 10 transformations). The transformation reaction was set up by the sequential addition of the following: 50 µl cells in lithium acetate, 300 µl 40% (w/v)

PEG 3350 in 100 mM lithium acetate, 5 µl 10 mg.ml^{-1} heat denatured herring sperm DNA and plasmid in a volume of 50 µl. The mixture was briefly vortexed and incubated at 42°C for 30 minutes with gentle agitation. Thereafter the transformation reaction was centrifuged for 2 minutes at 2000 *xg* and the supernatant discarded. The cells were resuspended in 500 µl sterile milliQ water and plated out on SDgal-URA. After incubation at 30°C for three days transformed colonies were used for further experiments.

4.2.5.4. Enzyme extraction and TPS activity determinations

Soluble proteins were extracted from cells collected from 50 ml liquid cultures. The extraction buffer contained 50 mM imidazole (pH 6.3), 1 mM EDTA, 100 mM $MgCl_2$ and 10 mM phenylmethylsulfonylfluoride. Prior to enzyme assays all samples were desalted using Sephadex G25 pre-equilibrated with the 200 mM Tricine (pH 7). TPS activity was determined by quantification of UDP production in a stop assay (Hottiger et al. 1987), modified to microtiter plate format. Final TPS assay conditions were 50 mM Tricine (pH 7), 12.5 mM $MgCl_2$, 5 mM UDP-glucose and 3.3 mM glucose-6-phosphate. Each assay contained 30 µl desalted protein extract. Reactions were incubated at 30°C for 30 minutes and then boiled for 5 minutes to stop any enzyme activity. Control reactions without glucose-6-phosphate were run in parallel. Production of UDP was measured by coupling to the oxidation of NADH via the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH). Twenty µl of the TPS assay was included in the UDP assay containing 70 mM Tricine (pH 7.6), 1.76 mM phosphoenolpyruvate, 0.25 mM NADH and PK/LDH at one unit per assay.

Proteins were quantified using the Bradford method (Bradford 1976) and IgG as a standard. SDS-PAGE separation of proteins was carried out as previously described (Laemmli 1970).

4.2.6. Extraction and assay of enzymes from sugarcane internodal tissues

Enzymes were extracted from sugarcane internodal tissues and desalted as previously described (Rose & Botha 2000). Partially purified enzyme samples used for determination of TPS activity had been separated on an anion exchange column (HiTrapQ Sepharose, Pharmacia Biotech) and eluted with 200 mM NaCl.

4.2.6.1. TPS assay using GCMS determination of trehalose

The TPS assay was run under final assay conditions of 40 mM Hepes (pH 7.5), 16 mM $MgCl_2$, 20 mM UDP-glucose, 10 mM glucose-6-phosphate and one mM NaF in a final volume of 125 µl. The reaction was incubated at room temperature for one hour and stopped by boiling for 10 minutes. Sixty µl of the reaction was dried down under vacuum, derivatised and analysed by GCMS as described in section 3.2.3.

4.2.6.2. TPS assay using ^{14}C radiolabel

Proteins extracted from young internodal tissue of *Saccharum* variety N19 (1:2 w/v 100 mM Tris (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 10% (w/v) glycerol, 2% (w/v) polyvinylpolypyrrolidone and Complete® protease inhibitors) were saturated to 95% ammonium sulphate and centrifuged at 13 000 *xg* for 25 minutes at 4°C to collect all precipitated proteins. The pellet was resuspended in 100 mM Tris (pH 7.5) and desalted on a pre-equilibrated 5 ml Sephadex G-25 column. Assay conditions for TPS activity were 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM UDP-glucose, 5 mM glucose-6-phosphate, 10 mM 6-phosphogluconate and 10 mM NaF. Each reaction contained 10 kBq UDP-D-[U-^{14}C]glucose (Amersham Biosciences, UK Limited), resulting in a specific activity of 40 $Bq.nmol^{-1}$ UDP-glucose. Assays were set up in duplicate and stopped after either one or two hour reaction times by boiling for 10 minutes. Each sample was treated with 10 U alkaline phosphatase for one and a half hours. Reactions were boiled for 10 minutes to denature all proteins. The samples were centrifuged at 13 000 *xg* for one minute, and the supernatant transferred to a 1.5 ml Eppendorf tube, whereafter they were dried down under partial vacuum (Speed Vac ® Plus SC110A, Savant Instruments, Inc., Holbrook, NY, USA). The reaction products were then resuspended in 10 mM Tris (pH 7.5) containing 10 mM trehalose, glucose, fructose and sucrose each. Separation of sugars was achieved by anion exchange HPLC using an RCX-10 column (Hamilton Company, Reno, Nevada, USA). The flow rate of the eluant (60 mM NaOH) was 1 $ml.min^{-1}$ and the total separation time of the abovementioned sugars 12

minutes. Retention times for trehalose, glucose, fructose and sucrose were 4.0, 5.3, 5.9 and 8.8 minutes respectively. These peaks were collected and counted on a Tri-Carb® scintillation analyzer (PerkinElmer Life Sciences, Boston, MA, USA) to determine levels of radio-activity.

4.2.6.3. TPP and Trehalase activity assays

Both TPP and trehalase activities were shown to be linear over time period of one hour. Trehalase was assayed by measuring the production of glucose. Trehalase was assayed in 50 mM K-Mes (pH 6.5) with 60 mM trehalose for one hour at 37°C. The reaction was stopped by boiling and glucose determined by the method of Bergmeyer and Bernt (Bergmeyer & Bernt 1974). TPP was assayed in 50 mM K-Mes (pH 6), 10 mM $MgCl_2$ and 1.6 mM trehalose-6-phosphate. After the reaction had run for an hour it was stopped by boiling. After 5 minutes it was allowed to cool to room temperature where after 0.02 U trehalase (Sigma-Aldrich, Missouri, USA) was added and the reaction allowed to continue to completion before assaying for glucose as above.

4.2.6.4. Hexose phosphate isomerase (HPI) and sucrose-6-phosphate synthase (SPS) activity assays

HPI was assayed with 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1.75 mM NADP, 1 U per assay glucose-6-phosphate dehydrogenase, 10 mM NaF and 4.4 mM fructose-6-phosphate in a final volume of 250 µl. The reaction was followed real-time at 340 nm using a spectrophotometer. SPS was assayed as previously described (Botha & Black 2000).

4.3. Results

Identification and expression profiling of the transcripts and enzymes involved in trehalose metabolism was the first step in the description of the mechanism of production of this disaccharide in sugarcane.

4.3.1. Partial cDNAs coding for enzymes of trehalose metabolism

4.3.3.1. Identification by sequence homology

TPS and TPP

EST clones I2-245 (TPS) (Appendix 4.A1.) and I7-154 (TPP) (Appendix 4.A2.) (Carson et al. 2002) were sequenced and their identities confirmed by homology to known sequences of functional plant *TPS* (trehalose-6-phosphate synthase) and *TPP* (trehalose-6-phosphate phosphatase) genes. The cDNA I2-245 displayed 41% and 45% nucleotide identity to *Arabidopsis thaliana AtTPS1* (accession number Y08568) and *Selaginella lepidophylla SlTPS1* (accession number SLU96736) in the area of homology, respectively. The cDNA I7-154 had 49.5% and 47.9% nucleotide identity to the corresponding segments of *AtTPPA* (accession number 2944177) and *AtTPPB* (accession number 2944179) respectively. To facilitate specific amplification of these two genes primers were designed to amplify fragments of 307 bp of the *TPS* gene and 262 bp of the *TPP* gene from sugarcane cDNA. The results clearly indicate a highly specific amplification of specific cDNA of the expected sizes (figure 4.1A and B).

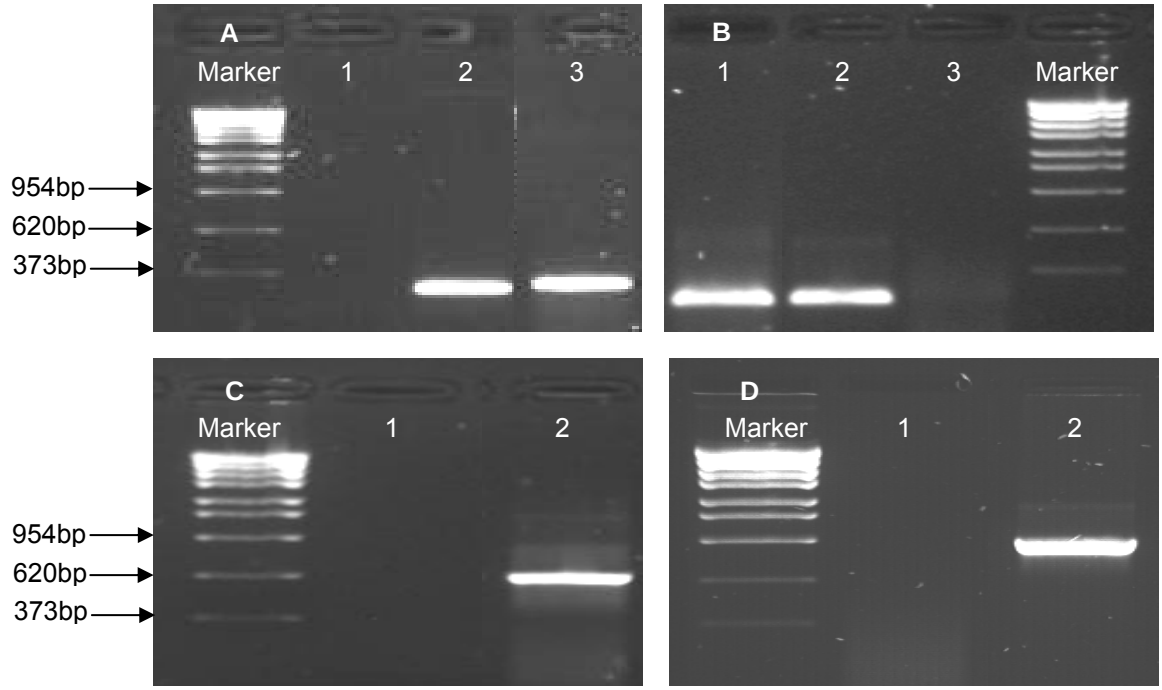


Figure 4.1. Amplification of partial cDNA's putatively involved in trehalose metabolism from sugarcane internodal tissue. Panel A: *TPS* (307 bp fragment) amplified from water, plasmid (containing the I2-245 EST) and single stranded cDNA were used as templates in lanes 1, 2 and 3 respectively. Panel B: *TPP* (262 bp fragment) amplified form first strand cDNA, and plasmid (harbouring the I7-154 EST) and water in lanes 1,2 and 3 respectively. Panel C: Amplification of an *actin* fragment (approximately 660 bp) from sugarcane first strand cDNA in lane 2, lane 1 is the water control. Panel D: Approximately 900 bp *tre* fragment was amplified from first strand cDNA in lane 2, lane 1 is the water control.

Trehalase

In order to amplify sugarcane trehalase, primers were designed against a consensus sequence constructed from two sugarcane ESTs (accession numbers CA148153.1 and CA292414.1) identified on the NCBI database using the functional *GMTreI* (accession number 4559291) as a query. These primers enabled the amplification of a cDNA fragment of approximately 900 kb. The sequence of this fragment, designated *tre,* was compared with other known trehalase sequences (table 4.3.). On both nucleotide and amino acid level there was a high level of sequence identity between the *Saccharum* sequence and both the *Oryza sativa* (accession number 31433100) and the *Glycine max* (accession number 4559291) sequences. It was therefore assumed that this fragment represented sugarcane trehalase (figure 4.1C and Appendix 4.A3.).

Table 4.3. Sequence homology of a novel partial *Saccharum officinarum* trehalase sequence with that of trehalases from *Oryza sativa* (accession number 31433100) and *Glycine max* (accession number 4559291). Sequence alignments for both nucleotide and amino acid similarity were carried out using ClustalW multiple alignment.

	% identity (nucleotide bases)		% identity (amino acid residues)	
	S. officinarum	***G. max***	***S. officinarum***	***G. max***
S. officinarum		79.9		60.5
O. sativa	63.5	63.3	75.5	58.8

Actin

For the purposes of including an internal control in the RT-PCR expression analysis, a fragment of approximately 600 bp was amplified using *actin* primers. The resulting sugarcane fragment was identified as coding for actin by sequence homology. On amino acid level the fragment showed over 95% identity with another sugarcane sequence (accession number AY742219) and one from *Zea mays* (accession number BT018135) (table 4.4.).

Table 4.4. Comparison of actin sequences with the novel EST isolated from *S. officinarum*. % identity on both nucleotide and amino acid level were determined using ClustalW multiple alignments. Sequences from previously described actin genes were used: *S. officinarum (So)* (accession number AY742219) and *Zea mays* (accession number BT018135).

	% identity (nucleotide bases)		% identity (amino acid residues)	
	S. officinarum	***Z. mays***	***S. officinarum***	***Z. mays***
S. officinarum		92.8		95.2
So AY742219	95.4	93.7	96.0	99.2

The above-described transcripts identified for the three enzymes involved in trehalose metabolism in sugarcane, along with an internal control, provided the molecular tools for the expression analyses described below.

4.3.2. Transcript expression of trehalose metabolizing enzymes in the sugarcane culm

4.3.2.1. Optimisation of the detection method

Total RNA was extracted from field grown N19, N31, N27 and two ancestral *Saccharum* species: *spontaneum* (US6656-15) and *robustum* (NG7794). Transcript levels of *TPS* and *TPP* were determined by both Northern blot analysis and RT-PCR. Comparison of the two methods on the same samples gave equivalent results (figure 4.2.). The sensitivity of the RT-PCR procedure was higher and thus more efficient, and all further transcript expression analyses were all done using RT-PCR.

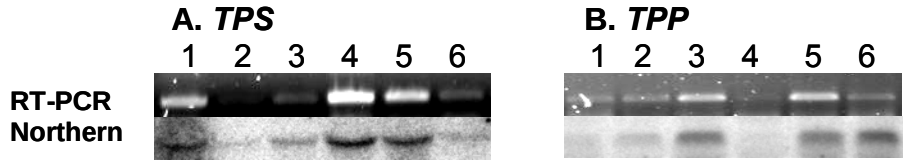


Figure 4.2. Northern blot and RT-PCR analysis of TPS and TPP for transcript expression. A: Trehalose phosphate synthase (*TPS*) transcript levels were detected from US6656-15: Lanes 1 through 3 and 4 through 6 are from separate plants. Internode 3 is represented in lanes 1 and 4, internode 8 in lanes 2 and 5, internode 11 in lanes 3 and 6. B: Trehalose phosphate phosphatase (*TPP*) transcript levels were detected from N27: Lanes 1 through 3 and 4 through 6 are from separate plants. Internode 3 is represented in lanes 1 and 4, internode 8 in lanes 2 and 5, internode 11 in lanes 3 and 6.

4.3.2.2. Transcript levels in different tissues

TPS and TPP

Transcript abundance of *TPS* by RT-PCR showed that in N19 there was a trend of decreasing expression from young to older internodes (figure 4.3A). In this variety, levels of the *TPP* transcript showed no particular pattern between internodes. N31 and N27 showed similar transcript expression trends for both *TPS* and *TPP* (figure 4.3B and C). *TPS* levels in the *S. spontaneum* (US6656-15) and *S. robustum* (NG7794) showed markedly higher levels in internode 3 than the older internodes sampled (figure 4.3D and E). In the latter two varieties *TPP* transcript levels were not detectable using the established experimental parameters.

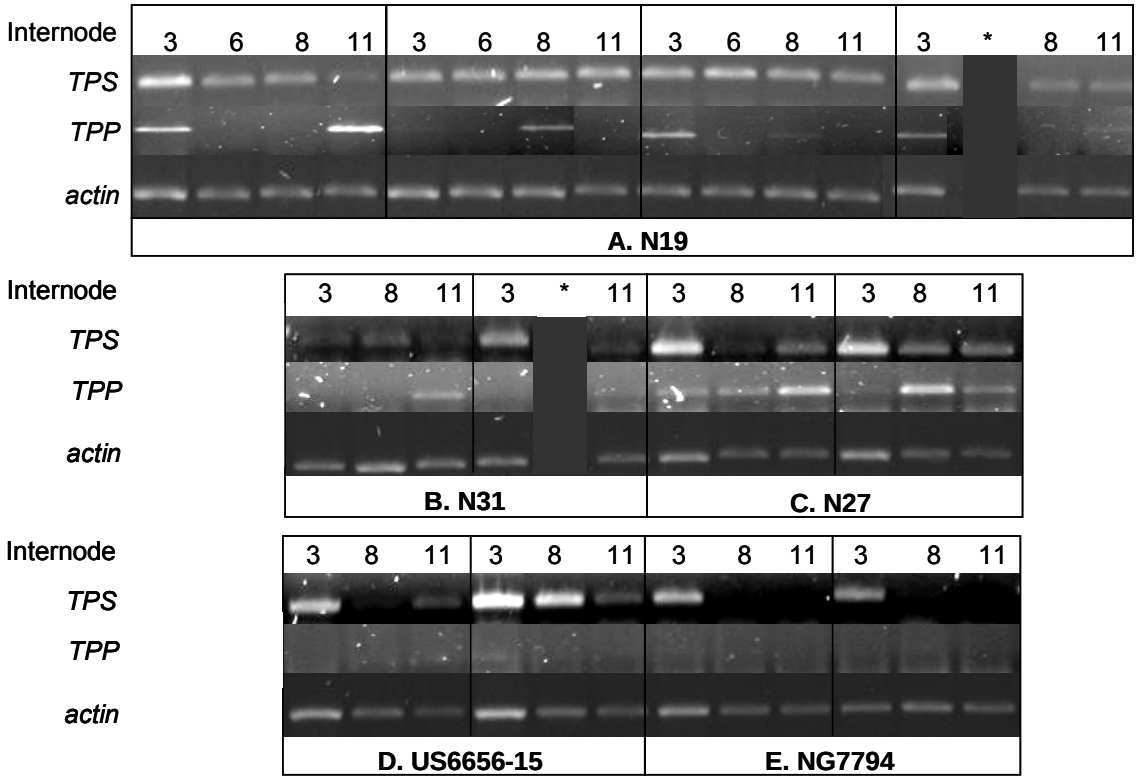


Figure 4.3. RT-PCR analysis of transcript expression of trehalose-6-phosphate synthase (*TPS*), trehalose-6-phosphate phosphatase (*TPP*) and actin from 5 *Saccharum* genotypes. *Saccharum* interspecific hybrids are represented by N19, N31 and N27 (A, B and C). *S. spontaneum* and *S. robustum* clones sampled were US6656-15 (D) and NG7794 (E) respectively. Tissues were sampled from internodes of increasing maturity from 3 to 11. Each set is representative of an individual plant. * indicate samples where reverse transcription was unsuccessful.

Trehalase

Trehalase transcript levels were highest in intermediate internode 8 in all plants across two genotypes (figure 4.4).

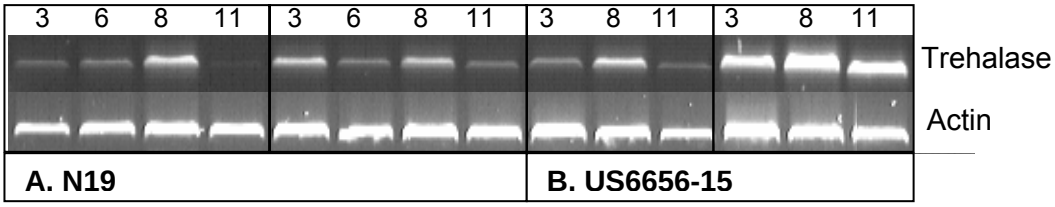


Figure 4.4. Transcript levels of *trehalase* and *actin* in interspecific *Saccharum* hybrid N19 (A), and *S. spontaneum* variety US6656-15. Internodes were sampled in increasing maturity from 3 to 11. Each set represents an individual plant.

Transcript levels varied between individuals of the same variety. This was observed for all three genes in figures 4.3. and 4.4., demonstrating the biological variation of individuals of the same genotype.

4.3.3. Amplification of a putative full-length trehalose-6-phosphate synthase cDNA

4.3.3.1. Isolation

Primers (TPS_ATG and TPS_3) were designed against sequences with homology to known *TPS* transcripts and were used for the amplification of a novel sugarcane cDNA (figure 4.5.). A cDNA of 2597 bp was amplified from cDNA prepared from sugarcane variety, N19 total RNA. Sequencing of this fragment indicated an open reading frame (ORF) of 2487 bp, and encoding a protein of 92.2 kDa. The deduced amino acid sequence is listed in Appendix 4.B.

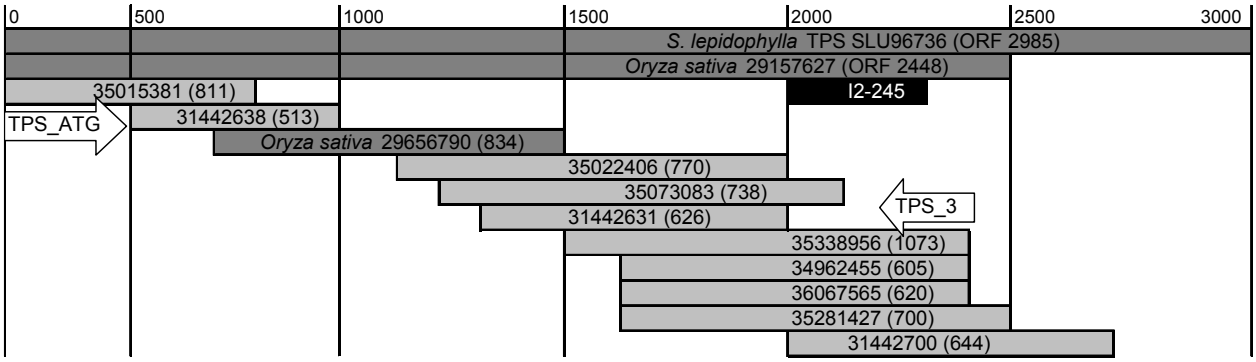


Figure 4.5. The *S. lepidophylla* TPS cDNA (accession number 4100324) was used to identify putative sugarcane TPS ESTs. Sequences were aligned and primers designed for amplification of a full length sugarcane TPS. Accession numbers are indicated for each sequence, with their sizes in parenthesis.

4.3.3.2. Molecular characterization by in silico sequence analysis

The *A. thaliana* genome contains 11 potential isoforms of *TPS* that have been divided into two classes (Leyman et al. 2001). The sugarcane sequence here (*SugTPS*) had highest residue identity on both nucleotide and amino acid level with *AtTPS11* (57% in both cases) (figure 4.6.). The amino acid sequences of all 11 *A. thaliana* and the sugarcane sequence were aligned using the ClustalW program, and then the consequent aligned identities used to cluster them in a Neighbour joining tree. The two classes of *TPS*s clustered apart, and this indicates that the *SugTPS* should have TPP-like properties. The deduced amino acid sequence indicated that two conserved phosphatase boxes were present in the sugarcane sequence (Appendix 4.B).

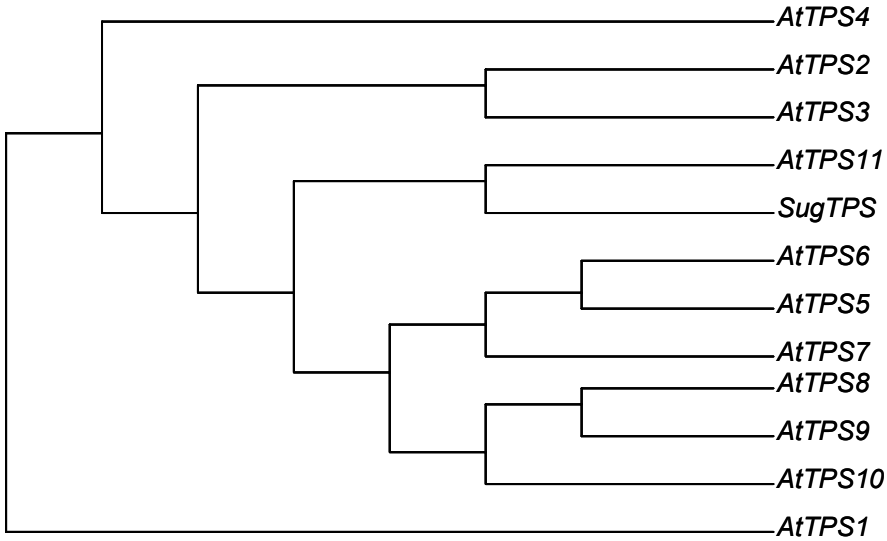


Figure 4.6. Relationship of novel *SugTPS* cDNA to the eleven isoforms of *TPS* identified from *A. thaliana* (Leyman et al. 2001) on amino acid level. Sequences were aligned using the ClustalW program and Neighbour joining method for construction of the tree.

4.3.3.3. Functional analysis of the putative sugarcane TPS in the yeast tps1 deletion mutant

Even though the isolated sugarcane transcript had been identified as a *TPS* by sequence analysis, its *in vivo* function still needed to be ascertained. For this purpose the *S. cerevisiae Δtps1* deletion mutant was used.

S. cerevisiae Δtps1 mutants transformed with the *SugTPS* under the control of a Cu^{2+} promoter in yeast expression vector pSAL4, were selected and streaked out onto minimal media -URA with either 2% (w/v) galactose or 2% (w/v) glucose as carbon source. These media were duplicated with or without 100μM Cu^{2+}. Both untransformed *Δtps1* and *Δtps1*pSAL4 (empty vector) were included as negative controls. The *ScTPS1* gene was included as a positive yeast control (*Δtps1*pSAL4*ScTPS1*), with full-length and truncated *AtTPS1* as positive plant controls (*Δtps1*pSAL4*AtTPS1* and *Δtps1*pSAL4*ΔNAtTPS1*). All transformed lines grew with galactose as sole carbon source (figure 4.7A and B). However, the *SugTPS* gene did not complement growth on glucose, with or without Cu^{2+} in the media.

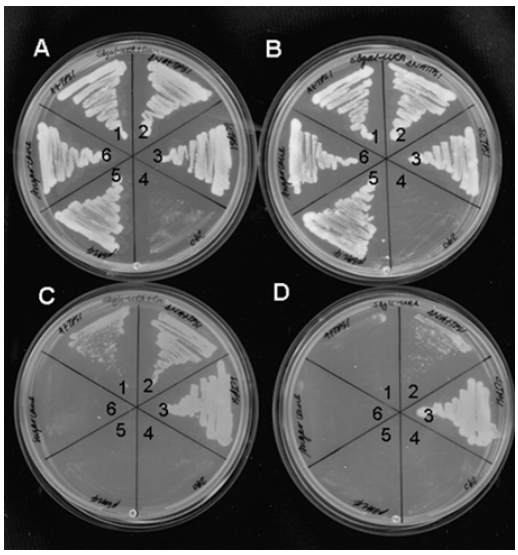


Figure 4.7. *S. cerevisiae tps1Δ* mutant with trehalose-6-phosphate synthase genes under the control of the CUP1 Cu^{2+} inducible promoter. Colonies were streaked out on minimal media without URA supplemented with 2% (w/v) galactose + 100μM $CuSO_4$ (A), 2% (w/v) galactose (B), 2% (w/v) glucose + 100μM $CuSO_4$ (C), 2% (w/v) glucose (D). Transformed lines were streaked out in the following order 1: *tps1Δ*pSAL4*AtTPS1*, 2: *tps1Δ*pSAL4ΔN*AtTPS1,* 3: *tps1Δ*pSAL4*ScTPS1*, 4. : *tps1Δ*, 5: *tps1Δ*pSAL4, 6: *tps1Δ*pSAL4*SugTPS*.

To further investigate reasons why the *SugTPS* did not complement the yeast mutant, transformants were cultured in different concentrations of Cu^{2+}, and galactose as carbon source. Proteins were extracted, assayed for TPS activity, and visualised by SDS-PAGE

and Coomassie staining (figure 4.8.). Both the untransformed *Δtps1* and *tps1Δ*pSAL4*SugTPS* lines had no detectable TPS activity. The *Δtps1*pSAL*ScTPS1* line displayed activity of 15 nmol.min^{-1}.mg^{-1}protein regardless of the concentration of Cu^{2+} in the medium (basal expression of the *ScTPS1* control was so high that it complemented the mutant without Cu^{2+} as an inducer (figure 4.7D)). It was therefore possible that the lack of complementation of the *Δtps1* mutant by the *SugTPS* gene was as a result of a lack of TPS activity. Visualisation of these protein extracts conveyed the possibility that the lack of activity could have been due to a lack of production of the protein, as the *Δtps1*pSAL4*SugTPS* line (figure 4.8., lanes 4, 5 and 6) showed no additional bands when compared with the untransformed control (figure 4.8., lanes 1, 2 and 3). The predicted molecular weight of the sugarcane protein is approximately 92k Da. The *ScTPS1* positive control showed production of an additional protein of 56.2 kDa corresponding to the expected gene product (figure 4.8., lanes 7 through 9).

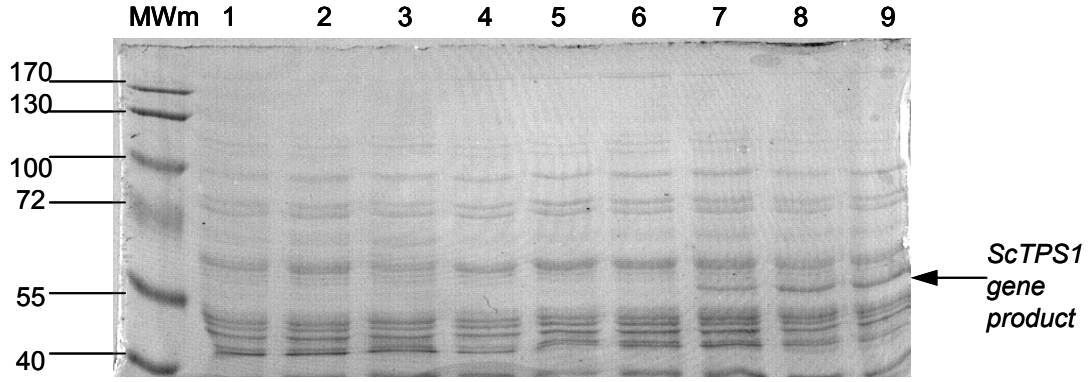


Figure 4.8. SDS-PAGE analysis of crude protein extracts from yeast mutant *Δtps1*. Untransformed controls cultured in 10, 50 and 100μM Cu^{2+}: lanes 1, 2 and 3. *Δtps1*pSAL4*SugTPS* cultured in 10, 50 and 100μM Cu^{2+}: lanes 4, 5 and 6. *Δtps1*pSAL4*ScTPS1* cultured in 10, 50 and 100μM Cu^{2+}: lanes 7, 8 and 9. The molecular weight marker is indicated by MWm, with sizes in kDa. The gel was stained with Coomassie blue.

Because of its conserved phosphatase boxes observed in the amino acid sequence (Appendix 4.B.) the *SugTPS* gene was tested for complementation of the *Δtps2 (*trehalose-6-phosphatase yeast deletion mutant) and the double deletion *Δtps1Δtps2* strain. However, there was no reversal of the lethal phenotypes resultant from deletion of these yeast genes. This suggests that either the transcript did not produce enough protein when translation was

induced, or that the protein produced did not have the ability to catalyse either the TPS or TPP reactions. It is also possible that the plant protein was not active in the cellular context of the host organism *S. cerevisiae*.

Amino acid alignment of the ORFs for functional TPS *AtTPS1* (accession no. Y08568), *SlTPS1* (accession no. SLU96736) and *SugTPS* revealed a difference in the 5' end of the protein. The *SugTPS* aligned 42 and 55 residues downstream from the *A. thaliana* and *S. lepidophylla* initial methionine residues respectively. This might imply that the *SugTPS* isolated above was not a full-length clone, but was in fact lacking its true start codon. A search for sugarcane ESTs on international databases using the 5' region of the *SugTPS* gene did not render any additional ESTs with upstream methionine residues in frame.

Regardless of the functionality of the isolated transcript, the fact that trehalose had been detected in sugarcane culm tissues (Chapter 3) meant that there must be enzymes in the plant that can synthesise this disaccharide. Evidence for this is presented below.

4.3.4. Evidence for the presence of trehalose biosynthetic and degradative enzymes in two sugarcane varieties

4.3.4.1. Trehalose-6-phosphate synthase (TPS)

TPS produces trehalose-6-phosphate from the substrates UDP-glucose and glucose-6-phosphate. The greatest challenge in measuring this activity from sugarcane internodal tissues is the abundance of enzymes that share these substrates. As sugarcane is a dedicated sucrose storer there is a high rate of flux from the hexose phosphate pool into sucrose in these tissues. The substrates for sucrose synthesis via sucrose-6-phosphate synthase (SPS) from this pool are UDP-glucose (common to the TPS reaction) and fructose-6-phosphate. Glucose-6-phosphate (common to the TPS reaction) is readily interconverted to fructose-6-phosphate by hexose phosphate isomerase (HPI). In tissues used for TPS analysis levels of SPS and HPI were determined at levels of 22 nmol.min^{-1}.mg^{-1}protein and 54 nmol.min^{-1}.mg^{-1}protein respectively. In order to avoid these interfering activities we assayed for inhibition of sugarcane HPI with the previously described HPI inhibitor; 6-phosphogluconate (Backhausen et al. 1997). Up to 70% of HPI activity was inhibited by 6-phosphogluconate (figure 4.9..). In light of this 10 mM 6-phosphogluconate was included in TPS reactions when it was desired that HPI competition for substrate should be lowered.

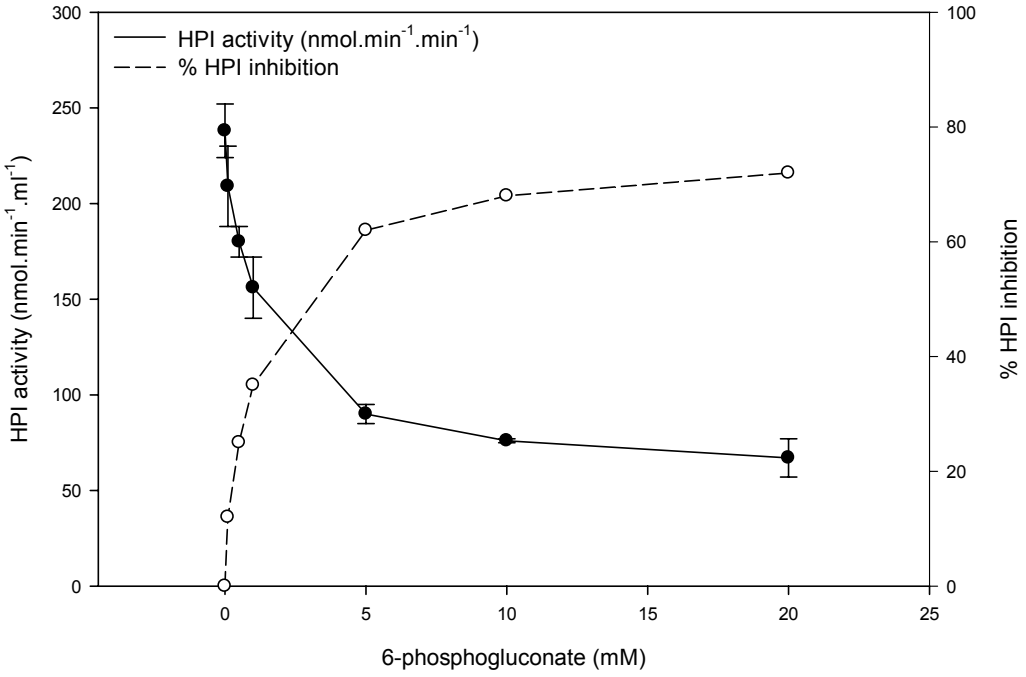


Figure 4.9. Inhibition of hexose phosphate isomerase (HPI) activity by 6-phosphogluconate. Values are the mean of three replicates ± standard deviation.

In spite of reduced HPI activity a few challenges remained, because there was still supply of fructose-6-phosphate to SPS from the remaining 30% of HPI activity. Alternate methods to those used in bacterial and yeast extracts (that do not contain SPS activity) were sought. Both the SPS and TPS reactions produce UDP. As a result measuring production of UDP as a representation of TPS activity did not allow us to distinguish between the two enzymes' activities. Trehalose-6-phosphate is also a product of the reaction, and three methods were optimised to measure it to identify and calculate TPS activity. The anthrone method (Handel 1968) was tested but found unsuitable because it detected both sucrose and trehalose, and once again the two activities could not be distinguished from each other.

Two methods using chromatographic determination of trehalose to calculate TPS activity were successfully developed. The first method used the combined separation and sensitivity of Gas Chromatography-Mass Spectrometry (GCMS). This was the first indication that sugarcane had the ability to synthesise trehalose from the substrates UDP-glucose and glucose-6-phosphate. Substrates were supplied to partially purified extracts of sugarcane internodal tissue, and the reaction terminated by boiling after a three-hour incubation at room temperature. The product of the reaction was derivitised prior to injection on the GCMS. Crude extracts showed no production of trehalose using this method. However, measurable levels of trehalose were detected from the partially purified extracts (figure 4.10.).

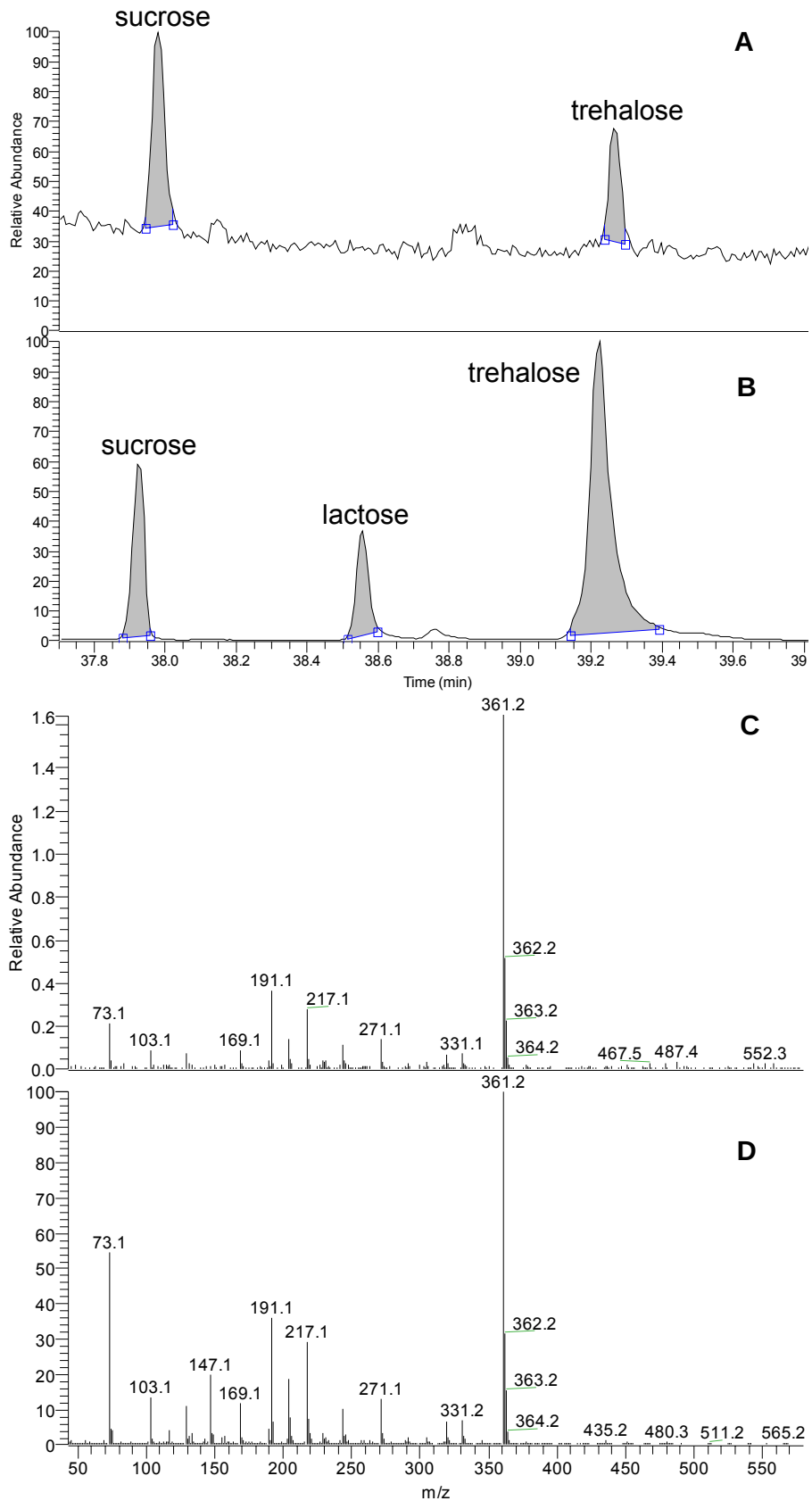


Figure 4.10. GCMS detection of trehalose resulting from trehalose-6-phosphate synthase activity of partially purified sugarcane proteins (A). Disaccharide standards were used as retention time references (B). Trehalose was identified as one of the products of the reaction from the match of its mass spectrum (C) to that of standard trehalose (D).

Both sucrose and trehalose were produced in the reaction analysed in figure 4.10. In a crude extract repetition of this experiment led to no detectable levels of trehalose, presumably because the levels of both HPI and SPS were too high and the sucrose produced interfered with the chromatographic detection.

In order to determine maximum catalytic activity of TPS from crude sugarcane extracts a technique employing ^{14}C radiolabel was optimised. Inhibition of HPI, phosphatases and invertase were inhibited in this assay by including 6-phosphogluconate, NaF and Tris respectively. HPI was maximally inhibited by 6-phosphogluconate levels of 10 mM (see figure 4.9). NaF inhibited approximately 40% glucose-6-phosphate and 100% trehalose-6-phosphate dephosphorylating activities in the protein samples used for the TPS assay. The TPS assay was carried out in a Tris buffer at 50 mM. Tris effectively inhibits sugarcane neutral invertase (Vorster & Botha 1998). These three inhibitors were included to minimise the return of label to trehalose and glucose by the enzymes HPI, SPP, TPP, invertase and trehalase. Enzyme preparations were supplied with 5 mM glucose-6-phosphate, 5 mM UDP-glucose (40 $Bq.nmol^{-1}$), 10 mM 6-phosphogluconate and 10 mM NaF and buffered at pH 7.5 by 50 mM Tris. After the reaction had run for a specified time all enzymes were denatured by boiling for 10 minutes. Reaction products were treated with alkaline phosphatase and spiked with 10 mM cold sugars (trehalose, glucose, fructose and sucrose). These samples were separated by HPLC (anion exchange) and peaks collected for determination of label incorporation by scintillation counting (table 4.5.). Return of label was detected in sucrose, glucose and trehalose peaks.

Table 4.5. Trehalose-6-phosphate synthase (TPS) activity determined by incorporation of ^{14}C from UDP-[U-^{14}C]glucose into trehalose after 1 hour. Abbreviations: UDP-glucose, UDPGlc; glucose-6-phosphate, G6P.

	Label incorporated into trehalose *(Bq)*		**Total trehalose produced *(nmol)***		**TPS activity *(nmol.min^{-1}mg^{-1}protein)***	
Substrates	***1h***	***2h***	***1h***	***2h***	***1h***	***2h***
UDPGlc + G6P	231	63	5.8	1.6	2.674	0.726
UDPGlc	91	35	2.3	0.9	1.053	0.403

Calculation of TPS activity as represented in table 4.5. was confounded by the complexity of carbohydrate metabolism in the plant. Firstly, the presence of trehalase in the extracts would mean that some of the label incorporated into trehalose in the hour long assay would have been hydrolysed to glucose, notable in the 2 hour reading that has less radio-label in than the one hour reading. However, the complications of the HPI and SPS activities as

described above, as well as the abundant invertases would contribute to return of label into glucose. In light of this the total rate of TPS activity determined by its products (and indeed those of TPP and trehalase) could not be entirely distinguished from some of the other reactions assumed to be simultaneously taking place. Therefore the TPS activity represented in table 4.5. as 2.7 $nmol.min^{-1}.mg^{-1}$protein was most likely an underestimation of the actual activity. Another interesting phenomenon was observed in the reaction that did not have glucose-6-phosphate, but only UDP-glucose as substrate. In this reaction there was also return of label into the trehalose pool. The only plausible explanation was the presence of an enzyme that catalysed the conversion of UDP-glucose to glucose-1-phosphate and UMP since there were no other cofactors or substrates available. The enzyme UDP-glucose pyrophosphatase that catalyses this reaction has been reported in both monocotyledons and dicotyledons (Rodriguez-Lopez et al. 2000). Preliminary measurement of this enzyme from sugarcane internodal tissues indicated an activity of 6 $nmol.min^{-1}.mg^{-1}$protein. In light of this there may have been more than one of the glucose moieties labelled in trehalose in the ^{14}C TPS assay the calculated activity (table 4.5.) could be an overestimation of the actual reaction rate.

Despite the complications of measuring TPS activity the data illustrates the existence of such an enzyme in sugarcane tissues, as well as estimated the maximum catalytic activity to the best of our abilities with the current technical limitations.

With the first of the enzyme's in the biosynthesis of trehalose described, the next important step was to measure both TPP and trehalase.

4.3.4.2. Trehalose-6-phosphate phosphatase (TPP)

TPP activity was determined by quantification of trehalose produced by the dephosphorylation of trehalose-6-phosphate. Internodes 3, 6 and 10 were sampled from both N19, and US6656-15 (figure 4.11.). Between tissues there was no significant difference in the amount of TPP activity. However, between varieties a significant difference was observed. The activity in N19 was at least four times higher in all three internodes than in US6656-15.

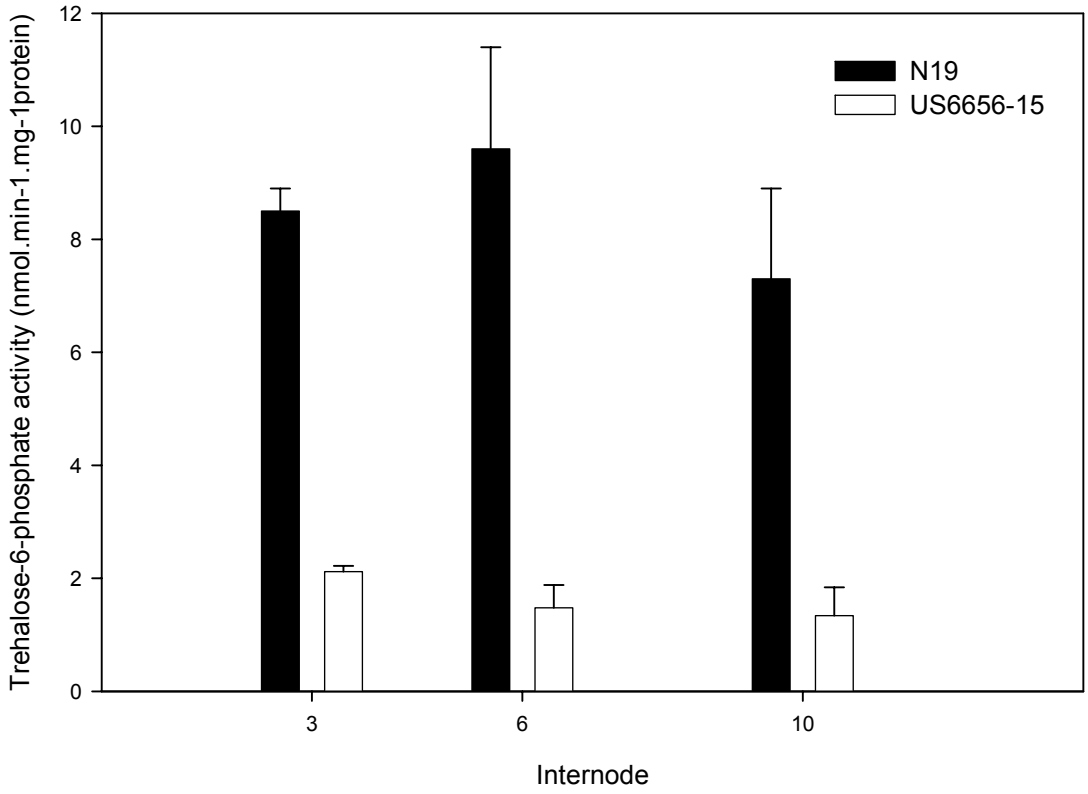


Figure 4.11. Trehalose-6-phosphate phosphatase activity (nmol.min^{-1}.mg^{-1}protein) from internodal tissues of *Saccharum* interspecific hybrid, N19 and *S. spontaneum* US6656-15. Data are the mean of 5 replicates ± standard error.

The measured TPP activity does not necessarily represent the activity of a single enzyme. Because of the nature of the protein extractions used any non-specific phosphatase may have contributed to the observed rate of reaction. The data gives us an indication of the total cellular ability to dephosphorylate trehalose-6-phosphate.

4.3.4.3. Trehalase

Trehalase hydrolyses trehalose to its to glucose moieties. Trehalase activity was determined in internodal tissues of sugarcane varieties N19 and US6656 (figure 4.12.).

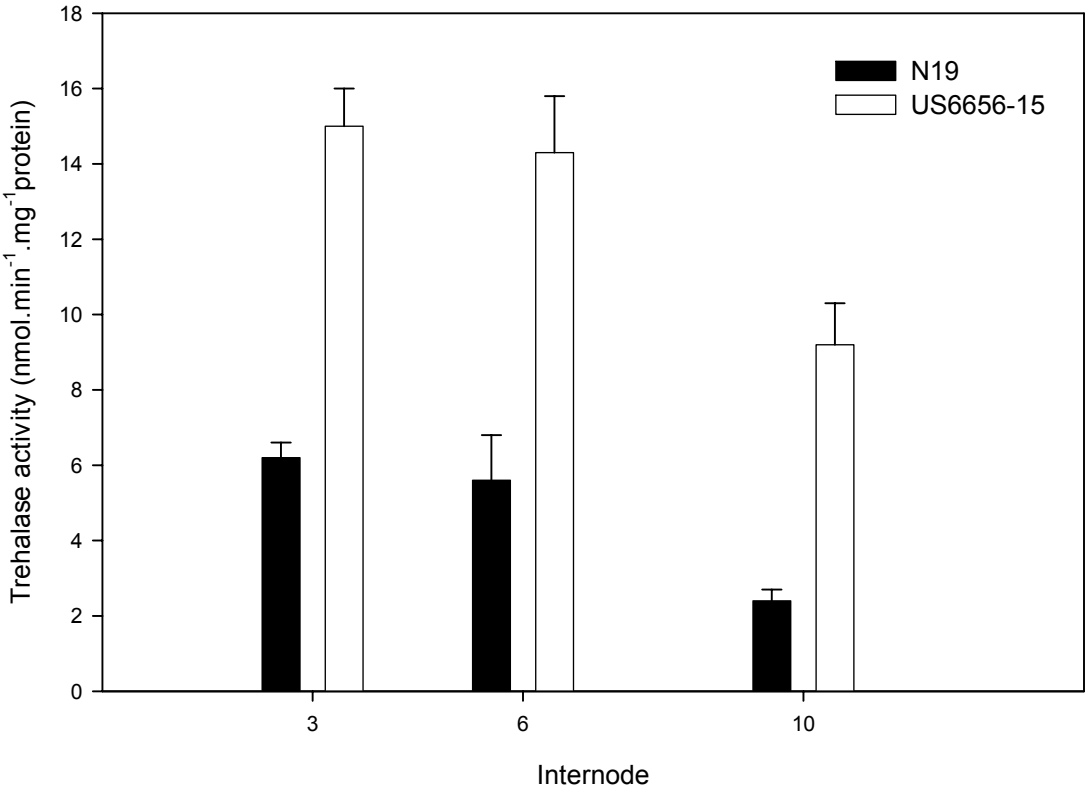


Figure 4.12. Trehalase activity (nmol.min^{-1}.mg^{-1}protein) from internodal tissues of *Saccharum* interspecific hybrid, N19 and *S. spontaneum* US6656-15. Data are the mean of 5 replicates ± standard error.

In both varieties sampled there was a trend of decreasing trehalase activity with increasing tissue maturity (figure 4.12.). In contrast with the measured TPP activity, trehalase activity in US6656-15 was higher in N19.

4.4. Discussion

Interest in the presence of the trehalose biosynthetic pathway in sugarcane originated from the discovery of putative sequences of *TPS* and *TPP* in EST projects from three separate sugarcane genomics projects (Carson et al. 2002; Casu et al. 2003), http://sucest.lad.doc.unicamp.br/en). Although the indication that there are a relative low abundance of these transcripts in sugarcane (between 1:11651 and 5:12806 www.tigr.org), it did not necessarily equate to an insignificant role of this pathway in sugarcane metabolism. This is particularly so because characteristics of the trehalose pathway, and specifically the steady state levels of trehalose-6-phosphate as a signalling molecule, have been implicated in the regulation of carbon partitioning in lower eukaryotes (Arguelles 2000).

Isolation of the sugarcane trehalase gene was needed in order to study transcript abundance of the known pathway of trehalose metabolism in other plants. This was achieved by the isolation of a partial transcript for trehalase. With this sequence and sequences isolated form other EST programmes we could complete an expression profile of this metabolic route in sugarcane internodal tissues. Levels of the *TPS* transcript decreased from young to old internodal tissues (figure 4.3.), *TPP* showed no obvious trend with tissue type (figure 4.3.), and *tre* transcript levels peaked in internode 8 (maturing culm) (figure 4.4.). The abundance of transcripts for these three trehalose metabolising genes were evidently regulated differently, perhaps by differential induction or turnover time, suggesting that the sugarcane trehalose pathway is not regulated as a unit, but rather that each step is finely controlled. Obvious differences between ancestral and commercial genotypes' transcript abundance were observed. Levels of *TPP* transcript were so low in US6656-15 and NG7794 that they were not detected using the RT-PCR parameters optimised for N19, N27 and N31 (figure 4.3.). Similarly, levels of TPP enzyme activity were lower in US6656-15 than in N19 (figure 4.11.). Trehalase transcript and enzyme levels were higher in US6656-15 than N19. These two enzymes are responsible for the degradation of trehalose-6-phosphate and trehalose, and as discussed in Chapter 2, trehalose-6-phosphate has been implicated in the regulation of carbon partitioning. It could therefore be hypothesised that the difference in carbon partitioning between genotypes (with specific reference to higher sucrose accumulation in N19, although the metabolite profiling showed higher organic and amino acids in US6656-15 (figure 3.4)) is linked to trehalose-6-phosphate levels. Enzyme kinetic characterisation of both TPP and trehalase will be described in Chapter 5. The control of steady-state levels of trehalose-6-phosphate due to TPS, TPP and trehalase activity is discussed in Chapter 6.

Isolation of a putative full-length sugarcane *TPS* (SugTPS) took the analysis of *TPS* further than the transcript abundance studies discussed above. Of the 11 *A. thaliana TPS*s (Leyman et al. 2001) only *AtTPS1* has been characterised to the enzyme activity level (Blazquez et al. 1998), but from sequence homology studies the others have been assigned with the same possible function and are divided into two classes. Class I (*AtTPS1* to *AtTPS4*) have been termed as "true" *TPS*s because of their homology with yeast and bacterial genes. The second class (*AtTPS5* through *AtTPS11*) have been described as *TPP* like *TPS*s due to the presence of conserved *TPP* regions in the 3' end of the genes. These conserved *TPP* motifs were identified upon sequence analysis of the putative full-length *SugTPS* (Appendix 4.B.), and suggested that it should fall into the class II *TPS* genes. Class II *AtTPS5* transcription was induced 21 fold by 100 mM sucrose (Schluepmann et al. 2004), in fact *AtTPS1* (a class I *TPS*) was also induced by sucrose, but to a lesser extent. Bearing in mind that *SugTPS* is a class II *TPS,* all the varieties used in this investigation *TPS* expression peaked in young internodal tissues where hexose levels were at their highest and sucrose levels at their lowest (figure 3.1.). This pattern of transcript abundance was also observed in the original study whose aim was to identify differentially expressed ESTs from sugarcane internodal tissues (Carson et al. 2002). It would therefore be possible that *TPS* levels are at their highest in these immature tissues because the sucrose concentration is optimal for transcriptional induction (sucrose content was 132.2 ± 7.1 $\mu mol.g^{-1}FW$ (table 3.1.), assuming equal distribution of sucrose between compartments, and a moisture content of 900 $\mu l.g^{-1}FW$, sucrose concentrations in N19 internode 3 were approximately 150 mM), and perhaps that higher levels of sucrose inhibit transcription in more mature internodes. Alternately, *TPS* transcription in sugarcane is not directly affected by the cellular sucrose concentration, but rather by other metabolic regulators such as transcriptional induction by high hexose levels in young internodes.

The observation that the most abundant putative *TPS* ESTs isolated from sugarcane genome projects were most likely class II *TPS*s is intriguing, as in *A. thaliana* these genes are expressed at very low levels under normal growth conditions (van Dijken et al. 2004). In *A. thaliana* the class I *AtTPS1* displays higher transcript levels in all tissues than any other *TPS* in its transcriptome. It is also the only one that has complemented the *S. cerevisiae Δtps1* mutant. This mutant can not grow on glucose as sole carbon source because of the alteration in the regulation of flux, caused by changes in the trehalose biosynthetic pathway. Complementation of this mutant restores its ability to grow normally on glucose. It can however grow on galactose, and it is assumed that entry of carbon via galactose avoided the altered regulation observed by the lack of growth on glucose. The system has been

successfully used to prove the function of both *AtTPS1* and *SlTPS1* genes from *A. thaliana* and *S. lepidophylla* respectively (Blazquez et al. 1998; Zentella et al. 1999). Two of the class II *AtTPS* genes were unable to complement the yeast *Δtps1* mutant, suggesting that they were either not active or had an alternate function (Vogel et al. 2001). This may also be the case with the *SugTPS* that did not complement the *Δtps1* mutant (figure 4.7.). It is possible that the transcribed *SugTPS* required other proteins with which to interact in order for its activity to be activated. Isolation of such a multi-enzyme complex, and sequence identification of the subunits involved would address this question. The *S. cerevisiae* TPS1 is 56.2 kDa, and the predicted molecular weight of the translated *SugTPS* is approximately 92 kDa. The C-terminal regions of the genes have high homology, leaving the remaining N-terminal region of the sugarcane TPS extending over the consensus. Such major differences in transcript product could indicate that even if the *SugTPS* were active it may not have had the ability to interact with the other subunits of the *S. cerevisiae* TPS complex that is responsible for TPS and TPP activity in this yeast. Another plausible explanation for the lack of activity is that the *SugTPS* described above was not a full-length clone, but that it was missing the correct start codon upstream of the identified one. The C-terminal region of *AtTPS1* holds an as yet unknown regulatory function. Transformation of *A. thaliana* mutants lacking the *AtTPS1* gene with the bacterial equivalent *OtsA* showed an only partially rescued phenotype (van Dijken et al. 2004), suggesting that the C-terminus of plant *TPS*s have an important role to play in the correct integration of this enzyme into metabolism.

Sugarcane: beyond the boundaries of a simple genome

As a model plant *A. thaliana* has been the subject of more investigation into trehalose metabolism than any other organism in this kingdom. Transcript expression profiles have been analysed for *AtTPS1* (van Dijken et al. 2004; Schluepmann et al. 2004), *AtTPS5* (Schluepmann et al. 2004), *AtTPPA, AtTPPB* (Vogel et al. 1998) and *AtTre* (van Dijken et al. 2004) across tissue type and developmental stages. A common feature of these profiles is that all these genes were expressed at the highest levels in flowering organs of the plants. Although basal expression was observed in stem tissues, their primary location of high transcription was in the reproductive organs. *AtTPS1* has been shown vital for embryo maturation (Eastmond et al. 2002), normal vegetative growth (van Dijken et al. 2004), transition to flowering (van Dijken et al. 2004), and involved in glucose, abscisic acid and stress signalling (Avonce et al. 2004). In the current study we have only investigated trehalose metabolism in the sugarcane culm, and not in other tissues. As a result of this, and the fact that it appears to be important for so many other processes in *A. thaliana*, it is

possible that we have missed other places of expression, and therefore inferred importance, simply by oversight as the purpose of this study was focussed on elucidating the role of trehalose metabolism in the context of sucrose accumulation in sugarcane internodal tissues.

The complexity of the sugarcane genome should also be taken into consideration. *A. thaliana* has a very simple, completely sequenced genome in comparison with that of any *Saccharum* species. Sugarcane is a polyploid, which implies that many of the enzymes important for metabolism may be present in more than one copy at genetic level. The expression profiles that we have investigated in this Chapter were limited to one transcript per enzyme. There are potentially many more copies of *TPS, TPP* and *tre* in sugarcane considering that there are 11 *TP*Ss, at least two *TPP*s and one identified *tre* in *A. thaliana.*

In addition to its complex genome, sugarcane has an intricate carbohydrate metabolism dedicated to accumulating sucrose. This presented a challenge when developing an assay system to determine TPS activity (section 4.4.3.1). Hexose phosphate isomerase (HPI) catalyses the readily reversible interconversion between glucose-6-phosphate and fructose-6-phosphate. Under conditions optimised for TPS activity HPI activity was 54 $nmol.min^{-1}.mg^{-1}$protein. This meant that besides the demand by TPS, glucose-6-phosphate was shared with another enzyme whose maximum catalytic activity was assumed to be higher. So when supplying substrate for the TPS reaction glucose-6-phosphate was readily being isomerised to fructose-6-phosphate. This in turn supplied substrate along with UDP-glucose for SPS activity. SPS activity in young internodal tissues was measured at 22 $nmol.min^{-1}.mg^{-1}$protein. This high SPS activity provided competition for the substrates of TPS, both directly (UDP-glucose) and indirectly (glucose-6-phosphate via HPI). HPI activity was maximally inhibited by 70% using 10 mM 6-phosphogluconate (figure 4.9.), still leaving approximately 16 $nmol.min^{-1}.mg^{-1}$protein available to supply fructose-6-phosphate from the glucose-6-phosphate intended for TPS. The radio-labelled TPS assay included the inhibitors for HPI (6-phosphogluconate), non-specific phosphatases (NaF) and invertase (Tris), in order to minimise the partitioning of substrate away from TPS via sucrose synthesis and breakdown. Despite this, return of label from UDP-D-[U-^{14}C]glucose was still observed in sucrose, indicating that the SPS route was not completely inhibited. This, coupled with the presence of trehalase in the extracts used for TPS activity determination meant that the calculated TPS rate (table 4.5.) was potentially an underestimation of TPS maximum catalytic activity. Despite the limitations of the system we have conclusively determined TPS activity from young internodal tissues of N19. In addition we have an estimated value of this activity that

is sufficient for inclusion in a kinetic model describing the metabolic control of trehalose metabolism in sugarcane sucrose accumulation (Chapter 6).

In the two sugarcane varieties that we have investigated for enzyme activity of trehalose metabolising enzymes the trehalose levels were approximately one nmol.g^{-1}FW (figure 3.2.). In young internodal tissue, protein content was 0.6 mg.g^{-1}FW, which therefore converts the trehalose content to 1.7 nmol.mg^{-1}protein. Assuming that its substrates are available, and that TPS is operating at 2.7 nmol.min^{-1}.mg^{-1}protein, the amount of trehalose observed would accumulate within 1.6 min.mg^{-1}protein. Extrapolated over a longer period of time one would assume that greater levels of trehalose would be accumulated unless there was an enzyme degrading it. This would appear to be the case (figure 4.12.), since in sugarcane trehalase is responsible for the hydrolytic cleavage of trehalose to its two glucose units.

Interpretation of the data presented here as the only representation of trehalose metabolism in sugarcane would be presumptuous. However, the present study has created the ground work for further research on the subject. Transcripts for the three enzymes in sugarcane trehalose metabolism have been identified and profiled in different genotypes. Enzyme assays for TPS, TPP and trehalase were developed used to determine maximum catalytic activities in different sugarcane tissues. These data will also be included in an *in silico* kinetic model (Chapter 6) that endeavours to describe the trehalose pathway in the context of sugarcane sucrose metabolism.

4.5. Conclusions

For the first time we have shown that sugarcane has the machinery to make and degrade trehalose. In this Chapter we have described expression of the enzymes responsible for trehalose synthesis and degradation on both transcript and activity level across sugarcane genotypes. Besides *S. lepidophylla* (Valenzuela-Soto et al. 2004) this is the only report of TPS activity isolated from a plant to date.

4.6. References

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signalling. Plant Physiology 136: 3649-3659

Backhausen JE, Jostingmeyer P, Scheibe R (1997) Competitive inhibition of spinach leaf phosphoglucose isomerase isozymes by erythrose 4-phosphate. Plant Science 130: 121-131

Bergmeyer HU, Bernt E (1974) Sucrose. In HU Bergmeyer, ed Methods of Enzymatic Analysis, Ed. 2. Verlag Chemie Weinheim, Academic press, New York, pp 1176-1179

Blazquez MA, Santos E, Flores C-L, Martinez-Zapater J-M, Salinas J, Gancedo C (1998) Isolation and molecular characterization of the *Arabidopsis TPS1* gene, encoding trehalose-6-phosphate synthase. The Plant Journal 13: 685-689

Bosch S, Grof CPL, Botha FC (2004) Expression of neutral invertase in sugarcane. Plant Science 166: 1125-1133

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Australian Journal of Plant Physiology 27: 81-85

Bradford MM (1976) A rapid sensitive method for hte quantification of microgram quantities of protein utilization the principle of protein-dye binding. Analytical Biochemistry 72: 248-254

Buckhout TJ, Thimm O (2003) Insights into metabolism obtained from microarray analysis. Current Opinion in Plant Biology 6: 288-296

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Gibson SI . Control of plant development and gene expression by sugar signalling. Current Opinion in Plant Biology 8, 1-10. 2005.
Ref Type: Journal (Full)

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Goddijn OJM, van Dunn K (1999) Trehalose metabolism in plants. Trends in Plant Science 4: 315-319

Hanahan D (1983) Studies on the transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166: 557-580

Handel EV (1968) Direct microdetection of sucrose. Analytical Biochemistry 22: 280-283

Hegde PS, White IR, Debouck C (2003) Interplay of transcriptomics and proteomics. Current Opinion in Biotechnology 14: 651

Hottiger T, Schmutz P, Wiemkien A (1987) Heat-Induced Accumulation and Futile Cycling of Trehalose in *Saccharomyces cerevisiae*. Journal of Bacteriology 169: 5518-5522

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Leyman B, Van Dijk P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends in Plant Science 6: 510-513

Meyers BC, Galbraith DW, Nelson T, Agrawal V (2004) Methods for Transcriptional Profiling in Plants. Be Fruitful and Replicate. Plant Physiology 135: 637-652

Rodriguez-Lopez M, Baroja-Fernandez E, Zandueta-Criado A, Pozueto-Romero J (2000) Adenosine diphosphate glucose pyrophosphatase: A plastidial phosphodiesterase that prevents starch biosynthesis. PNAS 97: 8705-8710

Rose S, Botha FC (2000) Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. Plant Physiology and Biochemistry 38: 819-824

Schluepmann H, van Dijken AJH, Aghdasi M, Wobbes B, Paul MJ, Smeekens SC (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate accumulation. Plant Physiology 135: 879-890

Valenzuela-Soto EM, Marquez-Escalante JA, Iturriaga G, Figueroa-Soto CG (2004) Trehalose 6-phosphate synthase from *Selaginella lepidophylla*: purification and properties. Biochemical and Biophysical Research Communications 313: 314-319

Van Dijck P, Mascorro-Gallardo JO, De Bus M, Royackers K, Iturriaga G, Thevelein JM (2002) Truncation of *Arabidopsis thaliana* and *Selaginella lepidophylla* trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochemical Journal 366: 63-71

van Dijken AJH, Schluepmann H, Smeekens SC (2004) Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegitative Growth and Transition to Flowering. Plant Physiology 135: 9

Vogel G, Aeschbacher RA, Muller J, Boller T, Wiemkien A (1998) Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of yeast *tps2* mutant. The Plant Journal 13: 673-683

Vogel G, Fiehn O, Jean-Richard-dit-Brussels L, Boller T, Wiemkien A, Aeschbacher RA, Wingler A (2001) Trehalose metabolism in *Arabidopsis*: occurence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. Journal of Experimental Botany 52: 1817-1826

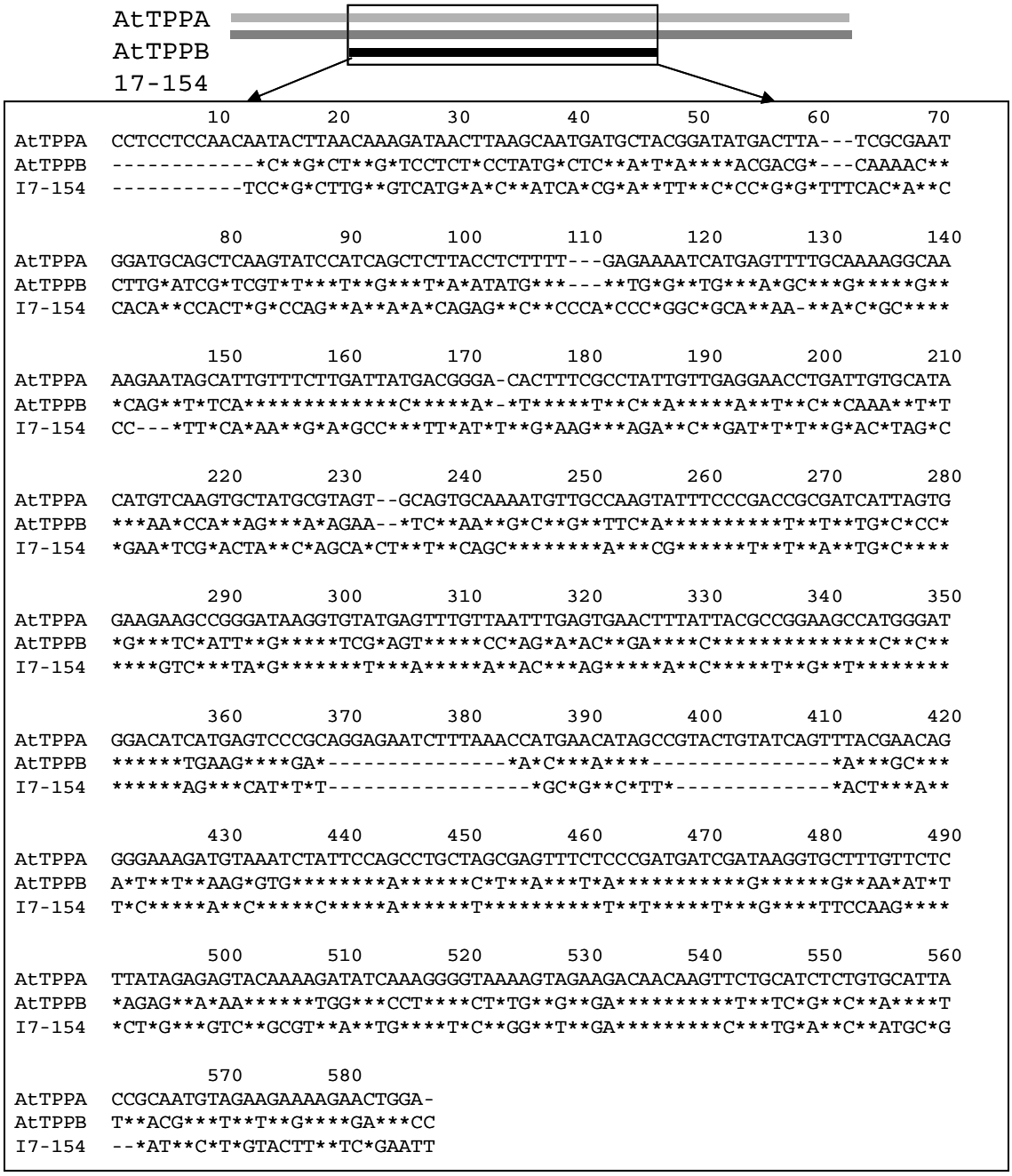
Vorster DJ, Botha FC (1998) Partial Purification and Characterisation of Sugarcane Neutral Invertase. Phytochemistry 49: 651-655

Zentella R, Mascorro-Gallardo JO, Van Dijck P, Folch-Mallol J, Bonini BM, Van Vaeck C, Gaxiola R, Covarrubias AA, Nieto-Sotelo J, Thevelein JM, Iturriaga G (1999) A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant. Plant Physiology 119: 1473-1482

4.7. Appendices


```
                    10        20        30        40        50        60        70
AtTPS1         AGCATGTTTTCAAGTACTTCACTGAGAGAACTCCCAGGTCACACTTTGAAACTCGCGATACTTCGCTTAT
SlATPS1        -----------G*T**T**TTG*****G**G**AC*C**TTTTG*C**G**C**T**G**G**T**GG*
SoTPS I2-245   -----*A**CGG*C**ACA*G--***GC***AGATG*T**CT**A*C**GCACAAG**G*GCG*CA*CG*

                    80        90        100       110       120       130       140
AtTPS1         TTGGAACTACAAATATGCAGATATCGAATTCGGGAGACTTCAAGCAAGAGATTTGTTACAACACTTATGG
SlATPS1        G********T**G*********G*T********C**GG*G**G***C*T***A**C****G***C*G***
SoTPS I2-245   C***C**C**C*CG*G**G**CCCG**C**T**TTCCTGC**G**C*AG**AC**C*CG*C**TC*CGAA

                    150       160       170       180       190       200       210
AtTPS1         ACAGGTCCAATCTCTAATGCATCAGTTGATGTTGTCCAAGGAAGCCGCTCTGTGGAAGTCCGTGCAGTTG
SlATPS1        **C**G**C**A**C**C**TG*C**C**C**C**G*****CG*AAAG**G**C**G*****CC*C**A*
SoTPS I2-245   *AC*TG*TCG---*C**C*A*C*C**G*TG**GAAGAG***CCAG*ATATC**C**G**TAACC*CCAG*

                    220       230       240       250       260       270       280
AtTPS1         GTGTCACAAAGGGAGCTGCAATTGATCGTATTCTAGGAGAGATAGTGCATAGCAAGTCGATGACTACACC
SlATPS1        *A***T*G*****GT**********C**G********C**A**C*****C*****ACAC*****G*T***
SoTPS I2-245   *CA***GC**A**C*TG*TTG*G**AA*CC*C**-------G*C**CT**G*TC*A--A*C*GGG*AG**

                    290       300       310       320       330       340       350
AtTPS1         AATCGATTACGTCTTGTGCATTGGTCATTTCTTGGGGAAGGACGAAGATGTTTACACTTTCTTCGAACCA
SlATPS1        C*****C******C*T*****A**A**C**T***A*C********G***A*C*****A**********G
SoTPS I2-245   GCCG**C*T***GC*******C**CG*CGA*C---**TC******G**CA*G*T*GAGAG*A***TGT--

                    360       370       380       390       400
AtTPS1         GAACTTCCATC-CGACATGCCAGCCATTGCACGATC--CAGACCATCATCTG
SlATPS1        **G**G***CTG*TGG*CAGGGA*TCG*CG****G*AA*G**GGGAA*C*--
SoTPS I2-245   *CC*C****A*AGC*G-C*TG*AGT*G*C*GAAT**----------------
```

Appendix 4.A1. Sequence alignment of Sugarcane *TPS* EST (I2-245) with functional TPS ORFs from *Arabidopsis thaliana* (acc. No. Y08568) and *Selaginella lepidophylla* (acc. No. SLU96736). In the area of homology the Sugarcane EST showed 41% and 45% identity with the *A. thaliana* and *S. lepidophylla* sequences respectively. Bases identical to the *A. thaliana* sequence are indicated by *, gaps by -.

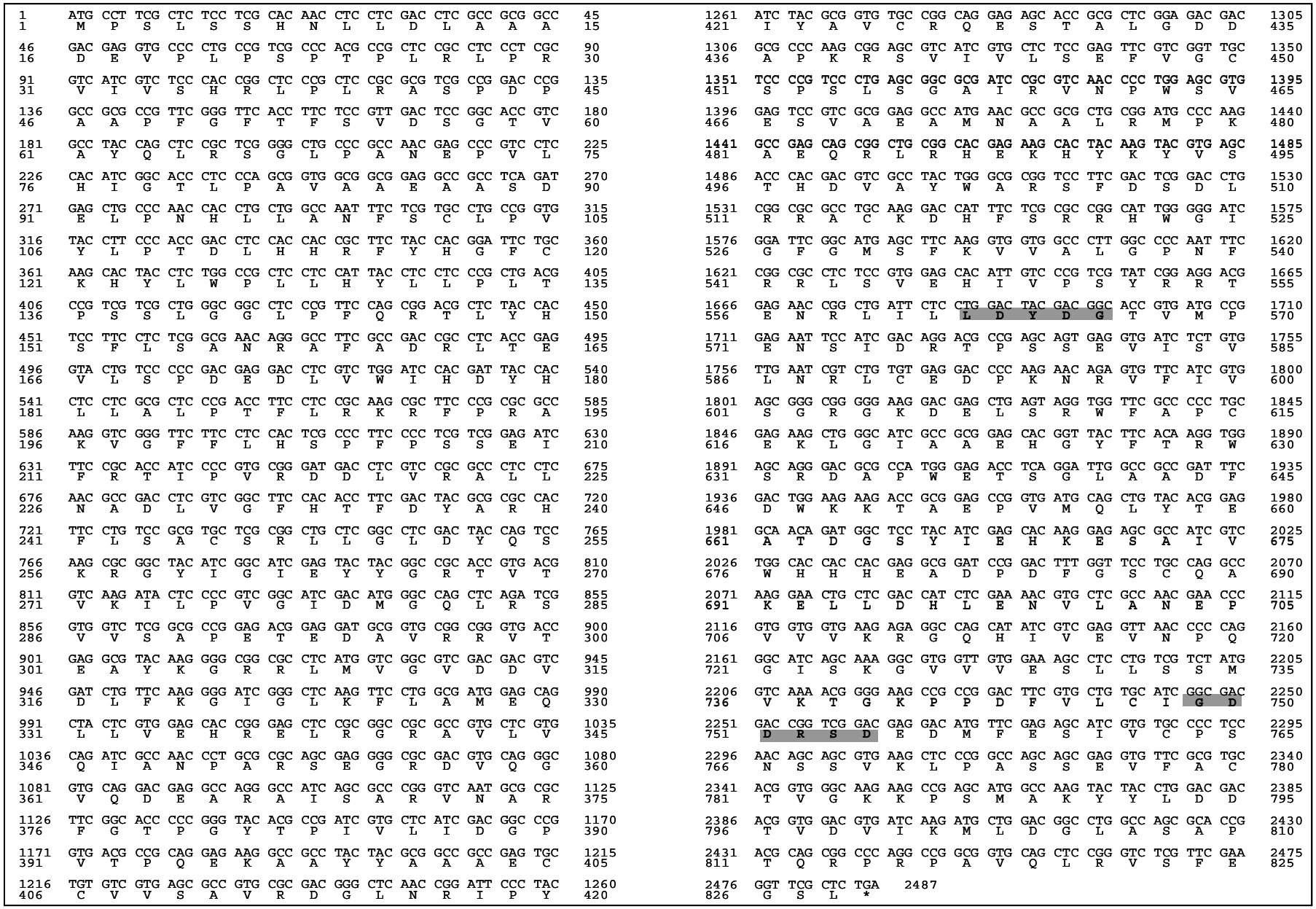



```
                10        20        30        40        50        60        70
AtTPPA  CCTCCTCCAACAATACTTAACAAAGATAACTTAAGCAATGATGCTACGGATATGACTTA---TCGCGAAT
AtTPPB  ------------*C**G*CT**G*TCCTCT*CCTATG*CTC**A*T*A****ACGACG*---CAAAAC**
I7-154  -----------TCC*G*CTTG**GTCATG*A*C**ATCA*CG*A**TT**C*CC*G*G*TTTCAC*A**C

                80        90        100       110       120       130       140
AtTPPA  GGATGCAGCTCAAGTATCCATCAGCTCTTACCTCTTTT---GAGAAAATCATGAGTTTTGCAAAAGGCAA
AtTPPB  CTTG*ATCG*TCGT*T***T**G***T*A*ATATG***---**TG*G**TG***A*GC***G*****G**
I7-154  CACA**CCACT*G*CCAG**A**A*A*CAGAG**C**CCCA*CCC*GGC*GCA**AA-**A*C*GC****

                150       160       170       180       190       200       210
AtTPPA  AAGAATAGCATTGTTTCTTGATTATGACGGGA-CACTTTCGCCTATTGTTGAGGAACCTGATTGTGCATA
AtTPPB  *CAG**T*TCA************C*****A*-*T*****T**C**A*****A**T**C**CAAA**T*T
I7-154  CC---*TT*CA*AA**G*A*GCC***TT*AT*T**G*AAG***AGA**C**GAT*T*T**G*AC*TAG*C

                220       230       240       250       260       270       280
AtTPPA  CATGTCAAGTGCTATGCGTAGT--GCAGTGCAAAATGTTGCCAAGTATTTCCCGACCGCGATCATTAGTG
AtTPPB  ***AA*CCA**AG***A*AGAA--*TC**AA**G*C**G**TTC*A**********T**T**TG*C*CC*
I7-154  *GAA*TCG*ACTA**C*AGCA*CT**T**CAGC********A***CG******T**T**A**TG*C****

                290       300       310       320       330       340       350
AtTPPA  GAAGAAGCCGGGATAAGGTGTATGAGTTTGTTAATTTGAGTGAACTTTATTACGCCGGAAGCCATGGGAT
AtTPPB  *G***TC*ATT**G*****TCG*AGT*****CC*AG*A*AC**GA****C**************C**C**
I7-154  ****GTC***TA*G*******T***A*****A**AC***AG*****A**C*****T**G**T********

                360       370       380       390       400       410       420
AtTPPA  GGACATCATGAGTCCCGCAGGAGAATCTTTAAACCATGAACATAGCCGTACTGTATCAGTTTACGAACAG
AtTPPB  ******TGAAG****GA*---------------*A*C***A****---------------*A***GC***
I7-154  ******AG***CAT*T*T-----------------*GC*G**C*TT*-------------*ACT***A**

                430       440       450       460       470       480       490
AtTPPA  GGGAAAGATGTAAATCTATTCCAGCCTGCTAGCGAGTTTCTCCCGATGATCGATAAGGTGCTTTGTTCTC
AtTPPB  A*T**T**AAG*GTG********A******C*T**A***T*A**********G******G**AA*AT*T
I7-154  T*C*****A**C*****C*****A******T**********T**T*****T***G****TTCCAAG****

                500       510       520       530       540       550       560
AtTPPA  TTATAGAGAGTACAAAAGATATCAAAGGGGTAAAAGTAGAAGACAACAAGTTCTGCATCTCTGTGCATTA
AtTPPB  *AGAG**A*AA******TGG***CCT****CT*TG**G**GA**********T**TC*G**C**A****T
I7-154  *CT*G***GTC**GCGT**A**TG****T*C**GG**T**GA*********C***TG*A**C**ATGC*G

                570       580
AtTPPA  CCGCAATGTAGAAGAAAAGAACTGGA-
AtTPPB  T**ACG***T**T**G****GA***CC
I7-154  --*AT**C*T*GTACTT**TC*GAATT
```

Appendix 4.A2. Sequence alignment of Sugarcane *TPP* EST (I27-154) with functional TPP ORFs from *Arabidopsis thaliana* AtTPPA (acc. no. 2944177) and AtTPPB (acc. no. 2944179). In the area of homology the Sugarcane EST showed 50% and 48% identity with the AtTPPA and AtTPPB sequences respectively. Bases identical to the AtTPPA sequence are indicated by *, gaps by-.

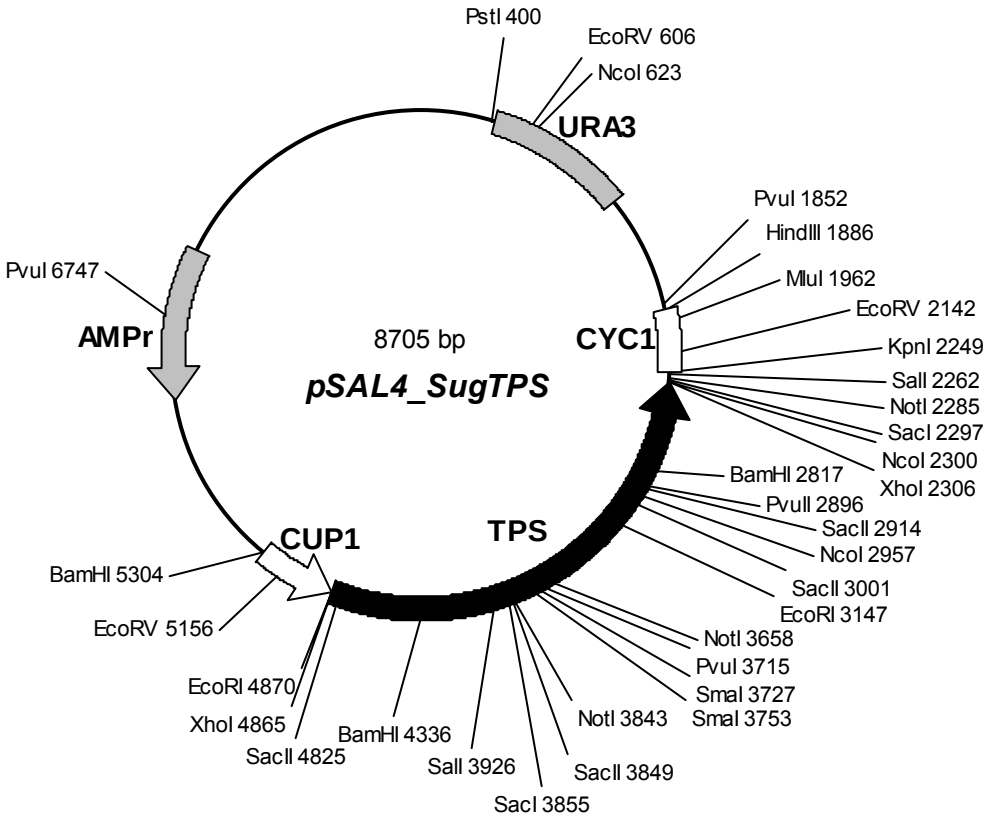
```
1     TCG AAG GTT AAT TCT ATG GCC GCT AAG GAA AAA CTG TAC CGC GAA   45
1      S   K   V   N   S   M   A   A   K   E   K   L   Y   R   E    15

46    ATT GCT TCC ACG GCA GAA TCA GGA TGG GAT TTT AGC TCT CGA TGG   90
16     I   A   S   T   A   E   S   G   W   D   F   S   S   R   W    30

91    ATG AGG AAT TCT ACT GAC ATG ACA ACA TTG GCA ACC ACT TAC ATC   135
31     M   R   N   S   T   D   M   T   T   L   A   T   T   Y   I    45

136   ATT CCT GTG GAC TTG AAC ACA TTC ATA TTT AAG ATG GAG CTG GAT   180
46     I   P   V   D   L   N   T   F   I   F   K   M   E   L   D    60

181   ATT GGT GCC TTG GCT AAA CTC GTA GGA GAT AAT GCA ACT TCA GAA   225
61     I   G   A   L   A   K   L   V   G   D   N   A   T   S   E    75

226   AAA TTT TTA AAG GCT TCA AAA GCA CGT CAT ATT GCA ATT GAC TCT   270
76     K   F   L   K   A   S   K   A   R   H   I   A   I   D   S    90

271   ATT TTG TGG AAC TCT GAG ATG GAA CAG TGG CTT GAC TAT TGG CTT   315
91     I   L   W   N   S   E   M   E   Q   W   L   D   Y   W   L   105

316   CCT GCT GAT GCA GAC TGC CAG GGA GTC CAC GAA TGG AAG TCT AAC   360
106    P   A   D   A   D   C   Q   G   V   H   E   W   K   S   N   120

361   TCA CAG AAC CGC AAC ATA TTT GCT TCT AAC TTC ATT CCG CTG TGG   405
121    S   Q   N   R   N   I   F   A   S   N   F   I   P   L   W   135

406   CTA AAT GCA TAC CAT TCC GGA TCG GTA CGC TTT GCT GAT GAG GCA   450
136    L   N   A   Y   H   S   G   S   V   R   F   A   D   E   A   150

451   AAA TCG AAG AGA GTT ATG GCG AGC CTC AAG GCA TCT GGA TTA CTT   495
151    K   S   K   R   V   M   A   S   L   K   A   S   G   L   L   165

496   CAT GCC GCA GGA ATA GCA ACT TCT CTG ATA AAC ACG GGC CAA CAA   540
166    H   A   A   G   I   A   T   S   L   I   N   T   G   Q   Q   180

541   TGG GAT TTC CCA AAT GGA TGG GCC CCA CTG CAG CAT CTT ATA GCT   585
181    W   D   F   P   N   G   W   A   P   L   Q   H   L   I   A   195

586   GAG GGA CTG CTG CAT TCT GGA TCA GAG GCC AAA ATA TTA GCT GAG   630
196    E   G   L   L   H   S   G   S   E   A   K   I   L   A   E   210

631   GAC ATT GCT ACG AGG TGG GTG AGG ACA AAC TAT GCC GCT TAC AAA   675
211    D   I   A   T   R   W   V   R   T   N   Y   A   A   Y   K   225

676   TTG ACG GGC GCG ATG CAT GAG AAG TAC AAT GTT ACC GCT TGT GGA   720
226    L   T   G   A   M   H   E   K   Y   N   V   T   A   C   G   240

721   GAA TCT GGA GGC GGT GGT GAA TAC AAG CCC CAG ACT GGT TTT GGC   765
241    E   S   G   G   G   G   E   Y   K   P   Q   T   G   F   G   255

766   TGG TCC AAT GGC GTG GTA TTG TCA TTC TTG GAA GAA TTG AGG TGG   810
256    W   S   N   G   V   V   L   S   F   L   E   E   L   R   W   270

811   CCA GAG GAC   819
271    P   E   D    273
```

Appendix 4.A3. Nucleotide and amino acid sequence of a novel partial sugarcane cDNA with a putative identity as trehalase



```
1     ATG CCT TCG CTC TCC TCG CAC AAC CTC CTC GAC CTC GCC GCG GCC   45
1      M   P   S   L   S   S   H   N   L   L   D   L   A   A   A   15

46    GAC GAG GTG CCC CTG CCG TCG CCC ACG CCG CTC CGC CTC CCT CGC   90
16     D   E   V   P   L   P   S   P   T   P   L   R   L   P   R   30

91    GTC ATC GTC TCC CAC CGG CTC CCG CTC CGC GCG TCG CCG GAC CCG  135
31     V   I   V   S   H   R   L   P   L   R   A   S   P   D   P   45

136   GCC GCG CCG TTC GGG TTC ACC TTC TCC GTT GAC TCC GGC ACC GTC  180
46     A   A   P   F   G   F   T   F   S   V   D   S   G   T   V   60

181   GCC TAC CAG CTC CGC TCG GGG CTG CCC GCC AAC GAG CCC GTC CTC  225
61     A   Y   Q   L   R   S   G   L   P   A   N   E   P   V   L   75

226   CAC ATC GGC ACC CTC CCA GCG GTG GCG GCG GAG GCC GCC TCA GAT  270
76     H   I   G   T   L   P   A   V   A   A   E   A   A   S   D   90

271   GAG CTG CCC AAC CAC CTG CTG GCC AAT TTC TCG TGC CTG CCG GTG  315
91     E   L   P   N   H   L   L   A   N   F   S   C   L   P   V  105

316   TAC CTT CCC ACC GAC CTC CAC CAC CGC TTC TAC CAC GGA TTC TGC  360
106    Y   L   P   T   D   L   H   H   R   F   Y   H   G   F   C  120

361   AAG CAC TAC CTC TGG CCG CTC CTC CAT TAC CTC CTC CCG CTG ACG  405
121    K   H   Y   L   W   P   L   L   H   Y   L   L   P   L   T  135

406   CCG TCG TCG CTG GGC GGC CTC CCG TTC CAG CGG ACG CTC TAC CAC  450
136    P   S   S   L   G   G   L   P   F   Q   R   T   L   Y   H  150

451   TCC TTC CTC TCG GCG AAC AGG GCC TTC GCC GAC CGC CTC ACC GAG  495
151    S   F   L   S   A   N   R   A   F   A   D   R   L   T   E  165

496   GTA CTG TCC CCC GAC GAG GAC CTC GTC TGG ATC CAC GAT TAC CAC  540
166    V   L   S   P   D   E   D   L   V   W   I   H   D   Y   H  180

541   CTC CTC GCG CTC CCG ACC TTC CTC CGC AAG CGC TTC CCG CGC GCC  585
181    L   L   A   L   P   T   F   L   R   K   R   F   P   R   A  195

586   AAG GTC GGG TTC TTC CTC CAC TCG CCC TTC CCC TCG TCG GAG ATC  630
196    K   V   G   F   F   L   H   S   P   F   P   S   S   E   I  210

631   TTC CGC ACC ATC CCC GTG CGG GAT GAC CTC GTC CGC GCC CTC CTC  675
211    F   R   T   I   P   V   R   D   D   L   V   R   A   L   L  225

676   AAC GCC GAC CTC GTC GGC TTC CAC ACC TTC GAC TAC GCG CGC CAC  720
226    N   A   D   L   V   G   F   H   T   F   D   Y   A   R   H  240

721   TTC CTG TCC GCG TGC TCG CGG CTG CTC GGC CTC GAC TAC CAG TCC  765
241    F   L   S   A   C   S   R   L   L   G   L   D   Y   Q   S  255

766   AAG CGC GGC TAC ATC GGC ATC GAG TAC TAC GGC CGC ACC GTG ACG  810
256    K   R   G   Y   I   G   I   E   Y   Y   G   R   T   V   T  270

811   GTC AAG ATA CTC CCC GTC GGC ATC GAC ATG GGC CAG CTC AGA TCG  855
271    V   K   I   L   P   V   G   I   D   M   G   Q   L   R   S  285

856   GTG GTC TCG GCG CCG GAG ACG GAG GAT GCG GTG CGG CGG GTG ACC  900
286    V   V   S   A   P   E   T   E   D   A   V   R   R   V   T  300

901   GAG GCG TAC AAG GGG CGG CGC CTC ATG GTC GGC GTC GAC GAC GTC  945
301    E   A   Y   K   G   R   R   L   M   V   G   V   D   D   V  315

946   GAT CTG TTC AAG GGG ATC GGG CTC AAG TTC CTG GCG ATG GAG CAG  990
316    D   L   F   K   G   I   G   L   K   F   L   A   M   E   Q  330

991   CTA CTC GTG GAG CAC CGG GAG CTC CGC GGC CGC GCC GTG CTC GTG 1035
331    L   L   V   E   H   R   E   L   R   G   R   A   V   L   V  345

1036  CAG ATC GCC AAC CCT GCG CGC AGC GAG GGG CGC GAC GTG CAG GGC 1080
346    Q   I   A   N   P   A   R   S   E   G   R   D   V   Q   G  360

1081  GTG CAG GAC GAG GCC AGG GCC ATC AGC GCC CGG GTC AAT GCG CGC 1125
361    V   Q   D   E   A   R   A   I   S   A   R   V   N   A   R  375

1126  TTC GGC ACC CCC GGG TAC ACG CCG ATC GTG CTC ATC GAC GGC CCG 1170
376    F   G   T   P   G   Y   T   P   I   V   L   I   D   G   P  390

1171  GTG ACG CCG CAG GAG AAG GCC GCC TAC TAC GCG GCC GCC GAG TGC 1215
391    V   T   P   Q   E   K   A   A   Y   Y   A   A   A   E   C  405

1216  TGT GTC GTG AGC GCC GTG CGC GAC GGG CTC AAC CGG ATT CCC TAC 1260
406    C   V   V   S   A   V   R   D   G   L   N   R   I   P   Y  420

1261  ATC TAC GCG GTG TGC CGG CAG GAG AGC ACC GCG CTC GGA GAC GAC 1305
421    I   Y   A   V   C   R   Q   E   S   T   A   L   G   D   D  435

1306  GCG CCC AAG CGG AGC GTC ATC GTG CTC TCC GAG TTC GTC GGT TGC 1350
436    A   P   K   R   S   V   I   V   L   S   E   F   V   G   C  450

1351  TCC CCG TCC CTG AGC GGC GCG ATC CGC GTC AAC CCC TGG AGC GTG 1395
451    S   P   S   L   S   G   A   I   R   V   N   P   W   S   V  465

1396  GAG TCC GTC GCG GAG GCC ATG AAC GCC GCG CTG CGG ATG CCC AAG 1440
466    E   S   V   A   E   A   M   N   A   A   L   R   M   P   K  480

1441  GCC GAG CAG CGG CTG CGG CAC GAG AAG CAC TAC AAG TAC GTG AGC 1485
481    A   E   Q   R   L   R   H   E   K   H   Y   K   Y   V   S  495

1486  ACC CAC GAC GTC GCC TAC TGG GCG CGG TCC TTC GAC TCG GAC CTG 1530
496    T   H   D   V   A   Y   W   A   R   S   F   D   S   D   L  510

1531  CGG CGC GCC TGC AAG GAC CAT TTC TCG CGC CGG CAT TGG GGG ATC 1575
511    R   R   A   C   K   D   H   F   S   R   R   H   W   G   I  525

1576  GGA TTC GGC ATG AGC TTC AAG GTG GTG GCC CTT GGC CCC AAT TTC 1620
526    G   F   G   M   S   F   K   V   V   A   L   G   P   N   F  540

1621  CGG CGC CTC TCC GTG GAG CAC ATT GTC CCG TCG TAT CGG AGG ACG 1665
541    R   R   L   S   V   E   H   I   V   P   S   Y   R   R   T  555

1666  GAG AAC CGG CTG ATT CTC CTG GAC TAC GAC GGC ACC GTG ATG CCG 1710
556    E   N   R   L   I   L   L   D   Y   D   G   T   V   M   P  570

1711  GAG AAT TCC ATC GAC AGG ACG CCG AGC AGT GAG GTG ATC TCT GTG 1755
571    E   N   S   I   D   R   T   P   S   S   E   V   I   S   V  585

1756  TTG AAT CGT CTG TGT GAG GAC CCC AAG AAC AGA GTG TTC ATC GTG 1800
586    L   N   R   L   C   E   D   P   K   N   R   V   F   I   V  600

1801  AGC GGG CGG GGG AAG GAC GAG CTG AGT AGG TGG TTC GCC CCC TGC 1845
601    S   G   R   G   K   D   E   L   S   R   W   F   A   P   C  615

1846  GAG AAG CTG GGC ATC GCC GCG GAG CAC GGT TAC TTC ACA AGG TGG 1890
616    E   K   L   G   I   A   A   E   H   G   Y   F   T   R   W  630

1891  AGC AGG GAC GCG CCA TGG GAG ACC TCA GGA TTG GCC GCC GAT TTC 1935
631    S   R   D   A   P   W   E   T   S   G   L   A   A   D   F  645

1936  GAC TGG AAG AAG ACC GCG GAG CCG GTG ATG CAG CTG TAC ACG GAG 1980
646    D   W   K   K   T   A   E   P   V   M   Q   L   Y   T   E  660

1981  GCA ACA GAT GGC TCC TAC ATC GAG CAC AAG GAG AGC GCC ATC GTC 2025
661    A   T   D   G   S   Y   I   E   H   K   E   S   A   I   V  675

2026  TGG CAC CAC CAC GAG GCG GAT CCG GAC TTT GGT TCC TGC CAG GCC 2070
676    W   H   H   H   E   A   D   P   D   F   G   S   C   Q   A  690

2071  AAG GAA CTG CTC GAC CAT CTC GAA AAC GTG CTC GCC AAC GAA CCC 2115
691    K   E   L   L   D   H   L   E   N   V   L   A   N   E   P  705

2116  GTG GTG GTG AAG AGA GGC CAG CAT ATC GTC GAG GTT AAC CCC CAG 2160
706    V   V   V   K   R   G   Q   H   I   V   E   V   N   P   Q  720

2161  GGC ATC AGC AAA GGC GTG GTT GTG GAA AGC CTC CTG TCG TCT ATG 2205
721    G   I   S   K   G   V   V   V   E   S   L   L   S   S   M  735

2206  GTC AAA ACG GGG AAG CCG CCG GAC TTC GTG CTG TGC ATC GGC GAC 2250
736    V   K   T   G   K   P   P   D   F   V   L   C   I   G   D  750

2251  GAC CGG TCG GAC GAG GAC ATG TTC GAG AGC ATC GTG TGC CCC TCC 2295
751    D   R   S   D   E   D   M   F   E   S   I   V   C   P   S  765

2296  AAC AGC AGC GTG AAG CTC CCG GCC AGC AGC GAG GTG TTC GCG TGC 2340
766    N   S   S   V   K   L   P   A   S   S   E   V   F   A   C  780

2341  ACG GTG GGC AAG AAG CCG AGC ATG GCC AAG TAC TAC CTG GAC GAC 2385
781    T   V   G   K   K   P   S   M   A   K   Y   Y   L   D   D  795

2386  ACG GTG GAC GTG ATC AAG ATG CTG GAC GGC CTG GCC AGC GCA CCG 2430
796    T   V   D   V   I   K   M   L   D   G   L   A   S   A   P  810

2431  ACG CAG CGG CCC AGG CCG GCG GTG CAG CTC CGG GTC TCG TTC GAA 2475
811    T   Q   R   P   R   P   A   V   Q   L   R   V   S   F   E  825

2476  GGT TCG CTC TGA   2487
826    G   S   L   *
```

Appendix 4.B. Putative sugarcane trehalose-6-phosphate synthase (*SugTPS*). Open reading frame (ORF) nucleotide sequence of transcript isolated from young internodal tissue RNA with its derived amino acid sequence. Two conserved trehalose-6-phosphate binding like domains are indicated by shading on the amino acid sequence.



Appendix 4.C. pSAL4_*SugTPS*. Yeast expression vector containing the putative Sugarcane trehalose-6-phosphate synthase transcript (TPS). The gene is under regulation of the CUP1 promoter sequence which is copper inducible. The vector contains and ampicillin resistance gene for bacterial selection (AMPr), and a uracil selection gene (URA3) for selection of yeast transformants.

CHAPTER 5

PARTIAL PURIFICATION AND KINETIC CHARACTERISATION OF SUGARCANE TPP AND TREHALASE

Abstract

Trehalose metabolism in plants has become the subject of much research in the last decade. Much of the work has been focussed on identification of genes that code for trehalose metabolising enzymes and their transcript profiles, with very little focus on the enzymes involved in trehalose biosynthesis and degradation. The purpose of this study was to partially purify and characterise trehalose-6-phosphate phosphatase (EC 3.1.3.12 TPP) and trehalase (EC 3.2.1.28) from sugarcane internodal tissues. The former is the enzyme responsible for the final step in trehalose biosynthesis, and the latter catalyses the degradation of this disaccharide. Kinetic analysis of two fractions of partially purified TPP revealed that there were three isoforms: TPPAI, TPPAII and TPPB. Both TPPA isoforms had pH optima of 6.0, and TPPB of pH 6.5. Apparent K_m values were determined as 0.447 ± 0.007 mM for TPPAI, 13.82 ± 1.98 mM for TPPAII and 1.387 ± 0.18 mM for TPPB. Partial purification of trehalase resulted in the separation of two fractions with trehalase activity: TREA and TREB. Both had similar pH profiles with dual optima at pH 3.5 and 6.0. The K_m values were 0.375 ± 0.032 mM and 0.345 ± 0.039 mM for TREA and TREB respectively. Both the pH dependence and kinetic constants of the trehalase fractions indicate that they are the same enzyme.

5.1. Introduction

Investigation of the role(s) of trehalose and its metabolism in plants has been prompted by four observations. Firstly, ESTs with putative identities for trehalose metabolising enzymes have been sequenced in plant transcriptomics studies (Leyman et al. 2001; Carson et al. 2002; Casu et al. 2003). Secondly, with the improved sensitivity of metabolite profiling technology, trehalose has been detected in a number of plant tissues at low levels (Roessner et al. 2000; Roessner et al. 2001; Roessner-Tunali et al. 2003), Chapter 3) when it previously had been hypothesised to be absent from the majority of plants. Thirdly, increasing trehalose levels *in planta* caused severe phenotypic changes, and alterations in carbon partitioning (Muller et al. 1995; Muller et al. 2001; Brodmann et al. 2002). Finally, in yeast the flux of carbon into glycolysis is regulated by the trehalose synthetic pathway, and specifically levels of the intermediate trehalose-6-phosphate (Thevelein & Hohmann 1995). These observations necessitated further investigation of trehalose metabolism in plants.

Current knowledge of plant trehalose metabolism is limited to the biosynthetic pathway via the enzymes trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), and the hydrolytic enzyme trehalase (Goddijn & van Dunn 1999). TPS uses the substrates glucose-6-phosphate and UDP-glucose to produce trehalose-6-phosphate and UDP. Trehalose-6-phosphate is dephosphorylated by TPP to trehalose, which is hydrolysed to two glucose units by trehalase. Little is known about the genes and particularly the proteins of these enzymes in plants, when weighted against the abundance of information available from yeast and bacteria (Thevelein & Hohmann 1995; Arguelles 2000; Voit 2003; Gancedo & Flores 2004). In order to elucidate the role of plant trehalose biosynthesis, much of the investigation has been on the genetic level, including both endogenous and transgenic transcript level profiling (Leyman et al. 2001; Eastmond et al. 2002; Schluepmann et al. 2003; van Dijken et al. 2004; Schluepmann et al. 2004; Avonce et al. 2004) and Chapter 4), and gene function studies in heterologous systems (Vogel et al. 1998; Zentella et al. 1999; Van Dijck et al. 2002; Wang et al. 2004). Although these data are useful in describing the system, they do not indicate the *in vivo* mechanism(s) of the regulation of trehalose synthesis and degradation or its interaction in carbon metabolism. For this purpose a detailed study of the enzymes involved is necessary. Kinetic analysis of an enzyme not only describe its characteristics, but also allows us to hypothesise on its role in the context of *in vitro* measurements of substrate, inhibitor and activator levels, as well as its interaction with other enzymes. *In silico* kinetic modelling of multiple enzyme reactions is a powerful tool used to both describe a system, and predict changes caused by perturbations

on the system. Such an example is the sucrose model of the sugarcane internode (Rohwer & Botha 2001). In order to include the trehalose pathway in this model and determine its role in sugarcane sucrose metabolism we require the kinetic parameters of the enzymes involved. These are presented in the current Chapter.

The study of trehalose metabolising enzymes in plants is limited by potentially low activities of synthesis, and substrates that are shared by other abundant enzymes (including hexose-phosphate isomerase and sucrose-6-phosphate synthase). TPS activity has been determined in *Selaginella lepidophylla* (Valenzuela-Soto et al. 2004), a resurrection plant that accumulates high levels of trehalose preferentially to sucrose. There are no other reports of endogenous TPS activity determinations in plants, besides that of sugarcane TPS (Chapter 4). Neither TPS nor TPP have been kinetically characterised from plants, leaving a large gap in our knowledge.

The ability of plant protein extracts to hydrolyse trehalose to its two glucose units was observed long before the genes and proteins involved in endogenous trehalose synthesis were discovered. Trehalase was therefore implicated in relieving a change in growth characteristic caused by exogenous supply of trehalose that could for instance result from pathogen attack (Brodmann et al. 2002).

Trehalases have been studied in a number of plants, including *Glycine max* (Aeschbacher et al. 1999), *Phaseolus vulgaris* (Garcia et al. 2005), *A. thaliana* (Muller et al. 2001) and sugarcane (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980), Chapter 4). In sugarcane, highest activity was detected in young internodal tissues where the sugarcane storage organ is rapidly growing. Other actively metabolising tissues such as leaves and roots displayed higher activity than maturing and mature internodes. The distribution of activity implies that trehalase is involved in active growth metabolism in some way. Levels of trehalase activity were consistently higher in *S. spontaneum* when compared with commercial interspecific hybrids that accumulated up to 10 times more sucrose. Whether trehalase activity is linked to genotype and their divergent phenotypes is still unclear. Interestingly 60 mM sucrose inhibited 22% of sugarcane trehalase activity. In some tissues trehalose hydrolytic activity was even higher than invertase activities, where the substrate for invertase (sucrose) far exceeded trehalose concentrations.

In view of the lack of kinetic data for TPP, and disparities in the literature regarding sugarcane trehalase kinetic parameters, we endeavour to complement what information is

available. Partial purification and kinetic characterisation of the two enzymes are discussed with the purpose of supplementing data for the construction of an *in silico* kinetic model for trehalose metabolism in the context of sugarcane sucrose accumulation as presented in Chapter 6.

5.2. Materials and Methods

5.2.1. Plant material

Sugarcane variety US6656-15 (*Saccharum spontaneum*) was grown outdoors in Stellenbosch, South Africa. Internodes were numbered sequentially with the internode attached to the first exposed dewlap designated number one. The first four internodes were used and represent young, growing tissues. The outer rind was removed and the tissue ground in liquid nitrogen and stored at –80°C until proteins were extracted.

5.2.2. Enzyme activity assays

5.2.2.1. Trehalose-6-phosphate phosphatase (TPP)

Routine TPP assays included samples from all extracts, purification steps and investigation of activity pH optimum. TPP was assayed at final conditions of 50 mM K-Mes/Tricine (pH 6), 1 mM NaF, 1 mM $MgCl_2$ and 1.6 mM trehalose-6-phosphate. Initial experiments showed that the reaction was linear over one hour. All subsequent assays were therefore terminated after one hour by boiling for 5 minutes. Pi released from the assay was determined by adding ammonium molybdate to a final concentration of 2 mM and ascorbic acid to a final proportion of 6.15 % (w/v). The Pi reaction was incubated for ten minutes at 37°C and absorbance read at 720 nm. Concentrations of Pi resulting from the reaction were determined from a standard curve of authentic Pi for each assay performed. Protein concentrations were determined as previously described (Bradford 1976) with IgG as standard protein.

5.2.2.2. Trehalase

Trehalase was measured at pH 6.5 (50 mM Citrate-Phosphate buffer) at a final trehalose concentration of 100 mM. The reaction was allowed to run for at least 30 minutes at 30°C (Preliminary assays showed that trehalase activity was linear between 30 and 60 minutes). Enzyme activity was stopped by heating to 98°C for 5 minutes. Glucose produced from trehalase hydrolysis was determined by the glucose oxidase/peroxidase method (Sigma kit).

5.2.2.3. Invertase, glucokinase and fructokinase

Invertase activities were measured at pH 5.0 (50 mM citrate/phosphate) and pH 7.0 (50 mM Hepes), at a final sucrose concentration of 100 mM. The assays were setup and run as for the trehalase activity determination and resulting glucose measured using the glucose oxidase/ peroxidase method (Sigma kit). Hexokinase activities (fructo- and glucokinases) were determined as previously described (Hoepfner & Botha 2003).

5.2.3. Partial purification of TPP and trehalase

5.2.3.1. TPP

Proteins were extracted in a 1:2 (gFW : ml extraction buffer) ratio. The extraction buffer contained 100 mM K-Mes (pH 6.5), 1 mM EDTA, 10 mM DTT, 10% (v/v) glycerol, 2% (w/v) PVPP (polyvynilpolypyrrolidine) and the Complete ® protease inhibitor cocktail (Roche). 200 grams of young internodal tissue were used for extraction, and the slurry stirred on ice for 15 minutes. Solid material was first separated from the soluble protein fraction by filtration through nylon mesh and then centrifuged at 12000 *xg* for 20 minutes, at 4°C. The supernatant was slowly brought to 60 % saturation with powdered ammonium sulphate and allowed to stir for 20 minutes at 0°C. Precipitated proteins were collected by centrifugation at 18000 *xg* for 35 minutes at 4°C. The supernatant was brought to 95 % ammonium sulphate saturation as above, and the pelleted proteins retained. This fraction was resuspended in 5 ml buffer (100 mM K-Mes (pH 6.5), 1 mM EDTA, 10% (v/v) glycerol). The sample was then desalted on a pre-equilibrated (50 mM Tricine, pH 7.0) Sephadex G25 column (Sigma, fine Sephadex). The desalted protein fraction was loaded onto a 5 ml ion exchange column (HiTrapQ sepharose) connected to a Pharamcia AktaPrime FPLC. Unbound proteins were washed off with 50 mM Tricine (pH 7.0). Bound proteins were eluted in a linear NaCl gradient (0 - 500 mM), over five column volumes at a flow rate of 3ml.min^{-1}. Fractions were collected at 2 ml intervals and those containing greater than 20% activity were pooled for kinetic analysis.

5.2.3.2. Trehalase

Proteins were extracted as for TPP. Total soluble proteins were slowly brought to 45% saturation with powdered ammonium sulphate and allowed to stir for 20 minutes at 0°C. Precipitated proteins were collected by centrifugation at 18 000 *xg* for 35 minutes at 4°C.

The supernatant was brought to 60% ammonium sulphate saturation as above, and the pelleted proteins retained. This fraction was resuspended in 3 ml desalting buffer (100mM K-Mes (pH 6.5), 1 mM EDTA, 10% (v/v) glycerol). Two and a half ml were desalted on a pre-equilibrated Sephadex G25 column (Sigma, fine Sephadex). The desalted protein fraction was loaded onto a 5 ml ion exchange column (HiTrapQ sepharose) connected to a Pharamcia AktaPrime FPLC. Unbound proteins were washed off with 50 mM K-Mes (pH 6.5). Bound proteins were eluted with a linear NaCl gradient (0 – 500 mM) at a flow rate of 3ml.min^{-1} over 5 column volumes. One ml fractions were collected, and those containing 20% or more of maximum activity were retained for kinetic analyses.

5.2.4. Kinetic analyses

SigmaPlot7 Enzyme Kinetics 1.1 (Systat, 2001) was used for analysis of data from enzyme kinetic experiments.

5.3. Results

5.3.1. Partial purification of TPP and trehalase

Construction of an *in silico* kinetic model describing trehalose metabolism in sugarcane requires both V_{max} and K_m values for the enzymes involved in the pathway. TPP and trehalase maximum catalytic activities have been determined in two sugarcane varieties (figure 4.11 and figure 4.12), and further kinetic characterisation of both enzymes are presented below.

5.3.1.1. Separation of two activities capable of dephosphorylating trehalose-6-phosphate

Two Sugarcane TPPs were partially purified in a two step protein separation. Total soluble proteins were extracted from young internodal tissues. Proteins that precipitated between 60 and 90 % ammonium sulphate saturation were subjected to anion exchange FPLC. In this purification step two isoforms of TPP were separated (figure 5.1). The peak activities were pooled separately and designated TPPA and TPPB.

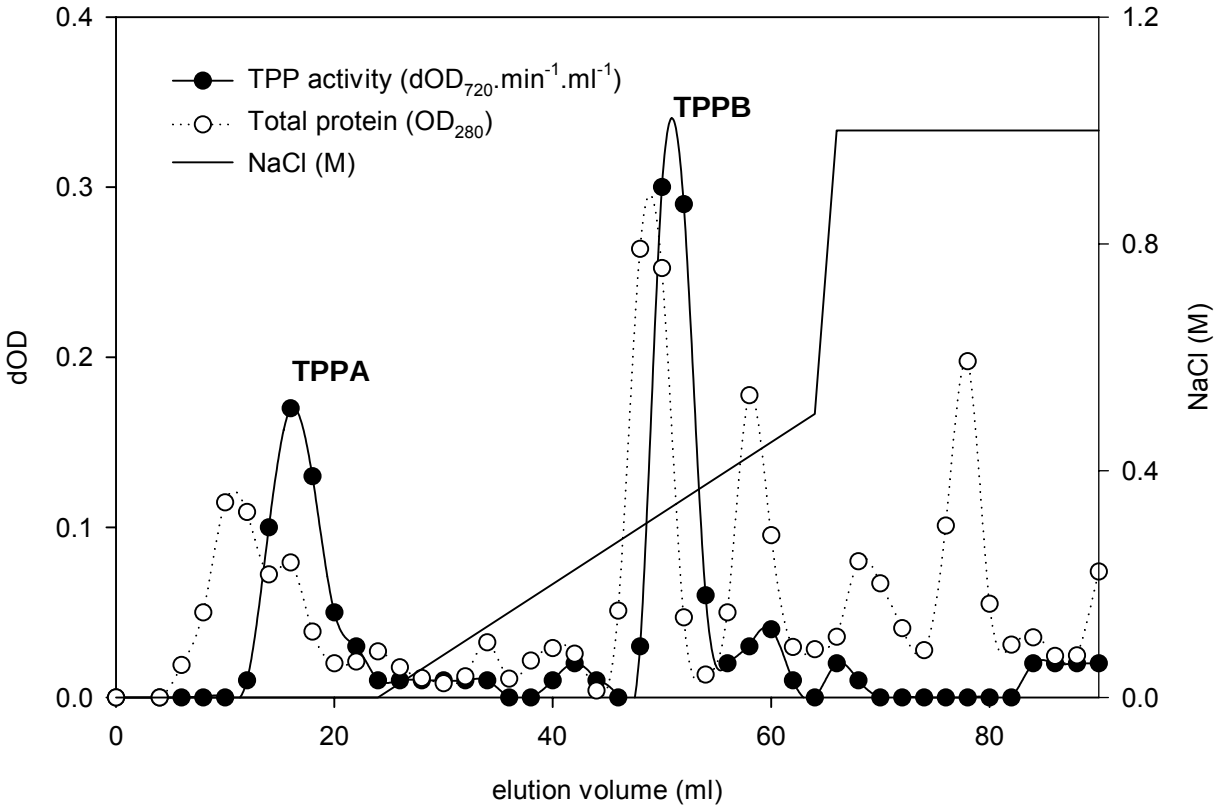


Figure 5.1. Separation of TPP isoforms by anion exchange chromatography (HiTrapQ Sepharose). The two peaks of TPP activity have been designated the identities TPPA and TPPB, eluting at 0 and ± 0.3 M NaCl respectively. TPP activity was measured over a 1 hour incubation period with trehalose-6-phosphate as substrate. Production of Pi was used to calculate TPP activity.

Details of the TPP purification are presented in table 5.1. TPPA and TPPB activities were 60.9 nmol.min^{-1}.mg^{-1}protein and 8.8 nmol.min^{-1}.mg^{-1}protein respectively, when measured with a substrate concentration of 30 mM trehalose-6-phosphate. This rendered a 90 fold purification of TPPA, and a 13 fold purification of TPPB from the original extract. Fifty five % of the total crude extract activity was recovered in TPPA, and 41 % in TPPB contributing to a 96 % of the total activity recovered in the purification.

Table 5.1. Purification of sugarcane TPP. Activities were determined with 30mM trehalose-6-phosphate as substrate

Fraction	***Protein*** *(mg.ml^{-1})*	***Total protein*** *(mg)*	***Total activity*** *(nmol.min^{-1})*	***Specific activity*** *(nmol.min^{-1}.mg^{-1}protein)*	***Purification*** *(fold)*	***Yield*** *(%)*
Crude	1.90	349.6	236.0	0.68	1	100
60-90 % $NH_4(SO_4)_2$	*n.d.*					
TPPA (HiTrap Q)	0.36	2.1	130.0	60.86	90	55
TPPB (HiTrap Q)	2.77	11.1	97.8	8.81	13	41

n.d. = not determined.

The two TPP isoforms displayed different pH optima, with highest activity in TPPA at pH 6.0, and TPPB at pH 6.5 (figure 5.2). This illustrates that the two fractions were indeed different isoforms of TPP.

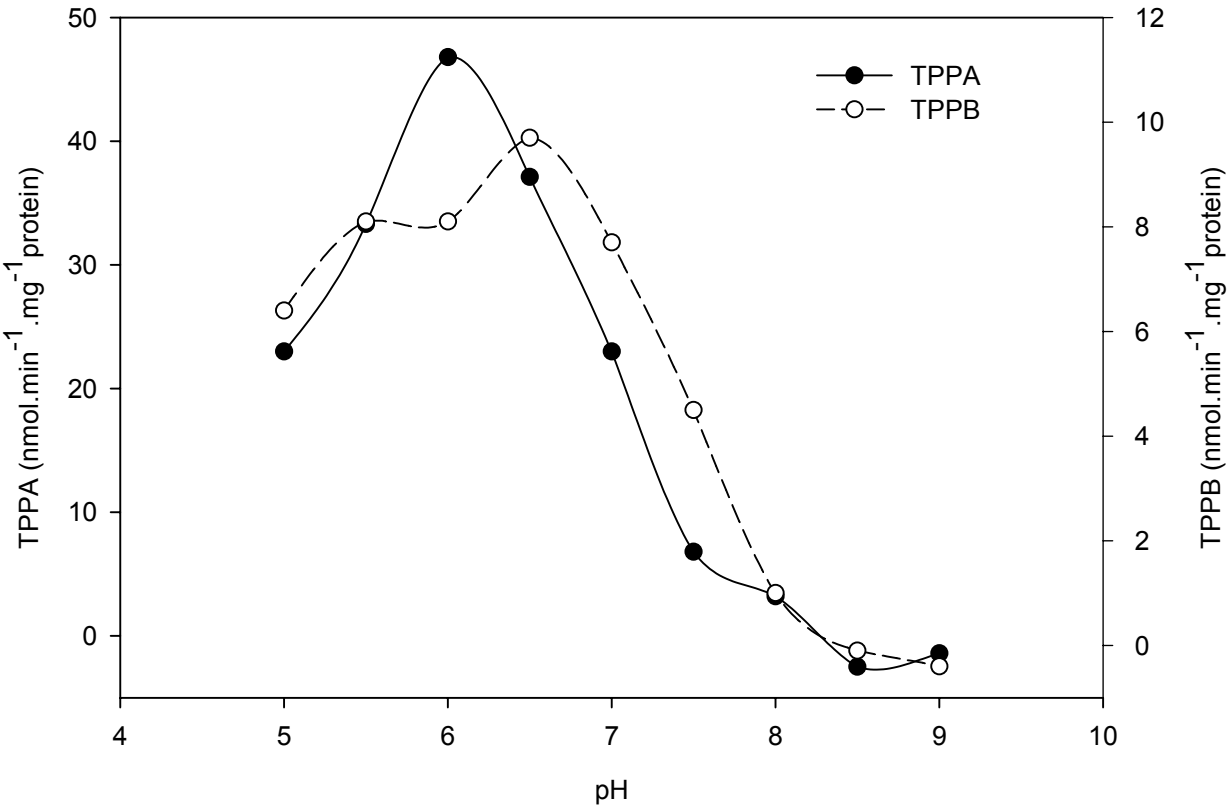


Figure 5.2. pH dependence of the TPP isoforms separated by anion exchange chromatography.

The substrate specificity of the partially purified TPPA and TPPB were analysed. Both preparations were able to dephosphorylate a range of sugar phosphate intermediates of glycolysis (figure 5.3). These included glucose-6-phosphate, fructose-6-phosphate, phosphoenolpyruvate and glucose-1-phosphate. The TPPA preparation had higher phosphatase activities than TPPB for all substrates assayed at 30 mM.

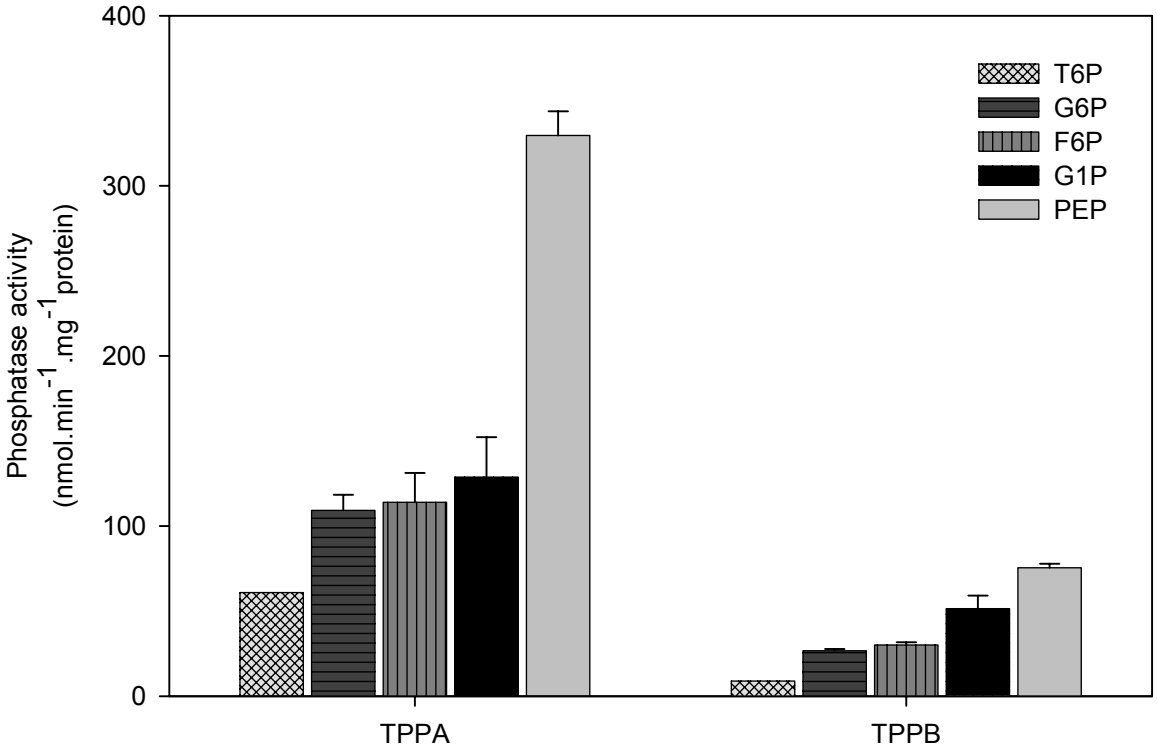


Figure 5.3. Phosphatase activities in anion exchange fractions TPPA and TPPB. Substrates tested were trehalose-6-phosphate (T6P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), glucose-1-phosphate (G1P) and phosphoenolpyruvate (PEP). Each sugar phosphate was supplied at 30 mM, and activity determined by quantification of Pi produced over time. Data are the mean of three independent replicates ± standard deviations.

5.3.1.2. Partial purification of trehalase

Discrepancies in previous investigations of sugarcane trehalase kinetic parameters (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980) prompted the partial purification and characterisation presented below.

Table 5.2. Purification of sugarcane trehalase

Fraction	***Protein*** *(mg.ml^{-1})*	***Total protein*** *(mg)*	***Total activity*** *(nmol.min^{-1})*	***Specific activity*** *(nmol.min^{-1}.mg^{-1}protein)*	***Purification*** *(fold)*	***Yield*** *(%)*
Crude	1.9	239.9	997.6	4.2	1.0	100
45-60 % $NH_4(SO_4)_2$	39.5	92.9	523.7	5.6	1.4	53
TREA (HiTrap Q)	0.4	1.3	100.2	76.6	18.4	10
TREB (HiTrap Q)	1.6	9.9	115.1	11.6	2.8	12

Total cellular soluble proteins were isolated from young internodal tissues of *S. spontaneum* US6656-15, as this was the tissue with the highest levels of activity (figure 4.12.). Preliminary experiments demonstrated that approximately 80% of total trehalase activity precipitated between 45 and 60% ammonium sulphate saturation. A second purification step, anion exchange, was included to further separate trehalase from other proteins in the ammonium sulphate cut. This step rendered two peaks of trehalase activity, designated TREA and TREB (figure 5.4). Details of the purification schedule are presented in table 5.2.

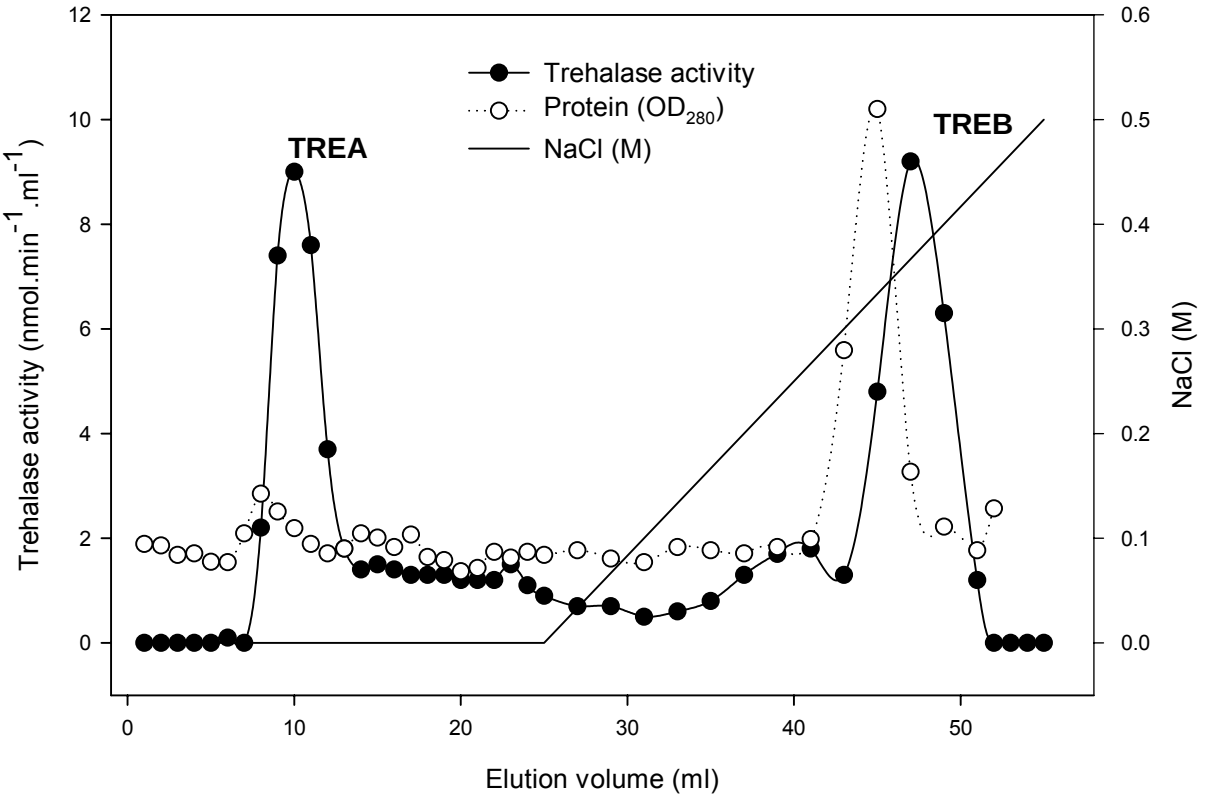


Figure 5.4. Separation of sugarcane proteins by anion exchange chromatography using a HiTrap Q sepharose column and a NaCl gradient. Two fractions displaying trehalase activity were detected and designated TREA and TREB.

Enzymes that shared substrates and products with the trehalase reaction, and could potentially interfere with kinetic assays, were assayed in all steps of the purification procedure (table 5.3.). In crude extracts trehalase activity exceeded that of both soluble acid and neutral invertase as well as glucokinase. The TREA fraction showed very little contamination of the four additional enzymes assayed, however there were significant levels of soluble acid invertase and both isoforms of fructokinase present in TREB.

Table 5.3. Enzyme activities in trehalase purification steps. Data is expressed as % trehalase activity in each fraction. The hexokinases assayed included the two fructokinase isoforms FK1 and FK2, as well as glucokinase (GK). FK1 was determined with 1 mM fructose as substrate and FK2 with 0.1 mM fructose. GK activity was determined with 2 mM glucose as substrate.

	Enzyme activity as a % trehalase activity					
	Trehalase	***Invertase***		***GK***	***FK1***	***FK2***
Sample		***Acid***	***Neutral***			
Crude	100	85	36	11	720	469
45-60% $(NH_4)_2SO_4$	100	42	24	1	7	7
TREA	100	3	3	0	0	0
TREB	100	1394	32	17	140	171

TREA and TREB activity displayed similar pH dependence (figure 5.5). pH optima were measured at both acid (pH 3.5) and close to neutral (pH 6.0). For the purposes of this study, which was to characterise trehalose metabolism in the cytosol, all kinetic parameters were determined at the neutral pH 6.0.

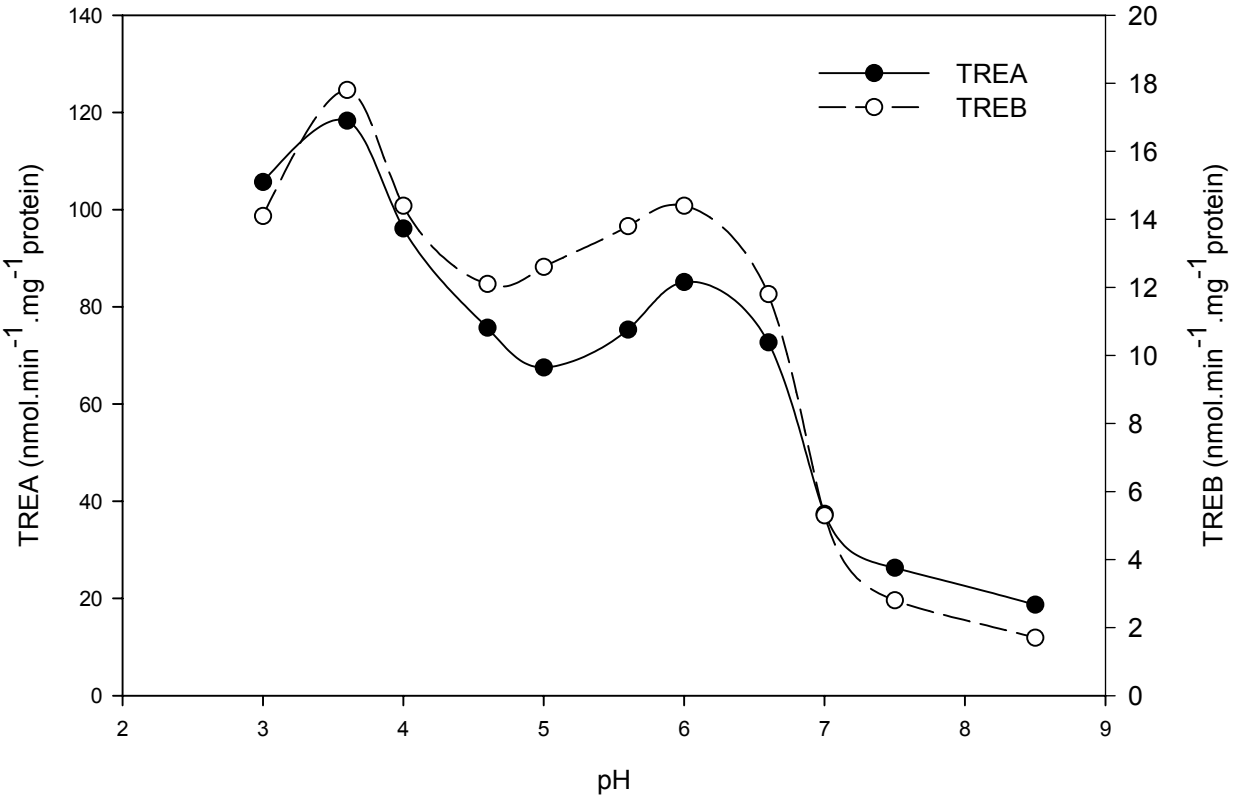


Figure 5.5. pH dependence of trehalase activity. Both TREA and TREB fractions from the anion exchange separation were assayed.

5.3.2. Kinetic characterisation of TPP and trehalase

5.3.2.1. Kinetic constants of partially purified TPPA and TPPB

Kinetic analysis of TPPA and TPPB showed that at least three isoforms of TPP were present in the sugarcane proteome. The TPPA fraction appeared to have two phosphatase isoforms able to dephosphorylate trehalose-6-phosphate. This was observed when the relationship between the trehalose-6-phosphate concentration supplied to the TPPA reaction and the resulting enzyme activities were best fit to a model that predicted an enzyme preparation containing more than one isoform. Figure 5.6A shows an Eadie-Hofstee plot of this relationship. These two activities in the TPPA fraction were termed TPPAI and TPPAII. The apparent K_m for TPPAI was 0.447 ± 0.007 mM, and 13.8 ± 2.0 mM for TPPAII (table 5.4.).

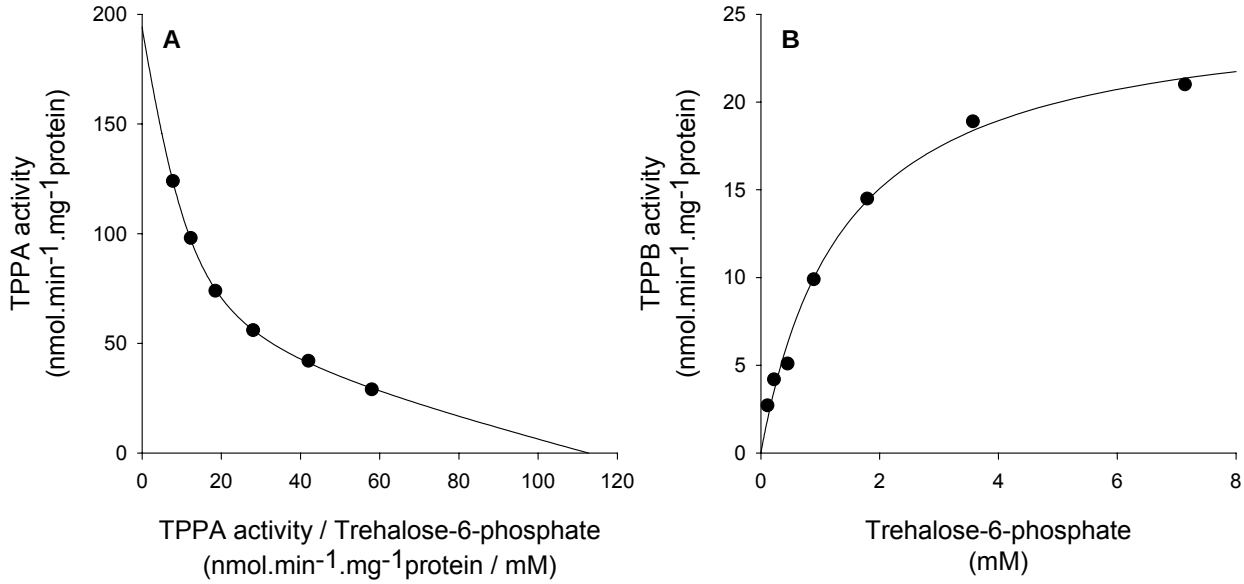


Figure 5.6. Kinetic relationship between TPP activity and substrate (trehalose-6-phosphate) concentration. TPPA fitted best with a mixed isozyme model, graphed on an Eadie-Hofstee plot (A). TPPB displayed Michaelis-Menten kinetics (B).

TPPB displayed classic Michaelis-Menten kinetics (figure 5.6B). TPPB had a V_{max} determined at 25.5 ± 1.2 nmol.min^{-1}.mg^{-1}protein, and an apparent K_m of 1.39 ± 0.18 mM (table 5.4.).

Table 5.4. Kinetic constants for TPPA and TPPB.

TPP isoforms	V_{max} *(nmol.min^{-1}.mg^{-1}protein)*	K_m *(mM)*
TPPAI	45.6 ± 4.0	0.447 ± 0.007
TPPAII	149.0 ± 4.7	13.8 ± 2.0
TPPB	25.5 ± 1.2	1.39 ± 0.18

Comparison of the kinetic constants of the three TPP activities in table 5.4., confirmed the presence of multiple enzymes in the sugarcane internode that dephosphorylated trehalose-6-phosphate. The three apparent K_m values for TPPAI, TPPAII and TPPB differed greatly. This is perhaps the most convincing evidence that the separated TPP activities (figure 5.1.) were in fact distinguishable isoforms of the enzyme.

5.3.2.2. Kinetic characteristics of trehalase

Trehalase is an enzyme with a single substrate and a single product. From kinetic analysis of the enzyme at different concentrations of the substrate, trehalose, classic Michaelis Menten saturation curves were best fit to the data (figure 5.7).

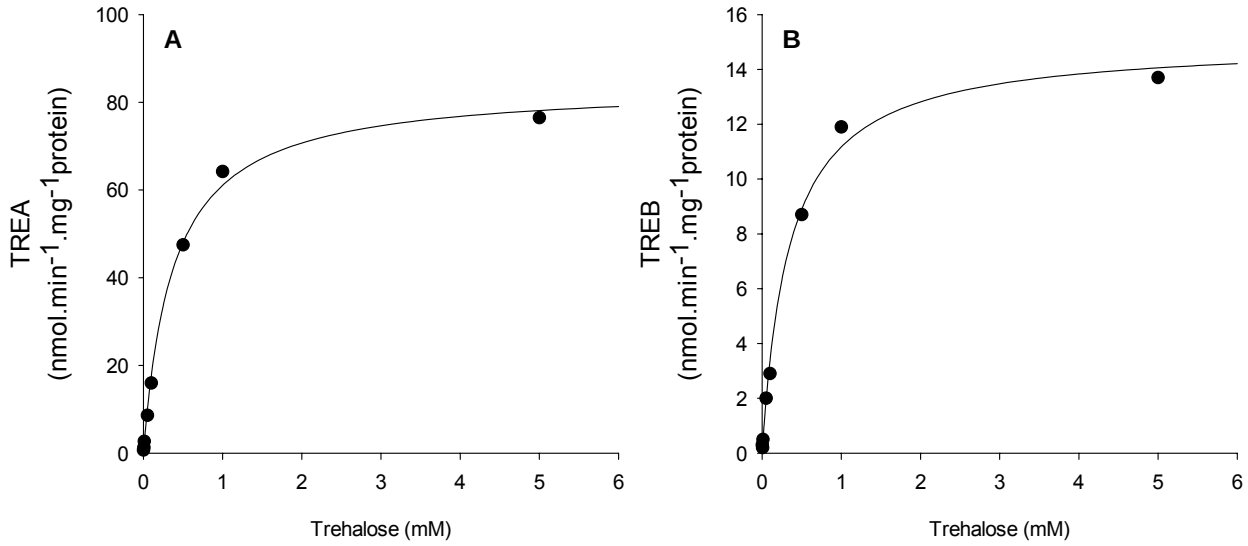


Figure 5.7. Substrate dependence of the trehalase reaction. TREA (A) and TREB (B) represent two peaks of trehalase activity separated by anion exchange chromatography.

Kinetic constants derived from fitting the data to the Michaelis Menten plot demonstrated that both TREA and TREB had K_m values between 0.345 mM and 0.375 mM that were not significantly different from one another (table 5.5.).

Table 5.5. Kinetic constants for TREA and TREB.

Trehalase isoforms	V_{max} *(nmol.min^{-1}.mg^{-1}protein)*	K_m *(mM)*
TREA	84.0 ± 2.0	0.375 ± 0.032
TREB	15.0 ± 0.5	0.345 ± 0.039

5.4. Discussion

In vivo flux through the trehalose biosynthetic pathway is crucial for the survival and reproduction of *A. thaliana* (Eastmond et al. 2002; Schluepmann et al. 2003; van Dijken et al. 2004). As a model system for plant molecular physiology and biochemistry, this organism has provided much valuable information concerning growth metabolism and carbon partitioning, with specific reference here to trehalose metabolism. However, there is still much to be elucidated about the enzyme biochemistry of this metabolism in this and other plants. In order to examine the control on the pathway of trehalose synthesis and breakdown in the context of sugarcane sucrose accumulation, kinetic constants (K_ms) and maximum catalytic activities (V_{max}s) are required for construction of an *in silico* model suitable for metabolic control analysis.

Enzyme activities of TPP, TPS and trehalase from two sugarcane genotypes were determined and reported in Chapter 4. This data was used to provide Vmax parameters for the enzyme kinetic model in Chapter 6. The current Chapter has described the kinetic characteristics of sugarcane TPP and trehalase, and their K_m values were used to complete the experimentally determined constants required for construction of the above mentioned kinetic model.

5.4.1. Multiplicity of TPP enzyme activity in sugarcane

It is not surprising that there were multiple enzymes, or isoforms of one enzyme, isolated from sugarcane internodal tissues that were able to dephosphorylate trehalose-6-phosphate (figure 5.1). Plants have many non-specific phosphatases that have the potential to use this disaccharide phosphate as a substrate. These isolated fractions used a variety of sugar phosphates as substrates (figure 5.3), again suggesting the non-specific nature of these phosphatases. Along with this the nature of the protein extraction used here combined all enzymes from all disrupted compartments into one pool used for the purification of TPP. So while we have described three isoforms of TPP, they may not all be active in the same cellular compartment *in vivo*. However, from the experimentally determined neutral pH optima (figure 5.2) one could deduce that the TPPs measured here were located in the cytosol, and therefore had access to trehalose-6-phosphate as we have assumed that the synthesis of trehalose occurred in this compartment.

Kinetic analysis of the TPP fractions revealed a third possible isoform. The three isoforms had distinct K_m values, ranging from 0.447 ± 0.007 mM (TPPAI), and 13.8 ± 2.0 mM

(TPPAII), with that of TPPB in between at 1.39 ± 0.18 mM (table 5.4.). Trehalose-6-phosphate levels have never been measured in sugarcane internodes. Levels of trehalose-6-phosphate from *A. thaliana* (Avonce et al. 2004) are at least 10 times lower than trehalose. If the same ratio were to hold in the sugarcane tissues used here, the cytosolic trehalose-6-phosphate concentration would be approximately 1-2 μM (see discussion below for estimation of cytosolic trehalose concentration). This value is well below any of the three K_ms calculated for the different TPP isoforms, and would imply that none of them operate at rates close to their V_{max} when trehalose-6-phosphate is the substrate.

Further analysis and discussion of TPP will be presented in context of TPS and trehalase in Chapter 6.

5.4.2. Trehalase: the enzyme responsible for trehalose degradation

An integral part of the trehalose pathway is the hydrolysis of trehalose by trehalase. Although it is known that there is substantial trehalase activity in sugarcane tissues (Glasziou & Gayler 1969) the endogenous pathway(s) involved in supplying substrate had not been established (Alexander 1973; Fleischmacher et al. 1980). Recent data from both genomic and metabolomic technologies indicate that the reactions required for the producing and degrading trehalose exists in plants. In Chapters 3 and 4 we discussed the distribution of trehalose (the substrate for trehalase) and the expression of its transcript and enzyme activity in different sugarcane genotypes and internodal tissues.

Two fractions of trehalase activity were separated by anion exchange chromatography (figure 5.4.), however subsequent characterisation indicated that they were not substantially different, and are potentially the same protein. Both TREA and TREB had similar biphasic pH optima (pH 3.5 and 6.0) (figure 5.5.). Apart from the pH dependence of TREA and TREB, their K_m values were not significantly different (table 5.5.). It is possible that the anion exchange column was overloaded, or that the flow rate at injection was too high for the amount of trehalase to bind to the column, resulting in one peak of trehalase activity eluting before the NaCl gradient (TREA), and one eluting at approximately 350 mM NaCl (TREB). Hence trehalase activity will be assumed to be represented by a single enzyme.

Sugarcane trehalase displayed classic Michaelis Menten kinetics with a K_m of approximately 0.36 mM (table 5.5.). Previous determination of sugarcane trehalase K_m values ranged between 0.1 mM (Glasziou & Gayler 1969) and 0.63 mM (Alexander 1973), with the value measured in this study almost exactly half way between the two. Trehalose levels in *S.*

spontaneum used for trehalase partial purification were 1.4 ± 1.2 nmol.g^{-1}FW (table 3.1.). The average fresh weight: dry weight ratio in young internodes was 10:1, indicating that approximately 90 % of the fresh weight was water. If one assumes that all trehalose was present in the cytosol, and that the cytosol was 10 % of the cell volume, the trehalose concentration in this compartment would have been approximately 15 µM. If the trehalose was distributed in other compartments the cytosolic trehalose concentration would be even less. This implies that the substrate availability for trehalase is at least 24 times lower than its K_m inferring that trehalase cannot operate at its maximum catalytic activity *in vitro*.

Similar to the sugarcane trehalase isolated in this study, an *A. thaliana* trehalase (accession no. 002343) also displays a biphasic pH optima (pH 4.5 and 6.3) when expressed in a yeast trehalase mutant (Muller et al. 2001). In soybean suspension cultures more than 80 % of total cellular trehalase activity is secreted into the media (Muller et al. 1992), indicating an apoplastic isoform of plant trehalases. At the outset we assumed that the trehalose pathway and its enzymes (TPS, TPP and trehalase) were located in the cytosol. However, with the evidence of trehalase activity pH dependence and the observation from soybean cultures, it is likely that plant trehalases are present in other cellular compartments such as the apoplast or even the vacuole. Immunohistochemical localisation of sugarcane trehalase would help to clarify these phenomena, and show where trehalose is present in the cell. If an apoplastic form is present, the original hypothesis of trehalase's role in protection from pathogen attack by the degradation of exogenous trehalose in order to avert its negative effects on growth, is plausible. Additionally, the presence of trehalase in the cytosol would mean that any trehalose synthesised in this compartment would be exposed to a degradative activity. This would support the hypothesis that flux through the trehalose pathway has a primary function in signalling, and that finely controlled low levels of its intermediates (especially trehalose-6-phosphate) can alter carbon partitioning by the regulation of cytosolic carbohydrate metabolism. In this way cytosolic trehalose should be rapidly hydrolysed in order to avoid phenotypic response to its presence, and to return carbon back into metabolism via the trehalase product, glucose.

The kinetic characteristics of the enzymes involved in the degradation of trehalose-6-phosphate (TPP) and trehalose (trehalase) have been established in this Chapter. Validity of these data do not depend on the separation of cell types or cellular compartments at extraction, as enzyme kinetic parameters are defined locally rather than globally, i.e. they are primarily dependant on substrate and product concentrations and not on the metabolite or protein complement in their immediate sub-cellular environment.

5.5. Conclusions

This is the first report of TPP activity extracted from plants, and specifically from sugarcane. Kinetic characterisation of sugarcane TPP and trehalase have made a significant contribution to our knowledge of trehalose metabolism in sugarcane. Moreover, three distinct isoforms of TPP have been separated and kinetically characterised. These analyses have provided useful data for the metabolic control analysis discussed in Chapter 6.

5.6. References

Aeschbacher RA, Muller J, Boller T, Wiemkien A (1999) Purification of Trehalase GMTRE1 from Soybean Nodules and Cloning of Its cDNA. *GMTRE1* Is Expressed at a Low Level in Multiple Tissues. Plant Physiology 119: 489-495

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signalling. Plant Physiology 136: 3649-3659

Bradford MM (1976) A rapid sensitive method for hte quantification of microgram quantities of protein utilization the principle of protein-dye binding. Analytical Biochemistry 72: 248-254

Brodmann D, Schuller A, Ludwig-Muller J, Aeschbacher RA, Wiemkien A, Boller T, Wingler A (2002) Induction of Trehalase in *Arabidopsis* Plants Infected With the Trehalose-Producing Pathogen *Plasmodiophora brassicae*. Molecular Plant-Microbe Interactions 15: 693-700

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Gancedo C, Flores C-L (2004) The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Research 4: 351-359

Garcia NAT, Iribarne C, Lopez M, Herrera-Cervera JA, Lluch C (2005) Physiological implications of trehalase from *Phaseolus vulgaris* root nodules: partial purification and characterization. Plant Physiology and Biochemistry in press:

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Goddijn OJM, van Dunn K (1999) Trehalose metabolism in plants. Trends in Plant Science 4: 315-319

Hoepfner SW, Botha FC (2003) Expression of fructokinase isoforms in the sugarcane culm. Plant Physiology and Biochemistry 41: 741-747

Leyman B, Van Dijk P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends in Plant Science 6: 510-513

Muller J, Aeschbacher RA, Wingler A, Boller T, Wiemkien A (2001) Trehalose and Trehalase in Arabidopsis. Plant Physiology 125: 1086-1093

Muller J, Boller T, Wiemkien A (1995) Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and nodules of soybean and cowpea. Planta 197 : 362-368

Muller J, Staehelin R, Mellor T, Wiemkien A (1992) Partial purification and characterization of trehalase from soybean nodules. Journal of Plant Physiology 140: 8-13

Roessner-Tunali U, Hegemann G, Lytovechenko A, Carrari F, Bruedigam C, Granot D, Fernie AR (2003) Metabolic Profiling of Transgenic Tomato Plants Overexpressing Hexokinase Reveals That the Influence of Hexose Phosphorylation Diminishes during Fruit Development. Plant Physiology 133: 84-99

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmetally Modified Plant Systems. The Plant Cell 13: 11-29

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. The Plant Journal 23: 131-142

Rohwer JM, Botha FC (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochemical Journal 358: 437-445

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Schluepmann H, van Dijken AJH, Aghdasi M, Wobbes B, Paul MJ, Smeekens SC (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate accumulation. Plant Physiology 135: 879-890

Thevelein JM, Hohmann S (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? Trends in Biological Sciences 20: 3-10

Valenzuela-Soto EM, Marquez-Escalante JA, Iturriaga G, Figueroa-Soto CG (2004) Trehalose 6-phosphate synthase from *Selaginella lepidophylla*: purification and properties. Biochemical and Biophysical Research Communications 313: 314-319

Van Dijck P, Mascorro-Gallardo JO, De Bus M, Royackers K, Iturriaga G, Thevelein JM (2002) Truncation of *Arabidopsis thaliana* and *Selaginella lepidophylla* trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochemical Journal 366: 63-71

van Dijken AJH, Schluepmann H, Smeekens SC (2004) Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegitative Growth and Transition to Flowering. Plant Physiology 135: 9

Vogel G, Aeschbacher RA, Muller J, Boller T, Wiemkien A (1998) Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of yeast *tps2* mutant. The Plant Journal 13: 673-683

Voit EO (2003) Biochemical and genomic regulation of the trehalose cycle in yeast: review of observations and canonical model analysis. Journal of Theoretical Biology 223: 55-78

Wang Y-J, Hao Y-J, Zhang Z-G, Chen T, Zhang J-S, Chen S-Y (2004) Isolation of trehalose-6-phosphate phosphatase gene from tobacco and its functional analysis in yeast cells. Journal of Plant Physiology in press:

Zentella R, Mascorro-Gallardo JO, Van Dijck P, Folch-Mallol J, Bonini BM, Van Vaeck C, Gaxiola R, Covarrubias AA, Nieto-Sotelo J, Thevelein JM, Iturriaga G (1999) A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant. Plant Physiology 119: 1473-1482

CHAPTER 6

METABOLIC CONTROL ANALYSIS OF SUGARCANE SUCROSE AND TREHALOSE METABOLISM

Abstract

Sucrose accumulation is the result of the difference between synthesis (including cytosolic production and vacuolar storage) and degradation. Sugarcane cytosolic sucrose metabolism has previously described using kinetic modelling and metabolic control analysis (MCA) (Rohwer & Botha 2001). This sucrose model has been expanded to include trehalose metabolism in this Chapter. The aim was to supplement the available information on cytosolic metabolism in sugarcane storage parenchyma, identify points of control between sucrose and trehalose metabolism, thus providing a basis for further experimental work and *in silico* kinetic modelling. pH values of sub-cellular compartments were determined with ^{31}P-NMR. The cytosolic pH was approximately 7.1, and both the apoplastic and vacuolar compartments had pHs below 5. A model of the linear trehalose pathway was constructed, and its parameters validated by experimentally determined trehalose levels. The model predicted trehalose in the same order of magnitude as the experiment. The majority of calculated flux control (>70%) over the pathway resided in the trehalose-6-phosphate synthase (EC 2.4.1.15, TPS) step. Incorporation of the trehalose branch into the original sucrose model showed that reactions from the original model significantly affected the steady-state attributes of the trehalose pathway. Due to the relatively low flux through the trehalose branch of the expanded model, complete recycling of trehalose, and the lack of allosteric regulation by trehalose-6-phosphate or trehalose on any of the reactions from the original sucrose model, incorporation of the trehalose branch had no significant effect on either steady-state cytosolic sucrose concentration or flux of sucrose into the vacuole. The expanded model is a useful tool for further investigation of trehalose metabolism in the context of plant sucrose accumulation.

6.1. Introduction

The metabolic complexities of biological systems demand investigation that goes beyond the quantification and characterisation of single enzymes or metabolites. Accumulated "end products" of metabolism are not the result of simple linear pathways, but rather networks of reactions that are tightly regulated. Sucrose accumulation in sugarcane internodes is an example of such integrated metabolism.

Commercial sugarcane varieties have higher sucrose yield than ancestral varieties (Zhu et al. 1997). The mechanisms that govern this phenotypic difference have not been sufficiently elucidated. Further investigations of the metabolic control of sucrose accumulation may indicate specific enzymes or metabolites that exercise significant control on sucrose levels in the sugarcane culm. This information can be used in screening progeny of traditionally crossed *Saccharum* hybrids, or integrated into a focussed genetic transformation program (reviewed in (Grof & Campbell 2001).

Sucrose accumulation in sugarcane internodes has been modelled as a tool to describe, understand and predict points control in this component of carbohydrate metabolism (Rohwer & Botha 2001). Enzymes able to both synthesise and degrade sucrose are located in the cytosol of plant cells (reviewed in (Hawker et al. 1991). Although sucrose is accumulated in the vacuole, and can be hydrolysed in both the apoplast and vacuole, the focus of this study is on cytosolic sucrose metabolism, with particular interest in its regulation. Kinetic modelling and metabolic control analysis (MCA) have indicated that hexose import, transport of sucrose into the vacuole, and neutral invertase could be three points in sucrose metabolism that were best suited to genetic manipulation with the aim of increasing sucrose yield (Rohwer & Botha 2001). Even though the current sucrose model has been validated with previously determined steady-state metabolite levels (Whittaker & Botha 1997) it does not claim to describe cytosolic sucrose metabolism in its entirety.

The study of the control of carbon partitioning in plants has spanned the disciplines of transcriptomics (Krapp & Stitt 1995; Buckhout & Thimm 2003), metabolomics (Fiehn et al. 2000; Kose et al. 2001; Fernie 2003), targeted genetic manipulation (Eastmond et al. 2002) and more traditional studies focussed on one or two enzymes (Lingle & Smith 1991; Lingle 1997; Obiadalla-Ali et al. 2004). Recent technological advances have allowed high-throughput production of data from transcriptomic and metabolomic studies. The indiscriminate nature of these technologies has led to the observation of many seemingly

unrelated, or undiscovered phenomena. Such an example is the prevalence of transcripts putatively coding for trehalose metabolising enzymes, and widespread identification of the metabolites trehalose and, to a lesser extent, trehalose-6-phosphate from plant transcriptomic (Wendler et al. 1990; Carson et al. 2002; Casu et al. 2003) and metabolomic studies (Whittaker & Botha 1997; Roessner et al. 2000; Roessner et al. 2001). In plants, trehalose is synthesised in a two step process via the enzymes trehalose-6-phosphate synthase (EC 2.4.1.15, TPS) and trehalose-6-phosphate phosphatase (EC 3.2.1.28, TPP). Degradation of trehalose is catalysed by trehalase (EC 3.2.1.28). Steady-state flux through the trehalose pathway, and levels of its intermediates, affect the partitioning of carbon in yeast and bacteria, primarily through the inhibition of hexokinase activity by trehalose-6-phosphate (reviewed in (Arguelles 2000; Gancedo & Flores 2004)). This has renewed interest in the identification of components of trehalose metabolism and their potential role(s) in plant metabolism (Eastmond et al. 2003; Eastmond & Graham 2003).

In light of the potential effects of trehalose metabolism in carbon partitioning, the aim of this Chapter is to integrate trehalose synthesis and degradation into the current sugarcane sucrose model (Rohwer & Botha 2001), using kinetic parameters of trehalose metabolising enzymes from Chapters 4 and 5. MCA of this sucrose-trehalose model will be used to indicate what effect(s) sucrose and trehalose metabolism have on each other.

6.2. Materials and Methods

6.2.1. Plant material

Sugarcane variety N19 (interspecific *Saccharum* hybrid) was grown under field conditions at Welgevallen experimental farm, Stellenbosch, South Africa. Internodes were numbered sequentially with the internode attached to the first exposed dewlap designated number one.

6.2.2. Hexokinase inhibition assays

Proteins for hexokinase inhibition studies were prepared from leaf roll tissue of sugarcane, variety N19. Enzymes were extracted and assayed as previously described (Hoepfner & Botha 2003). Trehalose-6-phosphate was included in the assays at concentrations of 0 to 10 mM.

6.2.3. ^{31}P NMR analysis of intracellular pH

6.2.3.1. Construction of pH vs. inorganic phosphate (Pi) shift titrations

The solution used for construction of the pH titration curve was identical to that used in the treatment of plant tissues before NMR analysis. The solution contained 25 mM K-Mes, 5 mM Na_2HPO_4, 5 mM glucose, 5 mM fructose, 250 mM mannitol, 1mM tri-ethyl phosphate (TEP) and 5 mM methylphosphonate (MeP) and pH adjusted to values between approximately 3.5 and 8 in increments of 0.5 units. TEP was used as an internal reference. MeP was used as a pH reference. ^{31}P shift was plotted against the pH for the calibration.

To determine whether the cellular environment had any effect on Pi shift, internodal extracts were added to the buffer before the pH was set and NMR performed. The extracts were prepared from internode 5 tissue. The tissue was ground to a fine powder in liquid nitrogen and added to the above-described buffer in a 2:1 (v/w) ratio. The extract was thoroughly mixed and passed through a Buchner funnel to get rid of the largest cellular waste particles. The suspension was then centrifuged at 10 000 rpm for 15 minutes at 4°C. The supernatant was divided into 10 aliquots and pH adjusted to values between approximately 3.5 and 8 in increments of 0.5 units. Once again, ^{31}P shift was plotted against the pH for calibration, and compared with the first calibration curve to determine if there were cellular components affecting Pi shift independent of the pH of the medium.

6.2.3.2. Preparation of plant tissues for in vivo biological NMR

Sugarcane, variety N19, was harvested from field grown plots at Welgevallen Stellenbosch. Cylinders of internode 5 tissue were bored out using a no. 4 cork borer. Sections were immediately placed in incubation buffer containing 25 mM K-Mes, 5 mM Na_2HPO_4, 5 mM glucose, 5 mM fructose, 250 mM mannitol, 1 mM TEP and 5 mM MeP. The buffer only partially covered the tissue and was agitated for 14 hours in the dark. NMR samples were prepared by mixing D_2O and incubation buffer in 1:4 (v/v) ratio, and insertion of the tissue cylinder into a 10 ml NMR tube.

6.2.3.3. NMR spectroscopy

^{31}P NMR spectra were collected on a 600 MHz Varian Unity Inova spectrometer equipped with an Oxford magnet (14.09 T) operating at 242.879 MHz for ^{31}P. Experiments were carried out at 20 °C on a 10 mm broad band (^{15}N - ^{31}P) probe. A standard one pulse sequence was used with a 1 sec pulse delay, 60° pulse angle, 1.6 sec acquisition time and inverse gated ^{1}H decoupling. Data were collected for 30 min per spectrum (690 transients).

6.3.4. In silico kinetic modelling and MCA

A kinetic model for the linear trehalose pathway was constructed using the rate equations below for TPS (6.1.), TPP (6.2.) and trehalase (6.3.).

$$v_{TPS} = \frac{\left[\frac{V_{max\ TPS}}{K_{m\ UDPglc} * K_{m\ G6P}}\right] * \left[[UDPglc][G6P] - \frac{[UDP]*[T6P]}{K_{eq\ TPS}}\right]}{\left[1 + \frac{[UDPglc]}{K_{m\ UDPglc}} + \frac{[UDP]}{K_{m\ UDP}}\right] * \left[1 + \frac{[G6P]}{K_{m\ G6P}} + \frac{[T6P]}{K_{m\ T6P}}\right]} \quad (6.1.)$$

$$v_{TPP} = \frac{V_{max\ TPPAI} * [T6P]}{K_{m\ TPPAI} + [T6P]} + \frac{V_{max\ TPPAII} * [T6P]}{K_{m\ TPPAII} + [T6P]} + \frac{V_{max\ TPPB} * [T6P]}{K_{m\ TPPB} + [T6P]} \quad (6.2.)$$

$$V_{trehalase} = \frac{V_{max\ trehalase} * [Tre]}{K_{m\ trehalase} * \left[1 + \frac{[Tre]}{K_{m\ trehalase}} + \frac{[Glc]}{K_{i\ Glc}} + \frac{[Suc]}{K_{i\ suc}}\right]} \quad (6.3.)$$

Enzyme kinetic parameters from Chapters 4 and 5 were used along with estimations for those not available from sugarcane, as detailed in table 6.3. Steady-state analysis and MCA were done using the modelling application PySCeS, the Python Simulator for Cellular Systems (Olivier et al. 2005).

The trehalose pathway was integrated into the current sucrose model as indicated in figure 6.5. (Rohwer & Botha 2001) including adjustments to sucrose synthase (EC 2.4.1.13, SuSy) parameters (Schäfer et al. 2004).

6.3.5. Moisture content of sugarcane internodal tissues

Sugarcane culms were harvested and internodal sections weighed for determination of fresh weight values. These sections were desiccated at 80°C to complete dryness, and their masses recorded. Moisture content was calculated from the difference between fresh and dry weights per internode.

6.3. Results

6.3.1. Intracellular pHs of sugarcane internodal tissue cells

^{31}P-NMR spectroscopy was used to ascertain *in vivo* pHs of internode 5 tissues of sugarcane variety N19.

6.3.3.1. Pi and MeP titration curves

Titration curves of buffers ranging from pH 3.8 to 8.3 (both including and excluding sugarcane internodal extract) were plotted to determine whether any intracellular components interfered with Pi shift (figure 6.1). Buffers contained Pi, TEP (internal shift standard) and MeP (internal pH standard). The chemical shift of the resonance of a pH dependant species is described by equation 6.4., where the chemical shifts of the acid and base are given as $^{a}\delta$ and $^{b}\delta$, respectively (Stewart et al. 1986).

$$\delta = \frac{[A^-]\,{}^{b}\delta + [HA]\,{}^{a}\delta}{[HA] + [A^-]} \qquad (6.4.)$$

Equation 6.4. can be rearranged and incorporated into the Henderson-Hasslebalch equation as in equation 6.5., where pK'_a is the apparent pK_a.

$$\delta = pK'a + \log_{10}\frac{(\delta - {}^{a}\delta)}{({}^{b}\delta - \delta)} \qquad (6.5.)$$

Titration curves for both phosphorous species; Pi (figure 6.1A) and MeP (figure 6.1B) fitted to equation 6.5. with R^2 values higher than 0.999. Constants for $^{a}\delta$, $^{b}\delta$ and pK'_a from both Pi and MeP are given in table 6.1. Plant cell extracts included in the titration buffers had no effect on the chemical shift of the resonance of ^{31}P.

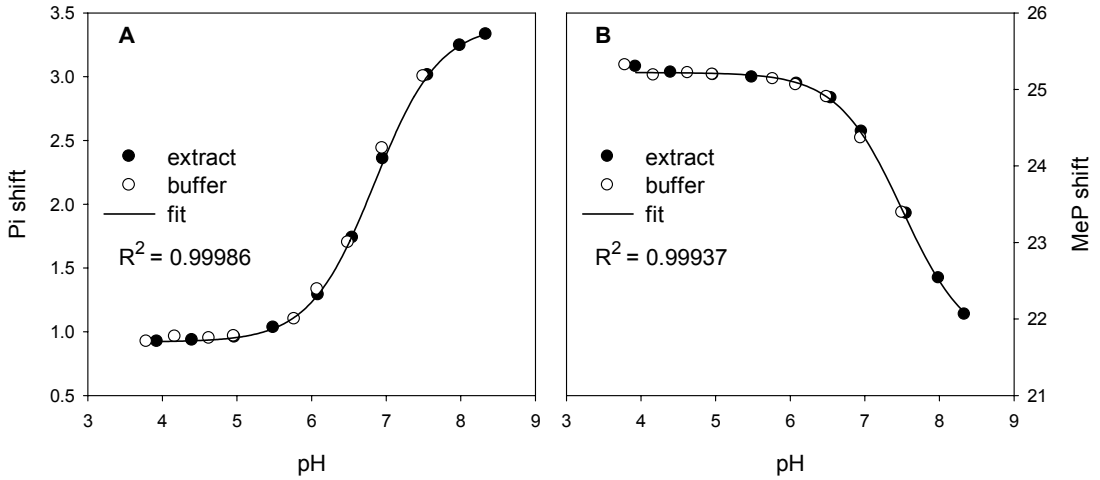


Figure 6.1. Titration curves of the chemical shift of the ^{31}Pi resonance of (A) Pi and (B) MeP, including extract (●) or with buffer alone (○).

Table 6.1. Parameters for fitting titration curves of Pi and MeP to equation 6.5.

Parameter	**Phosphorous species**	
	Pi	*MeP*
pK'_a	6.82	7.50
$^{a}\delta$	0.92	25.2
$^{b}\delta$	3.41	21.6

6.3.3.2. pHs of intracellular compartments using in vivo NMR

In vivo NMR analysis of ^{31}P resonance was used to determine the pHs of intracellular compartments of sugarcane internodal tissue (internode 5, sugarcane variety N19). The ^{31}P resonance shift of endogenous Pi gave only one peak δ = 0.960 on the spectrum (figure 6.2) which corresponded to pH ≤5.1.

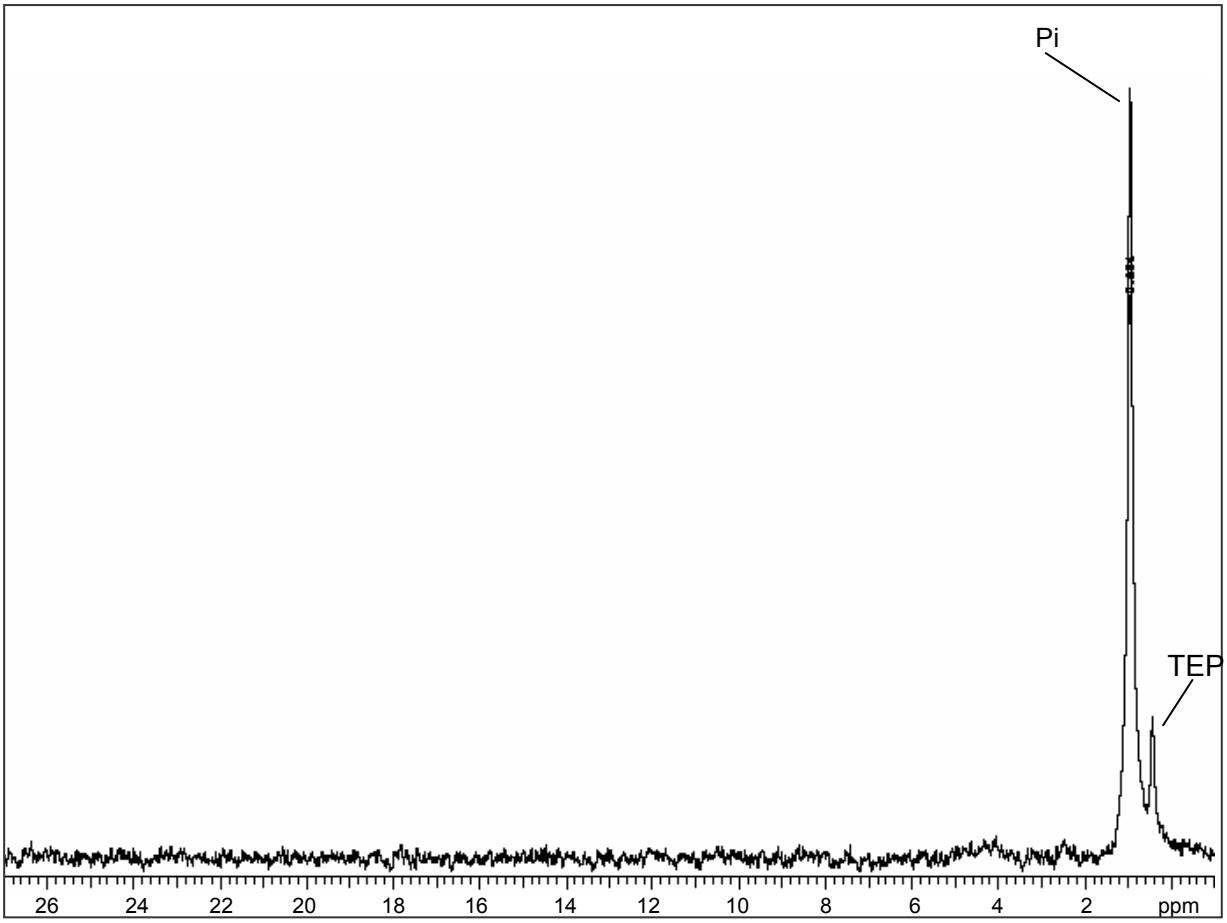


Figure 6.2. *In vivo* ^{31}P-NMR spectrum of endogenous Pi in sugarcane internode 5. Peaks corresponding to Pi and TEP (internal standard) are indicated.

It was necessary to increase the Pi concentration in the cytosol in order to detect a shift in resonance at a neutral pH. Four cylinders of internodal tissue were incubated in buffers at pH 4.2, 5.0, 6.1 and 7.0 and their ^{31}P-NMR spectra recorded (figure 6.3). Chemical shifts for Pi and MeP were assigned to cellular compartments (see figure 6.3), and fitted on the titration curves to determine pH (table 6.2)

Table 6.2. Intracellular pH determination using *in vivo* ^{31}P-NMR of Pi and MeP. pHs are determined from chemical resonance shift relative to TEP. Internodal tissues were treated at A, pH 4.2; B, pH 5.0; C, pH 6.1 and D, pH 7.0.

Treatment	**pH**					
	cytosol		*apoplast*		*vacuole*	
	Pi	*MeP*	*Pi*	*MeP*	*Pi*	*MeP*
A	7.2	7.2	§	<5.0	<5.0	§§
B	7.1	7.2	§	5.2	<5.0	§§
C	7.1	7.1	6.0	6.1	<5.0	§§
D	7.1	6.7	6.8	7.1	5.2	5.4

§ apoplastic Pi coinciding with vacuolar Pi. §§ vacuolar MeP coinciding with apoplastic MeP.

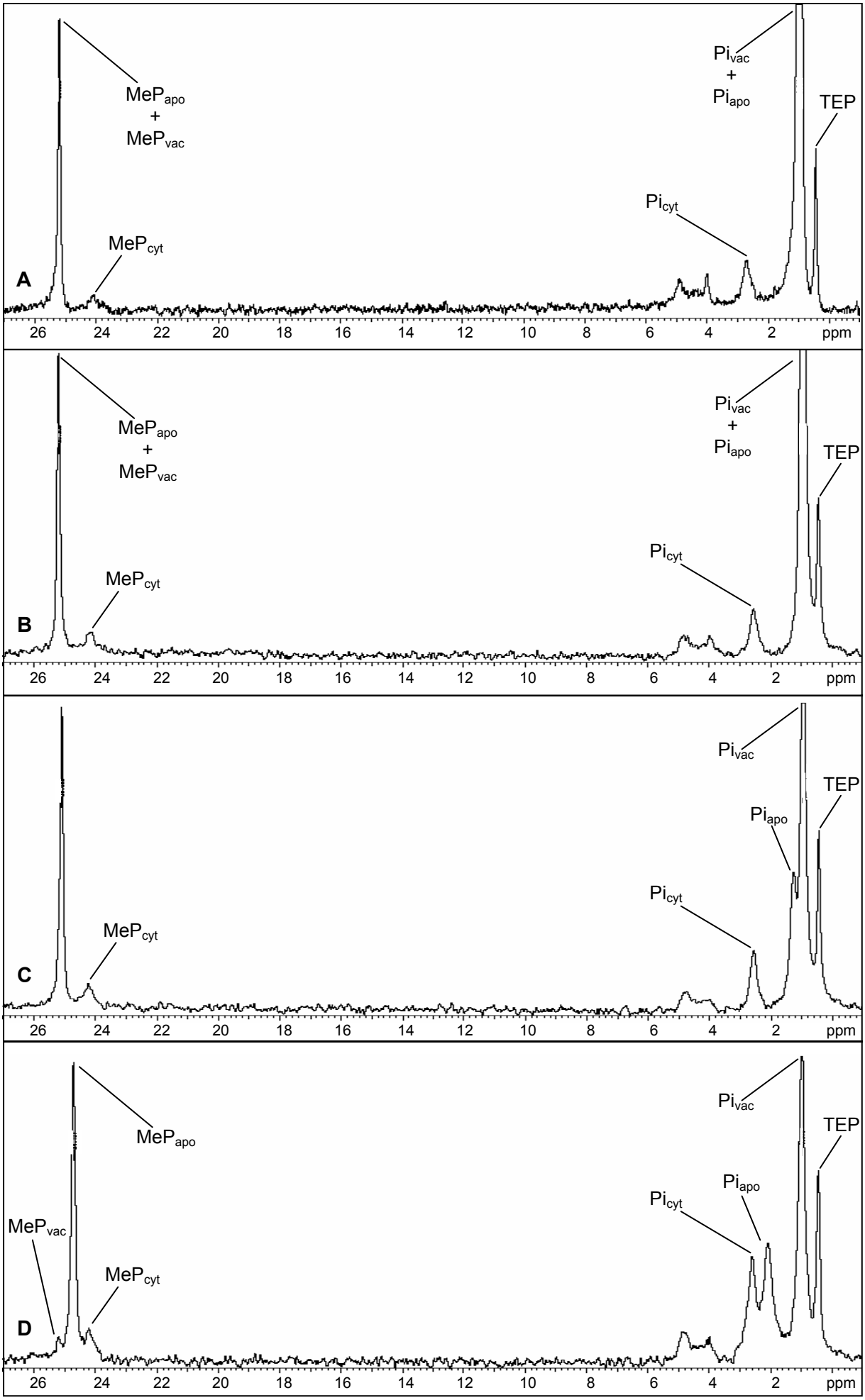


Figure 6.3. *In vivo* ^{31}P-NMR spectra of sugarcane internodal tissues incubated at A, pH 4.2; B, pH 5.0; C, pH 6.1; and D, pH 7.0. Chemical shifts for tri-ethylphosphate (TEP), phosphate (Pi) and methylphosphonate (MeP) were recorded in cytosol (cyt), apoplast (apo) and vacuole (vac).

6.3.2. Effect of trehalose-6-phosphate on sugarcane hexokinases

In order to identify points of control in sugarcane carbohydrate metabolism by trehalose pathway intermediates, the effect of trehalose-6-phosphate on sugarcane hexokinases was investigated, because trehalose-6-phosphate is an inhibitor of yeast hexokinase (Blazquez et al. 1993), inhibiting up to 60% of activity at a concentration of 1 mM. However, including trehalose-6-phosphate in concentrations up to 10 mM in sugarcane hexokinase assays had no effect on the phosphorylating activities using 0.1 mM fructose, 1 mM fructose or 2 mM glucose as substrate (figure 6.4.).

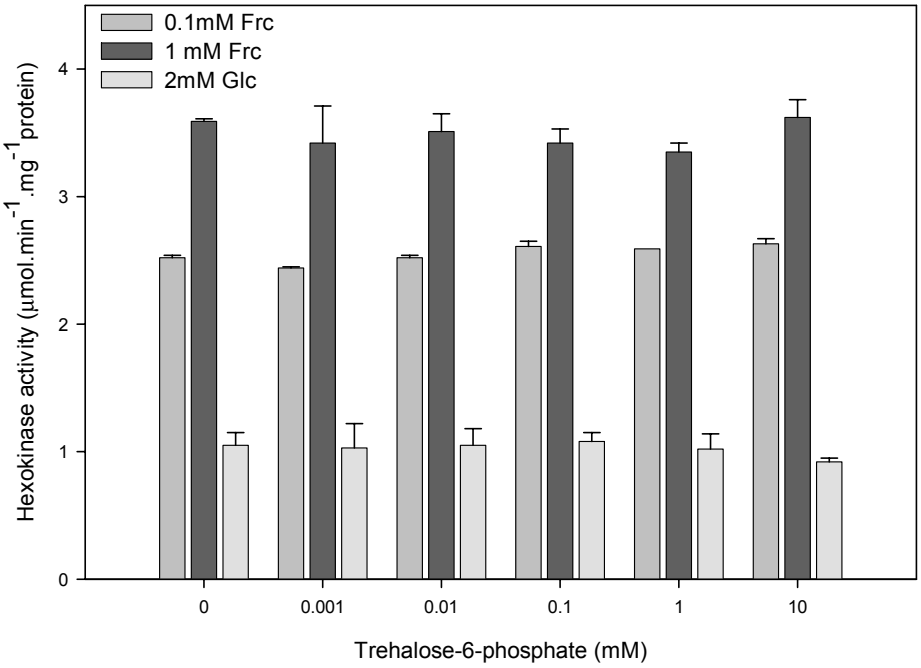


Figure 6.4. Sugarcane hexokinase activities measured in the presence of trehalose-6-phosphate. Phosphorylating activity was measured with 0.1 mM fructose, 1 mM fructose and 2 mM glucose separately as substrates. Data are the mean of four replicates ± standard deviation.

6.3.3. Modelling the linear trehalose synthesis/degradation pathway

Kinetic parameters for TPS, TPP and trehalase used in the linear trehalose pathway kinetic model are presented in table 6.3.

Table 6.3. Kinetic parameters of trehalose metabolising reactions. All V_{max} values are in $mM.min^{-1}$, and K_m and K_i in mM. Reaction numbers in parentheses are designated for the integration of the trehalose pathway into the sucrose metabolism model.

Reaction/parameter	Internode	Value	Reference	Comment
Reaction 1 (12) : trehalose-6-phosphate synthase				
$V_{max\ TPS}$ (1h)	3	0.0405	Table 4.5.	Determined using ^{14}C assay
$V_{max\ TPS}$ (2h)	3	0.0088	Table 4.5.	Determined using ^{14}C assay
$K_{mUDPGlc}$		0.5	(Vandercammen et al. 1989)	*S. cerevisiae*
K_{mG6P}		3.5	(Vandercammen et al. 1989)	*S. cerevisiae*
K_{mUDP}		0.3		Estimate
K_{mT6P}		0.1		Estimate
$K_{eq\ TPS}$		40	(Cabib & Leloir 1958)	
Reaction 2 (13) : trehalose-6-phosphate phosphatase				
$V_{max\ TPP\ total}$	3	0.0592	Figure 4.11.	
$V_{max\ TPPAI}$	3	0.00799		13.5% $V_{max\ TPP\ total}$
$V_{max\ TPPAII}$	3	0.0259		43.8% $V_{max\ TPP\ total}$
$V_{max\ TPPB}$	3	0.0253		43.0% $V_{max\ TPP\ total}$
$V_{max\ TPP\ total}$	6	0.0516	Figure 4.11.	
$V_{max\ TPPAI}$	6	0.00697		13.5% $V_{max\ TPP\ total}$
$V_{max\ TPPAII}$	6	0.0226		43.8% $V_{max\ TPP\ total}$
$V_{max\ TPPB}$	6	0.0220		43.0% $V_{max\ TPP\ total}$
K_{mTPPAI}		0.447	Table 5.4.	
$K_{mTPPAII}$		13.8	Table 5.4.	
K_{mTPPB}		1.39	Table 5.4.	
Reaction 3 (14) : trehalase				
$V_{max\ trehalase}$	3	0.0431	Figure 4.12.	
$V_{max\ trehalase}$	6	0.0164	Figure 4.12.	
K_{mTre}		0.360	Table 5.5.	average of $K_{m\ TREA}$ and $K_{m\ TREB}$
$K_{i\ Glc}$		13.2	(Fleischmacher et al. 1980)	Calculated from tabulated data
$K_{i\ Suc}$		16.0	(Fleischmacher et al. 1980)	

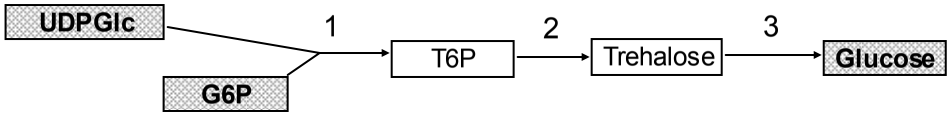
All enzymes' V_{max} values were calculated from the values in Chapter 4 as determined from extracts of *Saccharum* variety N19. Values in $mM.min^{-1}$ were calculated from the original $nmol.min^{-1}.mg^{-1}$protein, using protein contents specific for each tissue sampled, and assuming that moisture contents were 900 $\mu l.g^{-1}$FW for internode 3, and 700 $\mu l.g^{-1}$FW for internode. All enzymes and metabolites in this pathway were assumed to be located in the cytosol, whose volume was assumed at 10% of the total cellular volume. V_{max} values for TPS were calculated from both one and two hour time points from the ^{14}C assay described in

section 4.3.4.1. Due to a lack of available K_m values for sugarcane TPS, $K_{m\ substrates}$ from *S. cerevisiae* (Vandercammen et al. 1989) were used, and $K_{m\ products}$ were estimated. All three isoforms of TPP identified in Chapter 5 were included in the kinetic model. Proportions of the $V_{max\ TPP\ total}$ assigned to each isoform were calculated from tables 5.1. and 5.4. Inhibition of sugarcane trehalase by both its product glucose, and sucrose had been previously reported (Fleischmacher et al. 1980). The $K_{i\ Suc}$ is given as 16 mM, and $K_{i\ Glc}$ was calculated from the tabulated data as 13.2 mM using enzyme kinetics software (SigmaPlot v7.0 © with Enzyme Kinetics module v1.1, SPSS Inc.).

Steady state fluxes and metabolite values for the linear trehalose pathway (table 6.4.) were calculated using the model with both experimentally determined fixed metabolite levels (table 3.1.) and levels previously used (Rohwer & Botha 2001). Flux through the three enzymes in the linear pathway was always equal under a specific condition, and is indicated as a single steady state flux condition in table 6.4. Inhibition of trehalase by glucose affected steady state trehalose levels, and had no effect on either steady state flux through the pathway or trehalose-6-phosphate levels.

Predicted steady state trehalose levels were closest to those determined experimentally when the input metabolite values for UDP-glucose and glucose-6-phosphate were fixed as determined in the metabolite analysis of section 3.3.1. Without glucose inhibition on trehalase the predicted value for internode 3 was 0.0047 mM, 1.85 times lower than the experimentally determined value. Including inhibition on trehalase at a glucose concentration of 30 mM, the predicted trehalose level increased to 0.0155 mM, 1.8 times higher than the experimentally determined value. However, when using UDP-glucose and glucose-6-phosphate levels previously reported in the sucrose accumulation model (Rohwer & Botha 2001), predicted trehalose levels were consistently higher than experimentally determined levels.

Table 6.4. Steady state properties of the linear trehalose pathway model. Reactions TPS (1), TPP (2) and trehalase (3) represent the linear trehalose pathway. All analyses were performed with $V_{max\ TPS}$ determined experimentally from section 4.3.4.1. (1h, 0.0405 mM.min^{-1}). Data are reproduced for internodes 3 and 6 (N19), with varying levels of glucose inhibition on trehalase. Fixed input metabolite levels (UDPGlc and G6P) were used at different levels in the input file: **A** determined experimentally (Table 3.1), **B** (Rohwer & Botha 2001). Experimentally determined trehalose levels were taken from table 3.1. and calculated as indicated in the footnote.

Inhibition on trehalase	*Fixed (f) and steady- state (ss) metabolite levels (mM) and flux (mM.min^{-1}) determined with kinetic model*						*experimentally determined*	
								
A	***UDPGlc (f)***	***G6P (f)***	***T6P (ss)***	***Trehalose (ss)***	***Glucose (f)***	***Flux (ss)***	***Tre (cell)§***	***Tre (cyt)§§***
Internode3							0.00087	0.0087
no glucose inhibition	0.207	0.301	0.0150	0.0047	83.1	0.000560		
glc inhibition (cellular levels)	0.207	0.301	0.0150	0.0346	83.1	0.000560		
glc inhibition (30mM)	0.207	0.301	0.0150	0.0155	30.0	0.000560		
Internode6							0.00075	0.0075
no glucose inhibition	0.097	0.2	0.00650	0.0048	34.6	0.000215		
glc inhibition (cellular levels)	0.097	0.2	0.00655	0.0174	34.6	0.000215		
glc inhibition (30mM)	0.097	0.2	0.00655	0.0156	30.0	0.000215		
B	***UDPGlc (f)***	***G6P (f)***	***Tre6P (ss)***	***Trehalose (ss)***	***Glucose (f)***	***Flux (ss)***		
Internode3								
no glucose inhibition	2.46	0.34	0.0514	0.0159	83.1	0.00182		
glc inhibition (cellular levels)	2.46	0.34	0.0514	0.1160	83.1	0.00182		
glc inhibition (30mM)	2.46	0.34	0.0573	0.0521	30.0	0.00182		
Internode6								
no glucose inhibition	2.46	0.34	0.0573	0.0432	34.6	0.00176		
glc inhibition (cellular levels)	2.46	0.34	0.0573	0.1574	34.6	0.00176		
glc inhibition (30mM)	2.46	0.34	0.0514	0.1415	30.0	0.00176		

§ Tre (cell) = total trehalose measured (Table 3.1.)

§§ Tre (cyt) = 10% of Tre (cell), assuming all the trehalose is in the cytosol and the cytosol is 10% of the total cellular volume

6.3.3.1. Control analysis of the linear trehalose pathway

Table 6.5. Control analysis of the linear trehalose pathway. All analyses were done with experimentally determined parameters for internode 3 with $V_{max\ TPS}$ = 0.0405 mM.min^{-1}. Fixed metabolites UDP-glucose and glucose-6-phosphate determined experimentally (A), and from the literature (Rohwer & Botha 2001) (B) were used as input values. Including glucose inhibition (up to 85 mM) on trehalase had no effect on the control distribution.

	Flux control coefficients			***Concentration-control coefficients***					
	C^{J}_{TPS}	C^{J}_{TPP}	$C^{J}_{Trehalase}$	C^{T6P}_{TPS}	C^{T6P}_{TPP}	$C^{T6P}_{Trehalase}$	C^{Tre}_{TPS}	C^{Tre}_{TPP}	$C^{Tre}_{Trehalase}$
A	0.89	0.11	0.00	0.91	-0.91	0.00	0.90	0.11	-1.01
B	0.75	0.25	0.00	0.80	-0.80	0.00	0.78	0.27	-1.04

Both flux- and concentration-control coefficients for the linear trehalose pathway were calculated (table 6.5.). Flux control coefficients in a given pathway always sum to one, and are calculated for each enzyme catalysed step. Therefore, in the linear trehalose pathway, total control over the flux through the pathway is shared between the three enzymes TPS, TPP and trehalase. The rate of TPS catalysis exercised the greatest amount of control over flux, accounting for at least 74% of the total control. Regardless of the amount of inhibition on trehalase, the rate of the TPS reaction held the same level control over the pathway. Increased substrate levels of TPS (table 6.5B) reduced the flux control coefficient of TPS.

Concentration-control coefficients describe the effect of a reaction rate on a steady state metabolite level. Reactions that contribute to the pool of a particular metabolite are positive, and those that cause a decrease are negative. These features cause concentration-control coefficients in a pathway to sum to zero. Control over steady state trehalose-6-phosphate levels was equally shared between the rates of TPS and TPP, the former synthesising and the latter degrading. Trehalose-6-phosphate steady-state levels were unaffected by the rate of the trehalase reaction. Control of steady-state trehalose levels is shared between all three enzymes, with trehalase having the largest numerical concentration control coefficient.

6.3.3.2. Validation of experimentally determined V_{max}*TPS*

V_{maxTPS} was experimentally determined using a ^{14}C radio-labelling assay (table 4.5.) from internode 3 (N19), and the challenges regarding these and other TPS assays were discussed in depth in section 4.4. The kinetic model of the linear trehalose pathway, as described above, was used to fit $V_{max\ TPS}$ for the validation of the assayed activity and prediction of values for internode 6 tissues. $V_{max\ TPS}$ was fitted by adjusting its value until trehalose steady-state concentrations corresponded to 0.0087 mM (for internode 3) and 0.0075 mM (for internode 6) (table 6.4.). Glucose inhibition on trehalase was included to observe its effect on the reaction rate of TPS.

Table 6.6. $V_{max\ TPS}$ fitted to experimentally determined trehalose levels

		$V_{max\ TPS}$	
	Inhibition on trehalase	*internode 3*	*internode 6*
experimentally determined		0.0405	nd
fitted on model	none	0.0811	0.0657
	glucose at cellular levels[§]	0.0093	0.0179
	glucose at 30 mM[§§]	0.0216	0.0191

[§]glucose at cellular levels as determined from table 3.1. 83.11 mM in internode 3, and 34.86 mM in internode 6, assuming equal distribution of glucose concentration between cellular compartments. [§§](Rohwer & Botha 2001). nd = not determined

In internode 3 the fitted $V_{max\ TPS}$ without inhibition on trehalase was double that of the experimental determination (table 6.6.). Including glucose inhibition on trehalase at cellular levels of glucose caused a $V_{max\ TPS}$ over 4 times lower than the measured one. With glucose levels at 30 mM (close to the levels in the sucrose model (Rohwer & Botha 2001)), the fitted $V_{max\ TPS}$ was lower with a factor of approximately 2. Trehalase is inhibited by glucose (Fleischmacher et al. 1980) and this could be an important factor when integrating the linear trehalose pathway into the current sucrose model, for this reason the fitted $V_{max\ TPS}$ values for both internodes 3 and 6 determined with 30 mM glucose in the fitting were used for all subsequent analyses where stated.

6.3.4. Integration of the trehalose pathway into the sugarcane sucrose model

The linear trehalose pathway described above was incorporated into a kinetic model of sugarcane sucrose metabolism (Rohwer & Botha 2001; Schäfer et al. 2004).

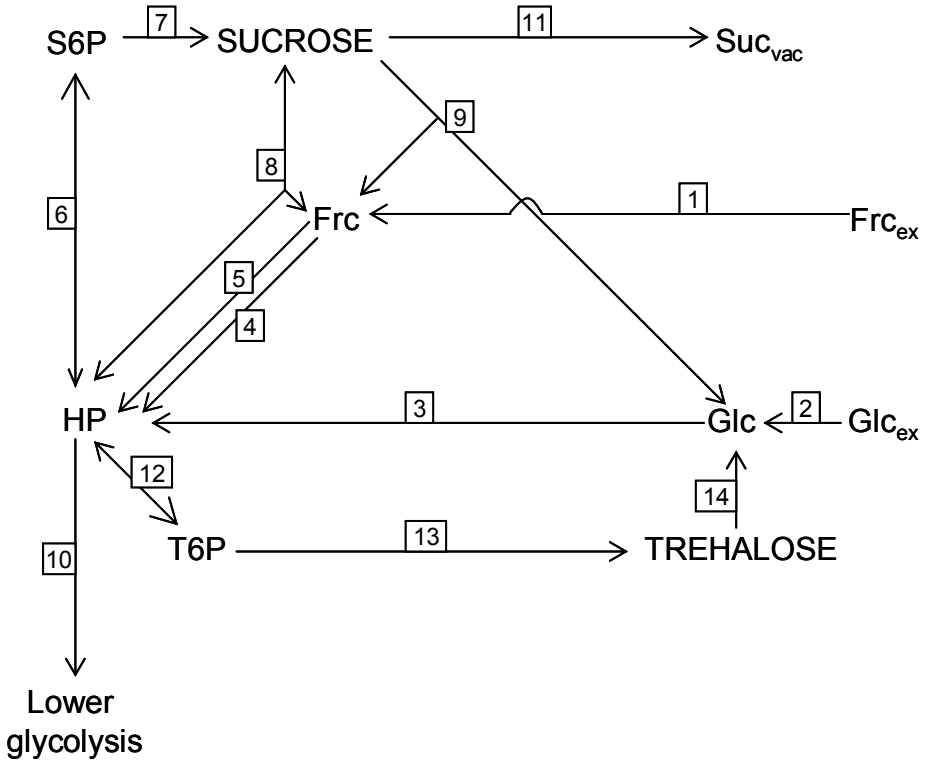


Figure 6.5. Sucrose and trehalose metabolism. Reactions 1 through 11 as in (Rohwer & Botha 2001), reactions 12, 13 and 14 have been integrated to represent the trehalose pathway. Reactions: 1, fructose (Frc) uptake; 2, glucose (Glc) uptake; 3, hexokinase (Glc); 4, hexokinase (Frc); 5, fructokinase; 6, sucrose phosphate synthase; 7, sucrose phosphate phosphatase; 8, sucrose synthase; 9, invertase; 10, glycolysis; 11, sucrose import into the vacuole; 12, trehalose-6-phosphate synthase; 13, trehalose-6-phosphate phosphatase; 14, trehalase. Abbreviations: Frc, fructose; Glc, glucose; HP, hexose phosphates; S6P, sucrose-6-phosphate; Suc_{vac}, sucrose in the vacuole; T6P, trehalose-6-phosphate. Subscript $_{ex}$ assigned to extracellular metabolites.

6.3.4.1. Steady state properties of the current sucrose model

Steady-state attributes of the sucrose model (Rohwer & Botha 2001) as corrected in (Schäfer et al. 2004) (without the trehalose branch) are presented in table 6.7. The following corrections to the kinetic parameters of SuSy were proposed (Schäfer et al. 2004): $K_{eq\ SuSy}$ = 0.50, $K_{i\ UDP}$ = 0.3, $K_{i\ fructose}$ = 3.92 mM. From this point on the corrected sucrose model will be referred to as the 'current sucrose model'.

Table 6.7. Steady state attributes of the current sucrose kinetic model.

A		***Flux***
Reaction	***Enzyme***	***mM/min***
R1	Fructose uptake	0.147
R2	Glucose uptake	0.118
R3	Hexokinase (Glc)	0.156
R4	Hexokinase (Frc)	0.001
R5	Fructokinase	0.052
R6	Sucrose phosphate synthase	0.018
R7	Sucrose phosphate phosphatase	0.018
R8	Sucrose synthase	0.132
R9	Neutral invertase	0.038
R10	Lower glycolysis	0.041
R11	Vacuolar sucrose accumulation	0.112

B	
Partitioning	**%**
Sucrose futile cycling	25.2
Hexoses to sucrose	84.4
Hexoses to glycolysis	15.6

C	***Concentration***
Metabolite	***mM***
Fructose	22.6
Glucose	34.6
Sucrose	12.6
Sucrose-6-phosphate	0.00367
UDP-glucose	2.02
Glucose-6-phosphate	0.277
Glucose-1-phosphate	0.0157
Fructose-6-phosphate	0.141

The kinetic model was validated by comparing of predicted steady-state metabolite levels (table 6.7.) with experimentally determined levels (table 6.8.) from Chapter 3. Assuming equal concentrations of hexoses in all cellular compartments, glucose and fructose were predicted in the same order of magnitude as experimental determinations. Measured sucrose levels were higher than those predicted, however assuming sucrose is actively accumulated to the vacuole the cytosolic sucrose concentration could be less than the total cellular concentration. It was assumed that all hexose phosphates were located in the cytosol. Glucose-6-phosphate and fructose-6-phosphate levels were similar between the model prediction and the experiment. However, predicted UDP-glucose levels were 20 times higher and glucose-1-phosphate ten times lower than the experimentally determined values.

Table 6.8. Experimentally determined metabolite levels. Data were calculated from table 3.1. (N19 internode 6), assuming 700 µl.g^{-1} moisture and a 10 % cytosolic volume of the total cell.

	nmol/g FW	*mM*	
Metabolite	***cell***	***cell***	***cytsol***
Fructose	24300	34.8	
Glucose	24400	34.9	
Sucrose	212000	303	
UDP-glucose	6.80	0.0097	0.097
Glucose-6-phosphate	14.0	0.0200	0.200
Glucose-1-phosphate	7.62	0.0109	0.109
Fructose-6-phosphate	12.4	0.0177	0.177

6.3.4.2. Steady-state properties of the combined sucrose-trehalose model

Incorporation of the trehalose branch into the current sucrose model created an additional cycle from the hexose phosphate pool to glucose via the enzymes TPS (R12), TPP (R13) and trehalase (R14) (figure 6.5.). The model was simulated using PySCeS 0.1.6 (http://pysces.sourceforge.net) (Olivier et al. 2005) leading to the calculation of steady-state properties of the sucrose and trehalose pathways (table 6.9.). Values for kinetic parameters ($V_{max\ TPS}$ and K_i for trehalase) were varied in querying the sucrose-trehalose model. $V_{max\ TPS}$ from both experimental (ex) and fitted (fit) determinations were used, showing that the higher its value the higher the resulting steady-state trehalose-6-phosphate and trehalose levels were, as well as increased flux through the trehalose branch. Including K_i on trehalase resulted in higher steady-state trehalose levels, with no change in steady-state trehalose-6-phosphate levels or flux through the pathway. Resulting steady-state conditions were compared with the current sucrose model (figure 6.6.).

Inclusion of the trehalose branch into the current sucrose model did not result in large changes in either steady-state metabolite or flux levels. Glucose steady-state levels increased at least 2%, influenced more by $V_{max\ TPS}$ than inhibition on trehalase. There was a decrease between 2 and 4% in the hexose-phosphate and sucrose-6-phosphate pools. When the model was queried with the higher experimentally determined levels of $V_{max\ TPS}$, a 1% decrease in cytosolic sucrose levels was observed, and similarly there was a 1% decrease in sucrose cycling. With regard to steady-state fluxes the greatest effect was on the SPP and SPS reactions (R6 and R7), with a 4% decrease in flux through this route of sucrose synthesis. No change in partitioning of carbon to either vacuolar sucrose or glycolysis was observed. Fluxes through the three trehalose metabolising steps (R12, R13 and R14) were always the same, causing a 100% cycling of trehalose. Increasing $V_{max\ TPS}$ ten times from its measured level to equal $V_{max\ SPS}$ caused a 16% decrease in flux through

SPS, but only a 3% decrease in cytosolic sucrose (data not shown). Model variant D (including glucose inhibition on trehalase) was regarded as the description of sucrose-trehalose metabolism closest to authenticity because all trehalose pathway parameters were optimised for internode 6 tissues, and integrated into the current sucrose model whose parameters were optimised for the adjacent internode 5.

Table 6.9. Analysis of steady-state conditions of the sucrose-trehalose model. $V_{max\ TPS}$ values were from internode 3, 1h (In3_ex), internode 3 fitted (In3_fit), internode 3, 1h (In6_ex), internode 6, fitted (In6_fit). A + B had all experimentally determined trehalose pathway kinetic parameters for internode 3, and C + D from internode 6. Inhibition on trehalose was included as indicated.

	Inhibition on trehalase															
	A: none				***B: glucose***				***C: sucrose***				***D: glucose + sucrose***			
	In3_ex	***In3_fit***	***In6_ex***	***In6_fit***	***In3_ex***	***In3_fit***	***In6_ex***	***In6_fit***	***In3_ex***	***In3_fit***	***In6_ex***	***In6_fit***	***In3_ex***	***In3_fit***	***In6_ex***	***In6_fit***
$V_{max\ TPS}$	0.0405	0.0216	0.0405	0.0191	0.0405	0.0216	0.0405	0.0191	0.0405	0.0216	0.0405	0.0191	0.0405	0.0216	0.0405	0.0191
Steady-state metabolite levels (mM)																
T6P	0.041	0.024	0.046	0.025	0.041	0.024	0.046	0.025	0.041	0.024	0.046	0.025	0.041	0.024	0.046	0.025
Trehalose	0.013	0.008	0.034	0.018	0.047	0.028	0.127	0.067	0.023	0.014	0.061	0.033	0.057	0.034	0.153	0.081
% Tre/Suc	0.10	0.06	0.27	0.15	0.38	0.22	1.01	0.53	0.18	0.11	0.49	0.26	0.46	0.27	1.23	0.65
Steady state fluxes through trehalose pathway (mM.min^{-1})																
Flux	0.0015	0.0009	0.0014	0.0008	0.0015	0.0009	0.0014	0.0008	0.0015	0.0009	0.0014	0.0008	0.0015	0.0009	0.0014	0.0008

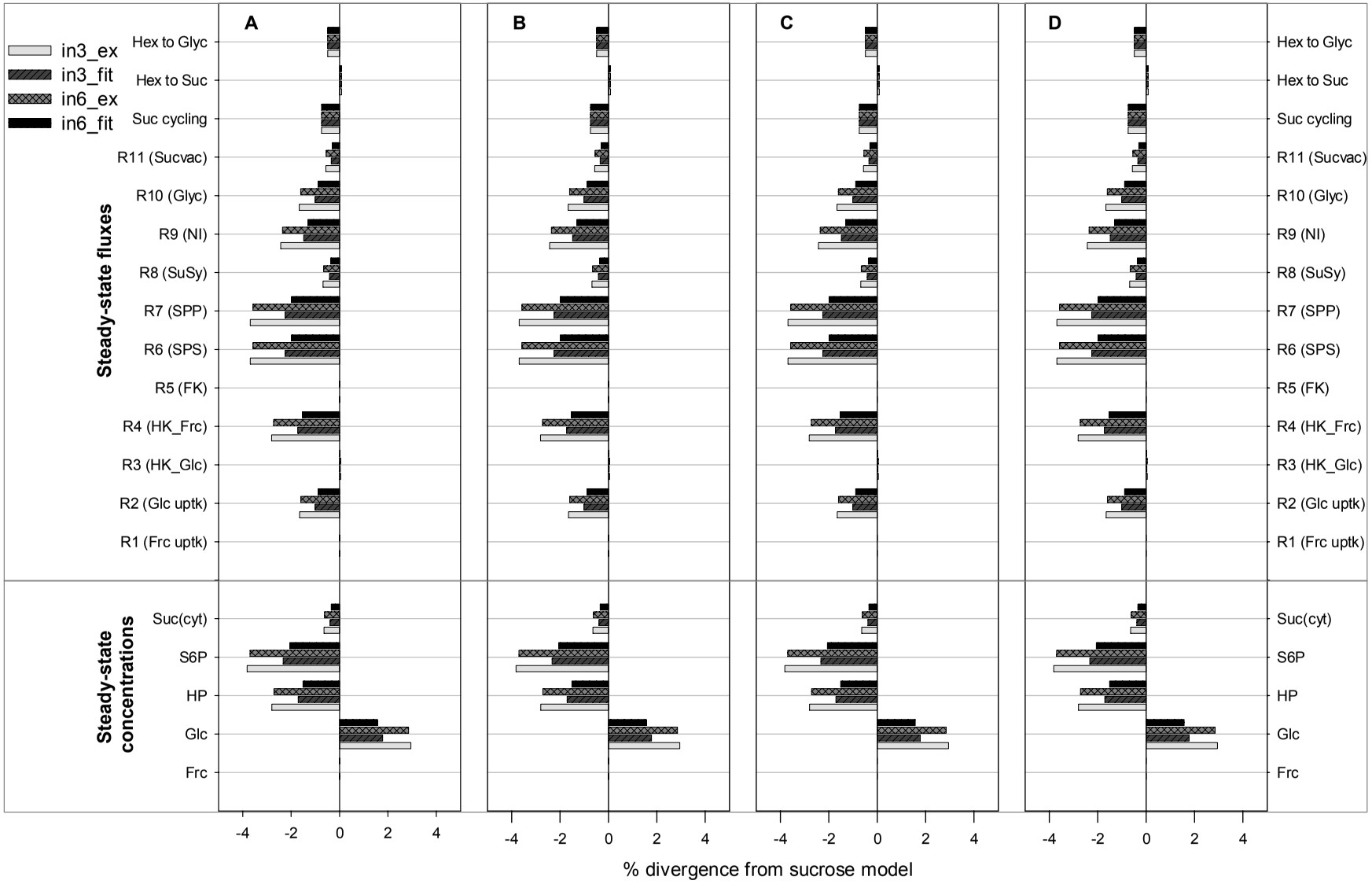


Figure 6.6. Effect of including the trehalose branch in the sucrose model. Data refer to the % divergence of the common metabolic variables (flux or concentration) between the expanded model including the trehalose branch and the original sucrose model. Variants of the expanded model included all TPS, TPP and trehalase parameters from either internodes 3 (in3) or 6 (in6). $V_{max\ TPS}$ was used at either experimentally (ex) determined levels or fitted (fit) levels. Inhibition on trehalase was (A) not included; (B) glucose; (C) sucrose; and (D) glucose and sucrose.

6.3.4.3. MCA of the sucrose-trehalose model

Metabolic control analysis of the sucrose-trehalose model was carried out to ascertain which steps in the combined model held the greatest level of control over the trehalose branch, as well as to determine whether the trehalose metabolising reactions exercised control over the accumulation of sucrose. The sucrose-trehalose model was queried with two variants, both of which had all the reaction rates and inputs of the current sucrose model, with parameters for the trehalose reactions determined for internode six. Suc-tre6 had a V_{maxTPS} = 0.0191 (fitted), and suc-tre3's V_{maxTPS} = 0.0405 (experimentally determined, 1h).

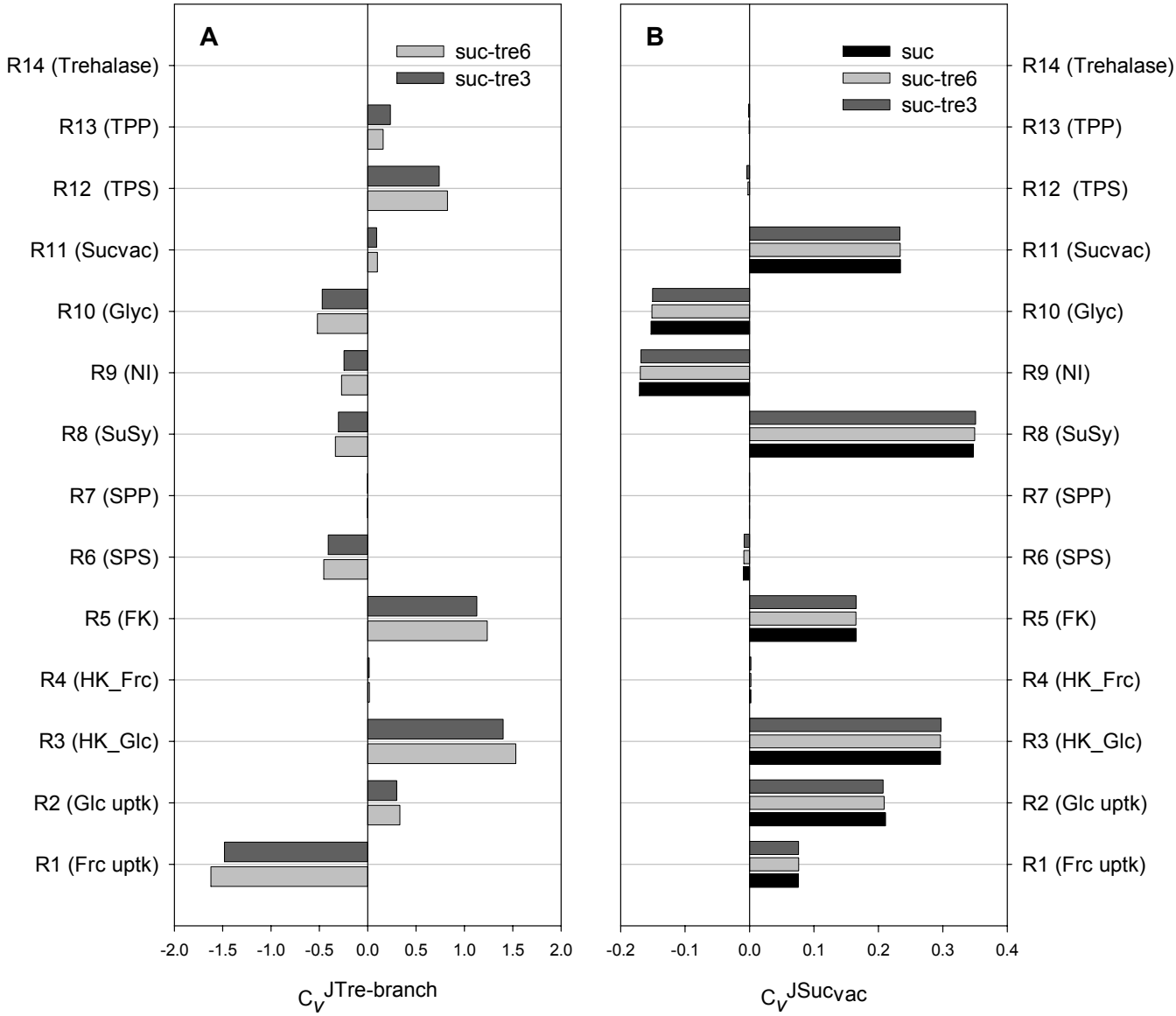


Figure 6.7. Flux-control coefficients from sucrose and sucrose-trehalose kinetic models. (A) $C_v^{JTre\text{-}branch}$, control coefficient of flux through trehalose branch (TPS, TPP and trehalase). (B) $C_v^{JSucvac}$, control coefficient of flux into vacuolar sucrose (R11). Model variants: suc = current sucrose model; suc-tre6 = sucrose-trehalose model with Vmax TPS fitted for internode 6; suc-tre3 = sucrose-trehalose model with Vmax TPS experimentally determined from internode 3 (1h).

Flux-control coefficients (C_v^J) describe the control over flux through a specific step by a change in the rate of a reaction within the model. Figure 6.7. represents such flux-control coefficients for the trehalose branch (Tre-branch, panel A) and R11 (accumulation of sucrose to the vacuole, panel B) in the combined sucrose-trehalose model. Examining $C_v^{JTre\text{-}branch}$ showed that rates of reaction through hexokinase (Glc), fructokinase, TPS, glucose uptake and TPP contributed to an increase in the flux through TPS, with the greatest control in the glucose phosphorylation step of hexokinase. Fructose uptake, glycolysis, SPS, SuSy and invertase reaction rates decreased flux through TPS, with the greatest control resting in the step of fructose uptake.

Control of the flux into the vacuolar sucrose pool (quantified by $C_v^{JSuc\ vac}$) between the three models (suc, suc-tre6 and suc-tre3) showed no variation (figure 6.7B). The greatest contributors of control to increased flux into Suc_{vac} were glucose phosphorylation by hexokinase and SuSy. Reactions causing a negative effect on flux into Suc_{vac} were invertase (NI) and the drain into glycolysis (glyc). None of the trehalose metabolising enzymes (TPS, TPP and trehalase) had any control over the flux of sucrose into the vacuole.

Concentration control coefficients ($C_v^{ss\_met}$, where ss_met represents a steady-state metabolite level) quantify the sensitivity of a steady-state metabolite level to a change in the rate of a specific reaction. C_v^{T6P}, $C_v^{Trehalose}$ and $C_v^{Suc\ cyt}$ were calculated for the three variants of the model described above (figure 6.8.). Like the control over flux through TPS ($C_v^{JTre\text{-}branch}$), the greatest control resulting in increased steady state levels of trehalose-6-phosphate (figure 6.8A) and trehalose (figure 6.8B) were from the reactions catalysed by hexokinase (glucose phosphorylating), fructokinase and TPS. Reactions that degrade the substrates trehalose-6-phosphate and trehalose, catalysed by TPP and trehalase respectively, had significant negative control coefficients. Varying the levels of $V_{max\ TPS}$ as represented by suc-tre6 and suc-tre3, did not lead to any major differences in the control of steady-state trehalose metabolism intermediates.

As with the control of flux into vacuolar sucrose, integration of the trehalose branch into the current sucrose model did not have any effects on $C_v^{Suc\ cyt}$. The major control of increasing steady-state cytosolic sucrose (Suc cyt) was observed in SuSy and glucose phosphorylating hexokinase activities. Transport of sucrose across the vacuolar membrane, glycolysis and invertase were the three rates that had significant negative control over cytosolic sucrose levels.

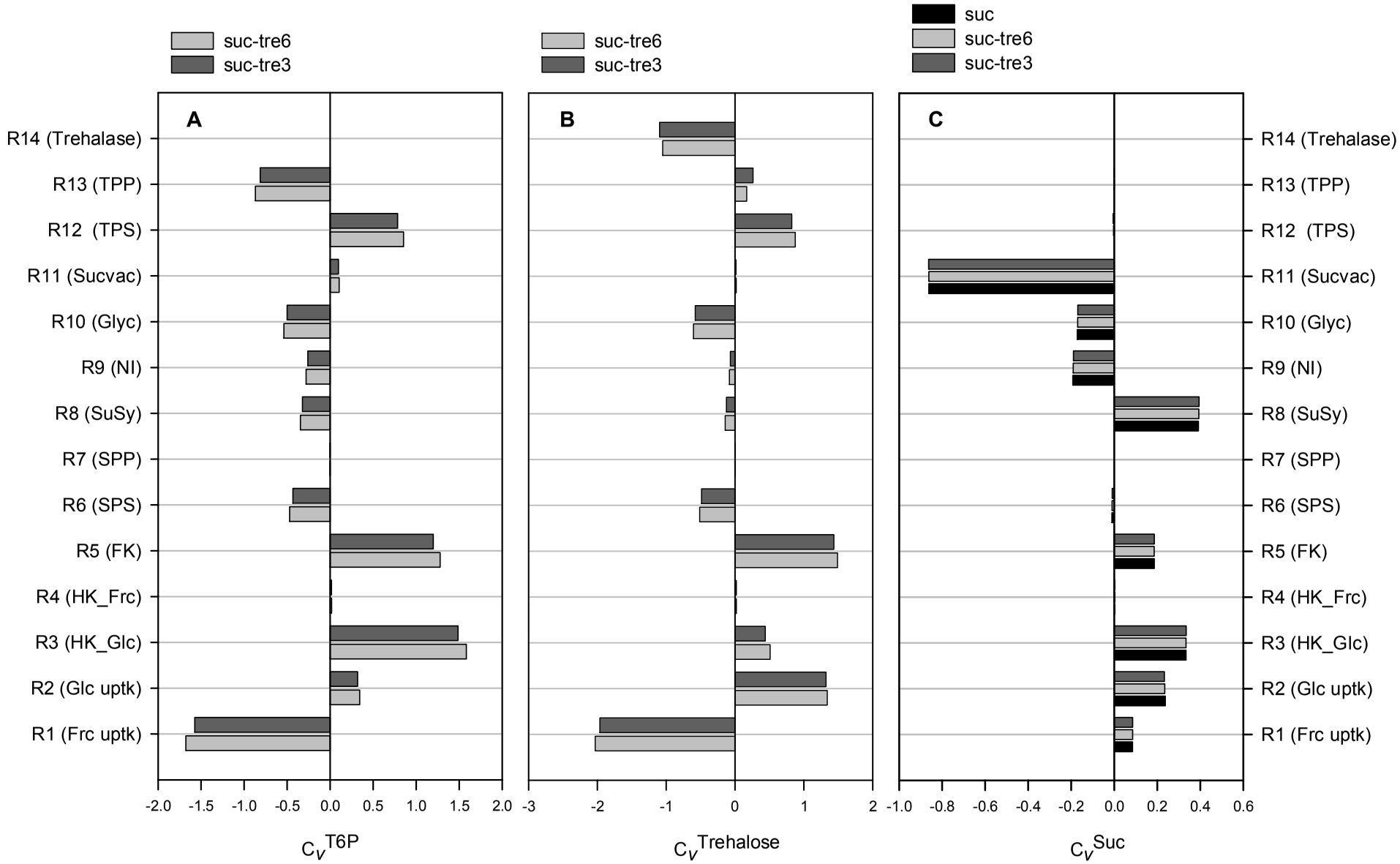


Figure 6.8. Concentration-control coefficients from sucrose and sucrose-trehalose kinetic models. (A) C_v^{T6P} , control coefficients of steady-state trehalose-6-phosphate (T6P) levels; (B) $C_v^{Trehalose}$, control coefficients of steady state trehalose levels; (C) C_v^{Suc}, control coefficients of steady-state cytosolic sucrose levels . Model variants: suc = current sucrose model; suc-tre6 = sucrose-trehalose model with Vmax TPS fitted for internode 6; suc-tre3 = sucrose-trehalose model with Vmax TPS experimentally determined from internode 3 (1h).

6.4. Discussion

Descriptions of biological systems should not only provide vast data sets and increased qualitative data, or be an end in themselves. Rather, they should serve to provide answers that cannot be visualised without the help of bioinformatic analyses, supply a mathematical framework which can be added to, and ultimately lay the foundation for future investigations. Although living organisms, and particularly plants, are metabolically complex, it should not deter biologists from quantitatively describing these systems of interest. In this Chapter we have investigated cytosolic sucrose and trehalose metabolism in sugarcane internodal tissues.

The two aims of this research were to increase the available information from which to interpret cytosolic sucrose and trehalose metabolism, as well as quantify the extent to which these two branches of carbohydrate metabolism influence each other. The following discussion will firstly describe the sub-cellular location sucrose and trehalose metabolism. Secondly, the trehalose synthesis and degradation pathway will be assessed from results of its kinetic modelling and discussed in the context of available plant trehalose metabolism literature. Finally, the interaction of sugarcane internodal sucrose and trehalose metabolism will be discussed in relation to one another.

6.4.1. Location of sucrose and trehalose synthesis

Vacuoles can make up to 90% of the total cell volume in sugarcane internodal tissues (Komor 1994), leaving the remaining 10% to house cytosol and organelles such as mitochondria and plastids. Sucrose is synthesised in the cytosol and is transported across the tonoplast into the vacuole, accumulating to levels of approximately 210 $\mu mol.g^{-1}FW$ in maturing internodes (table 3.1. N19, internode 6). Assuming at the vacuolar sucrose concentration to be greater or equal to that of the cytosol, and keeping the relative compartment size in mind, the implication is that the absolute cytosolic sucrose level is lower than that of the vacuole, yet this comparably small compartment synthesises all of the sucrose that is stored in internodal parenchyma cells. It is therefore evident that cytosolic sucrose metabolism is a dynamic process that requires strict control to provide sucrose for transport across the tonoplast and accumulation in the vacuole. Apart from being the site of sucrose synthesis, the cytosol is the location of complex primary (including the trehalose pathway) and secondary metabolism, and is the intermediate cell compartment between any two organelles. There is constant traffic at its membranes, allowing the transport of metabolites across compartmental boundaries, and despite this activity the cytosol is a highly

regulated and consistent environment. As an indication of this, determination of cytosolic pH using *in vivo* ^{31}P-NMR in sugarcane internodes (Figure 6.3. and table 6.2.), showed that regardless of the external (apoplastic) pH, the cytosol remained at approximately pH 7.1. The technique used for this purpose was not affected by any cellular component, and was accurate for determination of pHs between 5.5 and 8 (figure 6.1.). Sugarcane storage parenchyma vacuolar pH was shown to be ≤5, confirming its acidic nature. This was starting point for further investigation of the cytosollically located trehalose and sucrose pathways.

6.4.2. Analysis of trehalose metabolism

6.4.2.1. Kinetic modelling of the linear trehalose pathway

The components of the trehalose pathway have been presented in Chapters 3, 4 and 5. These have documented steady-state metabolite levels, transcript profiles and enzyme kinetics. The linear pathway was modelled using the parameters and variables for TPS, TPP and trehalase (tables 6.3. and 6.4.), employing PySCeS as a programming tool (http://pysces.sourceforge.net); (Olivier et al. 2005). Fixed substrate levels (UDP-glucose, glucose-6-phosphate and glucose) were used from two sources (table 3.1., (Rohwer & Botha 2001). Predicted steady-state trehalose levels were closest to experimentally determined values (table 3.1.) using the lower UDP-glucose and glucose-6-phosphate levels (table 6.4.). Steady-state flux through TPS, TPP and trehalase were always the same under any one condition. This was as a result of the linear nature of the trehalose branch, causing each reaction to have identical flux as they are in series and rely on supply of substrates from the others' products. The irreversibility of the TPP reaction (the rate equation was defined in the forward direction using the Michaelis-Menten relationship that defines reaction rate with only the substrate concentration included in the equation and not product concentration), caused an insensitivity of the TPS step to the trehalase step. For this reason product inhibition of trehalase by glucose could only affect steady-state trehalose levels.

MCA of the linear trehalose pathway also reflected these explanations. The majority of control of flux through the pathway rested with the rate of TPS reaction, with $C^{J}_{TPS} \geq 74\%$ of the summed flux-control coefficient of the pathway (table 6.5.). Concentration-control coefficients for trehalose-6-phosphate were numerically equal for TPS and TPP, only with opposite signs, indicating that the former increased the steady-state level, and the latter decreased it to the same extent. Control of steady-state trehalose levels was shared between TPS, TPP and trehalase. Trehalase had the largest absolute concentration control coefficient for trehalose, and is responsible for its degradation. The biosynthesis of trehalose

was insensitive to its steady-state concentration, and control of its synthesis was shared between TPS and TPP, with TPS having the greatest role. Trehalose levels would continue to increase unless the trehalase activity was high, as was the case here, where trehalase showed a relatively large negative concentration control coefficient. In light of the control analysis, the high levels of trehalase previously reported in sugarcane internodes (Glasziou & Gayler 1969; Alexander 1973; Fleischmacher et al. 1980) Chapter 4) would explain the low determined and predicted steady-state trehalose levels (tables 6.4. and 6.9.).

It is evident that TPS is the enzyme that exercises the greatest control over the biosynthesis of trehalose and flux in the pathway. In light of this, and the many challenges that faced the experimental determination of TPS activity (see section 4.3.4.1.), we mathematically fitted $V_{max\ TPS}$ (table 6.6.) to a model constructed with the experimentally determined parameters of TPP and trehalase (table 6.3.) and the steady state trehalose levels (table 3.1.). Including product inhibition on the trehalase step decreased the $V_{max\ TPS}$ because of a decreased demand for trehalose as substrate with the lowered (inhibited) trehalase reaction rate. Including glucose at 30 mM in the model led to a predicted $V_{max\ TPS}$ in the same order of magnitude as its experimental determination in internode 3 tissues. The fitting also provided an opportunity to predict a $V_{max\ TPS}$ for internode 6 tissues that we had not tested for experimentally, but was required for the integration of the trehalose pathway into the current sucrose model.

6.4.2.2. Do components of trehalose metabolism affect carbon partitioning?

Unlike yeast hexokinases that are inhibited up to 60% at 1 mM trehalose-6-phosphate (Blazquez et al. 1993), sugarcane hexokinase (assayed with both fructose and glucose) was insensitive to trehalose-6-phosphate (figure 6.4.). This lowered the possibility that trehalose-6-phosphate had a function of regulating the flux of carbon into glycolysis (and consequently carbon partitioning) at the step of hexose phosphorylation. However, as noted in *A. thaliana,* which contains six isoforms of hexokinase that cannot be distinguished from one another in biochemical assays of crude extracts (Schluepmann et al. 2003), the genetic complexity of sugarcane could lead to multiple homologues of hexokinase that are differentially expressed and have not been tested in isolation for trehalose-6-phosphate sensitivity. Therefore one cannot conclusively exclude the regulation of hexose phosphorylation by trehalose-6-phosphate, although it is evident that in total protein *in vitro* assays for sugarcane hexokinase there was no response to this sugar diphosphate.

Altered supply and steady-state levels of both trehalose and trehalose-6-phosphate in *A. thaliana* have affected growth phenotype and carbohydrate partitioning. Phenotypic response to high levels of trehalose (20 - 30 mM) included inhibition of root elongation and accumulation of starch in cotyledons and leaves of mature seedlings (Wingler et al. 2000; Bae et al. 2005). However, these phenomena were only detected when trehalose was supplied as sole carbon source, and at levels exceeding 25 mM. Investigation of such endogenous effects of trehalose on sugarcane culm carbohydrate metabolism was not within in the scope of this study. This was chiefly because experimentally determined trehalose levels in sugarcane internodes (table 3.1.) were almost three thousand times lower than the conditions that induced these responses in *A. thaliana*.

Correlation of trehalose-6-phosphate levels with over 6500 *A. thaliana* transcripts revealed relationships with transcripts involved in stress metabolism and signal transduction (Schluepmann et al. 2004). Increased *in planta* trehalose-6-phosphate levels, in seedlings grown on either sucrose or glucose, led to decreased glucose-6-phosphate and fructose-6-phosphate levels and increased photosynthetic capacity (Schluepmann et al. 2003). Decreased trehalose-6-phosphate levels resulted in exactly the opposite phenotype, with markedly increased respiratory intermediates. From these experiments it was proposed that trehalose-6-phosphate exerts control over carbon utilisation and partitioning. The site of this control is still unknown, although it was suggested to be further downstream in respiration than hexokinase.

The endogenous *A. thaliana* TPS gene (*AtTPS1*) knockout were unable to develop beyond the torpedo stage of embryo growth, making them an embryo-lethal phenotype (Eastmond et al. 2002). This same gene was shown to be essential for normal vegetative growth and transition to flowering (van Dijken et al. 2004). These phenomena could be important in sugarcane physiology too. Even though commercially grown sugarcane in subtropical climates (such a South Africa), is vegetatively propagated, breeding programs make use of artificial climatic conditions to induce flowering. If trehalose metabolism is so central to embryo development, growth metabolism and flowering in the model plant *A. thaliana,* it may play an important role in sugarcane too.

6.4.3. Integration of cytosolic trehalose and sucrose metabolism

The influence of the trehalose pathway on sugarcane cytosolic carbon partitioning was further investigated by integration of the linear kinetic model of the trehalose branch into the current sucrose model (Rohwer & Botha 2001), with particular interest in its effect on sucrose (figure 6.5.).

At this point it is important to note that the current sucrose model describes cytosolic sucrose metabolism. The model was constructed assuming that all carbon entered the cytosol as hexoses (glucose and fructose), and that these reactions' kinetics were identical. The model describes a sucrose accumulating cell where the sensitivity to carbohydrate uptake was estimated, as kinetic data was not available at the time. Radio-labelled hexose uptake studies have shown that the rate of glucose uptake is in fact higher than that of fructose uptake (Komor et al. 1996; Komor 2000). Uptake of sucrose across the plasma membrane, by either active transport or diffusion, was not accounted for, but has been experimentally shown (Whittaker & Botha 1997). The model does not account for transport of glucose or fructose into or out of the vacuole, or for sucrose re-entry into the cytosol from the vacuole. The flux of sucrose into the vacuole (R11) is also not sensitive to the levels of sucrose in the vacuole in the model. These simplifications of the model cause it to act as a potent sink, whose strength is almost largely determined by the net rate of sucrose turnover. The contribution of SuSy to sucrose synthesis is another inconsistency between the model and experimental data. Although SuSy has been shown to contribute to sucrose synthesis (Botha & Black 2000), within the high sucrose content cytosolic environment the energetics of the reaction should force it in the direction of breakdown. This is not reflected by the model (which predicted a relatively high rate of net synthesis, table6.7.), possibly because of the constant supply of fructose, as the modelled fructose uptake reaction that supplies substrate to the SuSy reaction. The sucrose-trehalose expanded model should interpreted in context of these limitations of this description of cytosolic sucrose accumulation, and the potential effect of the trehalose branch on the original model.

The current sucrose model (Rohwer & Botha 2001) was corrected in the SuSy step (Schäfer et al. 2004), and its steady-state properties determined (table 6.7). Comparison with experimentally measured metabolite levels (tables 3.1. and 6.8.) showed that predicted hexose and hexose-phosphate levels were similar to the experiment. Experimental sucrose concentration, calculated with the assumption that there was equal concentrations in all sub-cellular compartments, were 25 times higher than predicted levels. The difference can be explained by the fact that sucrose is actively transported out of the cytosol decreasing its

concentration, and this should be factored into the calculation of cytosolic sucrose concentrations. UDP-glucose levels were 20 times lower in the experimental determination. This could result from the nature of the model that does not include reactions that consume UDP-glucose, such as UDP-glucose dehydrogenase; or that UDP-glucose and glucose-1-phosphate are not in equilibrium *in vivo* as assumed in the construction of the model; or that the experimental quantification of UDP-glucose was not accurate.

Integration of the trehalose pathway into the current model as represented in figure 6.5. formed the basis of the expanded sucrose-trehalose model. Varying $V_{max\ TPS}$ values and product-inhibition states of trehalase were used to generate steady-state properties of the sucrose-trehalose model, relative to the current sucrose model (figure 6.6., table 6.9.). As with the linear trehalose pathway, inhibition on trehalase had no effect other than increasing trehalose levels. Small changes in steady-state metabolites were observed between the expanded and original sucrose models. These included an approximate 2% increase in glucose levels, probably due to the 100% cycling of trehalose; a 2-4% decrease in hexose- and sucrose-phosphates, attributed to substrate competition between SPS and TPS; a 1% decrease in sucrose cycling; and a 4% decrease in flux through SPS-SPP at the highest $V_{max\ TPS}$ levels. There was no observable change in cytosolic sucrose levels or in the partitioning from hexoses to either sucrose or glycolysis. Increasing $V_{max\ TPS}$ ten times to equal $V_{max\ SPS}$, led to a minimal 16% decrease in flux through SPS, a 3% decrease in cytosolic sucrose concentration, and a negligible decrease of flux of sucrose into the vacuole, presumably because of the 100% cycling of trehalose returning hexoses to be phosphorylated and added back into the sucrose synthesis substrate pool.

From these analyses it is apparent that within the constraints of the current expanded model, inclusion of the trehalose branch did not affect steady-state cytosolic sucrose levels, or flux of sucrose into the vacuole. MCA of the expanded model was done to determine which components of the system controlled steady-state properties of the trehalose branch and sucrose accumulation.

6.4.3.1. Control in the expanded model

MCA of the sucrose-trehalose model showed that the control over the trehalose branch was shared by its own reactions and some from the original sucrose model, and reiterated the lack of control over sucrose accumulation by the trehalose pathway reactions (figure 6.7. and 6.8.).

The majority of the control contributing to flux through the trehalose branch was shared between reactions supplying substrates for trehalose synthesis (hexokinase, fructokinase and TPS) (figure 6.7A), with fructose uptake exerting the most significant negative control. Steady-state concentrations of the trehalose branch (trehalose-6-phosphate and trehalose) were controlled in the same pattern as their flux counterparts (figure 6.8A and B). From these control coefficient analyses, it is clear that sucrose metabolism shares significant control over the trehalose pathway. This is also evident when comparing predicted steady-state trehalose levels between the linear trehalose model (table 6.4.), and those from the expanded model (table 6.9.). The linear trehalose model (including 30 mM glucose inhibition on trehalase, internode 6) predicted trehalose levels only twice as high as the experimentally determined value (table 6.4A). Fixing trehalose pathway substrate levels in the linear model at levels previously suggested (Rohwer & Botha 2001), predicted steady-state trehalose levels 19 times higher than the measured concentration (table 6.4B). However, the expanded model (whose TPS substrates are not fixed, but determined as steady-state intermediates of the system) predicted these trehalose levels approximately 9 times higher than experimentally determined (table 6.9.). From this we conclude that both TPS substrate concentration, and competition or control over the trehalose branch reactions by the reactions in the original sucrose model are contributing to steady-state properties of the trehalose branch in the expanded model. The apparent disparity of steady-state trehalose levels between the experiment and the model prediction could be as a result of erroneous experimental determination or the construction of the original model, that grouped the hexose phosphates together as one steady-state intermediate with the assumption that all the reaction were at equilibrium. Modelling each step explicitly could solve this discrepancy, but is beyond of the scope of this study.

The trehalose branch reactions and their parameters exercised no notable control over any of the reactions of the original sucrose model. This is due in part to the comparatively low $V_{max\ TPS}$ and hence low flux through the trehalose branch, the lack of trehalose accumulation to another cellular compartment, as well as the lack of known allosteric regulation by either trehalose-6-phosphate or trehalose on any of the sucrose metabolising enzymes. This is illustrated using the examples of control coefficients for the steady-state flux of sucrose into the vacuole (figure 6.7B), and cytosolic sucrose concentration (figure 6.8C). These both show that the flux of sucrose into the vacuole in the expanded model is controlled by the rate of hexose uptake (R1 and R2) and phosphorylation (R3 and R5), SuSy activity (R8) and transport of sucrose across the tonoplast (R11). The two reactions that decrease flux of sucrose to the vacuole (R9, invertase and R10, lower glycolysis) remain as previously

predicted (Rohwer & Botha 2001), and the reactions of the trehalose branch have no control at all.

Sites of allosteric control by components of the trehalose pathway may be uncovered by further experimental work, and these should be incorporated into the expanded model. However, with the current knowledge we conclude that the trehalose pathway does not significantly compete with cytosolic sucrose levels for carbon.

6.5. Conclusions

In this Chapter parameters for trehalose metabolism from Chapters 3, 4 and 5 have been incorporated into a kinetic model. The linear trehalose pathway has been considered, both in isolation and in the context of cytosolic sucrose metabolism with the use of *in silico* modelling and steady-state analyses. MCA has shown that the rate of TPS activity has the greatest flux-control over the linear trehalose pathway. Reactions of the current sucrose model that supply substrates for TPS, also exercised significant control over both steady-state flux and concentrations of trehalose metabolism. Without the identification of an allosteric control point of either trehalose-6-phosphate or trehalose on any of the reactions from the original sucrose model, and the relatively low reaction rate through the trehalose branch, caused both cytosolic sucrose concentrations and flux of sucrose into the vacuole to remain unchanged in the expanded model.

6.6. References

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Bae H, Herman E, Sicher R (2005) Exogenous trehalose promtes non-structural carbohydrate accumulation and induces chemical detoxification stress response proteins in *Arabidopsis thaliana* grown in liquid media. Plant Science 168: 1293-1301

Blazquez MA, Lagunas R, Gancedo C, Gancedo JM (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Letters 329: 51-54

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Australian Journal of Plant Physiology 27: 81-85

Buckhout TJ, Thimm O (2003) Insights into metabolism obtained from microarray analysis. Current Opinion in Plant Biology 6: 288-296

Cabib E, Leloir LF (1958) The biosynthesis of trehalose phosphate. Journal of Biological Chemistry 231: 259-275

Carson DL, Huckett BI, Botha FC (2002) Sugarcane ESTs differentially expressed in immature and maturing internodal tissue. Plant Science 162: 289-300

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Molecular Biology 52: 371-386

Eastmond PJ, Graham IA (2003) Trehalose metabolism: a regulatory role for trehalose-6-phosphate? Current Opinion in Plant Biology 6: 231-235

Eastmond PJ, Li Y, Graham IA (2003) Is trehalose-6-phosphate a regulator of sugar metabolism in plants? Journal of Experimental Botany 54: 533-537

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

Fernie AR (2003) Metabolome characterisation in plant system analysis. Functional Plant Biology 30: 111-120

Fiehn O, Kopka J, Dormann p, Altmann T, Trethewey RN , Willmitzer L (2000) Metabolite profiling for plant functional genomics. Nature Biotechnology 18: 1157-1161

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Gancedo C, Flores C-L (2004) The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Research 4: 351-359

Glasziou KT, Gayler KR (1969) Sugar Transport: occurrence of Trehalase Activity in Sugar Cane . Planta 85: 299-302

Grof CPL, Campbell JA (2001) Sugarcane sucrose metabolism: scope for molecular manipulation. Australian Journal of Agricultural Research 28: 1-12

Hawker JS, Jenner CF, Niemietz CM (1991) Sugar Metabolism and Compartmentation. Australian Journal of Plant Physiology 18: 227-237

Hoepfner SW, Botha FC (2003) Expression of fructokinase isoforms in the sugarcane culm. Plant Physiology and Biochemistry 41: 741-747

Komor E (1994) Regulation of futile cycles: the transport of carbon and nitrogen in plants. In E-D Schulze, ed Flux Control in Biological Systems. Academic Press, San Diego, CA, pp 153-201

Komor E (2000) The physiology of sucrose storage in sugarcane. In A Gupta, N Kaur, eds Carbohydrate Reserves in Plants, Ed. 26. pp 35-54

Komor E, Zingsheim O, Sprugel H (1996) Cycles of sugar transport and sucrose metabolism in sugarcane tissue: quantitative determination. In JR Wilson, DM Hogarth, JA Campbell, AL Garside, eds Sugarcane: research towards efficient and sustainable production. CSIRO Division of Tropical Crops and Pastures, Brisbane, pp 92-94

Kose K, Weckwerth W, Linke T, Fiehn O (2001) Visualizing plant metabolomic correlation networks using clique-metabolite matrices. Bioinformatics 17: 1198-1208

Krapp A, Stitt M (1995) An evaluation of direct and indirect mechanisms for the "sink-regulation" of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. Planta 195: 313-323

Lingle SE (1997) Seasonal Internode Development and Sugar Metabolism in Sugarcane. Crop Science 37: 1222-1227

Lingle SE, Smith RC (1991) Sucrose metabolism related to growth and ripening in sugarcane internodes. Crop Science 31: 172-177

Obiadalla-Ali H, Fernie AR, Kossmann J, Lloyd JR (2004) Developmental analysis of carbohydrate metabolism in tomato (*Lycopersicon esculentum* cv. Micro-Tom) fruits. Physiologia Plantarum 120: 196-204

Olivier BG, Rohwer JM, Hofmeyr J-HS (2005) Modelling cellular systems with PySCeS. Bioinformatics 21: 560-561

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmetally Modified Plant Systems. The Plant Cell 13: 11-29

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. The Plant Journal 23: 131-142

Rohwer JM, Botha FC (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochemical Journal 358: 437-445

Schäfer WE, Rohwer JM, Botha FC (2004) A kinetic study of sugarcane sucrose synthase. European Journal of Biochemistry 271: 3971-3977

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Schluepmann H, van Dijken AJH, Aghdasi M, Wobbes B, Paul MJ, Smeekens SC (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate accumulation. Plant Physiology 135: 879-890

Stewart IM, Chapman BE, Kirk K, Kuchel PW, Lovric VA , Raftos JE (1986) Intracellular pH in stored erthrocytes. Refinement and further characterisation of the ^{31}P-NMR methylphosphonate procedure. Biochimica et Biophysica Acta 885: 23-33

van Dijken AJH, Schluepmann H, Smeekens SC (2004) Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegitative Growth and Transition to Flowering. Plant Physiology 135: 9

Vandercammen A, Francois J, Hers H-G (1989) Characterization of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *Saccharomyces cerevisiae*. European Journal of Biochemistry 182: 613-620

Wendler R, Veith R, Dancer J, Stitt M, Komor E (1990) Sucrose storage in cell suspension cultures of *Saccharum* sp. (sugarcane) is regulated by a cycle of synthesis and degradation. Planta 183: 31-39

Whittaker A, Botha FC (1997) Carbon Partitioning during Sucrose Accumulation in Sugarcane Internodal Tissue. Plant Physiology 115 : 1651-1659

Wingler A, Fritzius T, Wiemkien A, Boller T, Aeschbacher RA (2000) Trehalose induces the ADP-Glucose Pyrophosphorylase Gene, *ApL3*, and Starch Synthesis in Arabidopsis. Plant Physiology 124: 105-114

Zhu YJ, Komor E, Moore PH (1997) Sucrose Accumulation in the Sugarcane Stem Is regulated by the Difference between the Activities of Soluble Acid Invertase and Sucrose Phosphate Synthase. Plant Physiology 115: 609-616

CHAPTER 7
GENERAL DISCUSSION

Trehalose and carbon partitioning in sugarcane internodal tissues

7.1. Introduction

This study has significantly contributed to a better understanding of some aspects of carbohydrate metabolism in the sugarcane culm. This is the first report on the identification and quantitation of trehalose in different sugarcane genotypes. Through a comprehensive metabolic profiling approach the prevailing trehalose levels could be placed it in the context of the metabolite profile of internodal tissues in two genotypes, which differ largely in their sucrose accumulation patterns.

Transcripts for all three enzymes of the trehalose pathway (trehalose-6-phosphate synthase (EC 2.4.1.15, TPS), trehalose-6-phosphate phosphatase (EC 3.1.3.12, TPP), and trehalase (EC 3.2.1.28)) have been isolated and their levels in internodal tissues determined. A putative full-length TPS cDNA was isolated and its functionality tested in a yeast *Δtps1* mutant. Enzyme activities of TPS, TPP and trehalase were assayed from internodal proteins in two sugarcane varieties. Both TPP and trehalase were partially purified and their basic kinetics characterised. A kinetic model of the linear trehalose pathway was constructed and validated using metabolite levels determined in this study. This model was integrated into the cytosolic sucrose model (Rohwer & Botha 2001; Schäfer et al. 2004), with the purpose of determining the effect of trehalose metabolism on sucrose accumulation. These contributions fulfil the four aims of this thesis as set out in Chapter 1.

7.2. Trehalose: Carbon source, stress protectant or the missing link in the regulation of carbon partitioning?

The diversity of biological systems is captured in part by the various roles that trehalose and its metabolism play in different organisms of divergent kingdoms. Prokaryotes and simple eukaryotes use this disaccharide as both a carbon source and stress protectant, and its derivative trehalose-6-phosphate, as a regulator of primary carbon metabolism (reviewed in (Arguelles 2000; Gancedo & Flores 2004). Besides those that photosynthesise, these organisms are heterotrophic, requiring carbohydrates from an exogenous source for survival. They are opportunistic and can move to more favourable environments in response to

external stimuli. In contrast, plants are sessile autotrophs that rely heavily on environmental conditions and resources for survival. By necessity plant metabolism has to be both adaptable and strictly controlled. In light of the central role of the trehalose pathway in yeast and bacteria, the question that demands answering is why does plant primary carbohydrate metabolism revolve around sucrose and not trehalose? I believe the answer lies in the in the understanding of metabolic control.

7.3. Complexity of plant metabolism

Plants are complex multi-cellular, multi-organellar organisms. Plant organs can act as either source or sink tissues. Source tissues provide carbohydrates to other parts of the plant, produced either *de novo* via photosynthesis (e.g. leaves with a net export of carbohydrates), or from stored reserves that can be mobilised on demand (e.g. cotyledons of germinating seeds). Sink tissues can use the carbohydrates supplied from the source for growth, respiration and storage metabolism, among others. Each organ is made up of multiple cell types that support both common and organ-specific functions. Plant cells are highly compartmentalised with organelles that sustain these diverse functions. Primary and secondary metabolic pathways can span compartments and in some cases even cell-types. Some metabolic pathways are duplicated in more than one compartment in a single cell. An example of this is glycolysis that has different isoforms of glycolytic enzymes in both the cytosol and plastid. Although the nuclear genome codes for many enzymes, the organelles also have their own genomes that code for some of the enzymes needed for their metabolism. Many enzymes are present in more than one copy in the plant genome, adding to the diversity of this intricate metabolism. Regulation of metabolism is crucial in light of its above-described complexity.

The multiplicity of plant enzymes adds to the complexity of the metabolic pathways that support the vast steady-state complements of metabolites. As with all living organisms plant enzyme activities are being controlled at a number of levels. These include transcriptional, translational and post-translational modification; as well as allosteric regulation of enzyme activity. It is not surprising that the regulation of plant metabolism is so diverse when one regards the complexity of the organism it must support. The regulation of plant metabolism requires strict control, and beyond strict control, it requires decentralised, shared control. The complexity of plant metabolism is the feature that promotes survival more than any other.

This is the point at which sucrose and trehalose metabolisms meet one another in plants. Even though sucrose metabolism is so dominant in most plants, pathways, like the one that produces and consumes trehalose, are present and seemingly indispensable. A greater potential for regulation of metabolism is opened up by distributing regulation of carbohydrate metabolism between various pathways. Sugarcane is an ideal plant to use as a case study for discussion of this radiation of metabolism. Sugarcane internodes accumulate vast quantities of sucrose, yet its metabolite profile contains many metabolites that are not directly involved in either sucrose or other routes of primary carbohydrate metabolism such as glycolysis, respiration or the oxidative pentose phosphate pathway (table 3.1.). Among these metabolites is trehalose.

In the overwhelming majority of plants, sucrose fulfils the roles of carbon source and stress protectant that trehalose plays in prokaryotes and lower eukaryotes. Experimental evidence suggests that trehalose metabolism is involved in signalling and the regulation of carbohydrate metabolism in plants, as is the case with fungi and bacteria (Schluepmann et al. 2003; Schluepmann et al. 2004; Avonce et al. 2004). However, the way in which this signalling is facilitated differs greatly between plants and other organisms. The discussion that follows critically assesses what we have learnt about sugarcane trehalose metabolism from this study, and suggests directions for further research.

7.4. Sugarcane trehalose metabolism experimental investigations

As mentioned previously, the concept of this project originated from the identification of differentially expressed transcripts from sugarcane internodal tissues that putatively coded for trehalose synthesising enzymes. The investment of energy required for transcription of such genes is great enough that one can consider the enzymes that they code for, as vital in some part of metabolism. The aim of this study was to identify and contextualise trehalose synthesis and degradation within the primary carbohydrate metabolism of the sugarcane culm. This encompassed a metabolite profile, transcript level analysis, enzyme activity study and construction of a kinetic model for metabolic control analysis (MCA).

Analysis of the sugarcane internodal metabolome of an ancestral and a commercial sugarcane variety (Chapter 3) has greatly increased available information of sugarcane metabolism. Even though the sucrose contents at the time of harvest were not significantly different there were still appreciable differences in other soluble metabolites. Higher levels of

amino and organic acids in the ancestral variety (that accumulates less sucrose early in the season) was most striking, perhaps indicating a greater allocation of carbon to respiration.

Levels of trehalose in sugarcane internodes were several orders of magnitude lower (between 80 000 and 700 000 times lower) than sucrose levels in the same tissue (table 3.1.), potentially excluding trehalose from acting as either carbon source or stress protectant in sugarcane. These nano- to micromolar amounts of trehalose occurring in internodal tissues would suggest its only possible function in its integration with carbohydrate metabolism, would be in a signalling capacity. Besides trehalose, the quantification of trehalose-6-phosphate would significantly supplement the available data from sugarcane tissues and allow for further speculation as to the role of the trehalose pathway in the *Saccharum* genus. Having tentatively ruled out the regulation of hexokinase activity by trehalose-6-phosphate (section 6.3.2.), other sites of regulation by allosteric modification of carbohydrate metabolising enzymes should be sought.

Isolation and identification of partial transcripts that coded for TPS, TPP and trehalase allowed for further investigation of their levels in different sugarcane genotypes and internodes. The most obvious result was the differential expression of all three transcripts' levels between genotypes and tissue types (figure 4.3. and 4.4.), indicating that the transcription of trehalose metabolising genes in sugarcane are specifically regulated and not just ubiquitously expressed. Fluctuations between individual plants within one variety could indicate the effect of micro-environmental stimuli, and the sensitivity of the sugarcane culm to adapt its metabolism under such conditions. In general all three transcripts were more highly expressed in young and maturing internodes (3, 6 and 8) than mature internodes (11), where sucrose contents were at their highest (table 3.1).

To date the only documented route of trehalose synthesis in plants is via TPS and TPP. Various other enzymes in yeast in and bacteria have the ability to make and degrade trehalose (figure 2.1.). It is possible that the description of trehalose synthesis and degradation in this study is not the one in operation in plants, and that these routes may play a role in the control of carbohydrate metabolism. However, the genes that code for the TPS-TPP pathway appear to be essential for plant embryo development, normal vegetative growth and transition to flowering, as shown in *A. thaliana* (Eastmond et al. 2002; van Dijken et al. 2004). Insertion of bacterial trehalose synthesising genes (TPS and TPP) into *A. thaliana* resulted altered trehalose-6-phosphate levels and marked changes in carbohydrate utilisation and partitioning (Schluepmann et al. 2003; Schluepmann et al. 2004), and

increased abiotic stress tolerance in rice (Jang et al. 2003). So clearly, this common route of trehalose synthesis is capable of affecting metabolism as a whole.

One of the most intriguing observations from plant trehalose metabolism investigations is the abundance of genes coding for TPS and TPP. In *A. thaliana* 11 TPS (Leyman et al. 2001) and 8 TPP (Schluepmann et al. 2004) genes have been proposed from the available complete genome. Equally intriguing is that only one copy of a gene coding for trehalase has been identified (Muller et al. 2001). From this one could speculate that the steady-state levels of trehalose-6-phosphate require more careful control than that of trehalose, once again implicating trehalose-6-phosphate as the potential regulator of plant carbohydrate metabolism and not trehalose. It is conceivable that the high levels of trehalase activity observed in plants (Alexander 1973; Fleischmacher et al. 1980; Muller et al. 2001; Gibon et al. 2002; Brodmann et al. 2002) (Chapter 4), are there to degrade any trehalose in the cell as fast as possible, assuming that the steady-state levels of trehalose-6-phosphate are more important as regulators. If such a state of gene radiation is present in *A. thaliana*, a simple diploid organism, what is the case in the complex polyploid genome of sugarcane? Once again we are faced with the concept of a highly regulated metabolism made possible by the allocation of control to more than one reaction in a metabolic network.

Profiling the sugarcane TPS, TPP and trehalase transcripts has allowed us to confirm their presence in divergent sugarcane genotypes, but in isolation did not allow for speculation of their role in metabolism. For us to be able to do this we needed to examine the next step in the molecular biological central dogma: the presence of their resultant enzymes and their kinetic characterisation.

At the outset of this project there were no accounts of trehalose synthesising enzyme activities from sugarcane. In fact, endogenous TPS activity has only been reported in one plant: *Selaginella lepidophylla*, a resurrection plant that preferentially accumulates trehalose. In Chapter 4 we have reported the activity of TPS from sugarcane protein extracts of young internodes (figure 4.10. and table 4.5.). Technical obstacles prevented a more detailed investigation of this enzyme in sugarcane (as discussed in section 4.3.4.1.), although with further development such experiments are recommended for future research. Section 4.3.4.2. documents the first report of sugarcane TPP activity. Kinetic analysis of this enzyme revealed at least three isoforms in sugarcane (figure 5.6.). Both TPP and trehalase activities had varying levels between internodes and genotypes, with highest TPP activity in commercial variety N19 (figure 4.11.), and highest trehalase activity in ancestral variety

US6656-15 (figure 4.12.). The fact that different tissues (both on internodal and genotypic level) do not display equivalent levels of enzymes' activities attests to the strict regulation of trehalose metabolism in sugarcane.

7.5. Integration of sugarcane trehalose and sucrose metabolism

Although we have gained significant ground in the investigation of trehalose metabolism in sugarcane, there are still many unanswered questions. With the data made available in Chapters 2, 3, 4 and 5 we integrated the linear trehalose pathway into a kinetic model describing cytosolic sucrose metabolism in the maturing sugarcane internode (figure 6.5.) (Rohwer & Botha 2001; Schäfer et al. 2004). This was done with the view to identifying the role(s) of the trehalose pathway in primary carbohydrate metabolism in sugarcane. This kinetic model was constructed with a few presuppositions of sugarcane trehalose metabolism. Firstly, all the substrates for trehalose were located in the cytosol. Secondly, all the enzymes of the trehalose pathway were also located in the cytosol. Thirdly, both trehalose and trehalose-6-phosphate were located in the cytosol and never exported to any other compartment. And finally, reactions via TPS, TPP and trehalase were the only route of trehalose synthesis and degradation. MCA of the integrated sucrose-trehalose model allowed a clear response to the most basic question in Chapter 2: is trehalose a carbon source, stress protectant or the missing link in the regulation of carbon partitioning?

As mentioned above trehalose levels in sugarcane internodes are too low to be considered a carbon source or stress protectant (table 3.1.). Modelling of the linear trehalose pathway predicted similar levels, validating the enzyme kinetic parameters used in its construction (table 6.4.). Flux through the trehalose branch was over 20 times lower than flux through sucrose synthesis via sucrose phosphate phosphatase (tables 6.7. and 6.9.). These low steady-state attributes of the trehalose pathway indicate that it does not significantly compete for carbon with sucrose synthesis (confirmed in the MCA of the integrated model (figures 6.7. and 6.8.)), and conclusively excludes the possible roles of carbon source or stress protectant for trehalose in the sugarcane culm. This leaves the only possible forecast role for the trehalose pathway in a signalling capacity that could regulate carbohydrate metabolism. Such signalling could be facilitated by either the metabolites or proteins of trehalose metabolism. Trehalose or trehalose-6-phosphate could act as gene specific inducers or allosteric enzyme regulators (preliminary investigation showed that, unlike in yeast, total sugarcane hexokinase activity is not affected by levels of trehalose-6-phosphate up to 10

mM, figure 6.4.). The enzymes of trehalose synthesis could affect other carbohydrate metabolising enzymes by protein-protein interaction.

7.6. Life alongside trehalose

One has to keep in mind that any one reaction or pathway (in this case trehalose synthesis), is not the sole determining factor in the complete phenotype of a plant. We also do not have a complete understanding of the intricacies of sugarcane sucrose metabolism, and there is still the potential for the discovery of novel sites of control, and even novel enzymes and metabolites. But within in the confines of what we do know there are many things that can be added to the current sucrose model.

Extension of the expanded sucrose model as presented in Chapter 6 would be of great benefit to increasing our understanding of cytosolic primary carbohydrate metabolism. This would require a substantial experimental effort to augment and refine the current parameters. Many of the parameters included in the current model were "borrowed" from other plants, and where those were not available, from yeast. Ascertaining these parameters from sugarcane internodal protein extracts would increase the accuracy and therefore the predictive power of the model. Including other metabolic pathways, such as starch and cell wall synthesis, oxidative pentose phosphate pathway, and explicit modelling of the steps in glycolysis would also add to accuracy and predictive power. Further improvements would include genotype, or tissue-type customisation of the model that could highlight other points of metabolic control previously overlooked.

7.7. Conclusion

This study has highlighted the intricacy of plant metabolism and its regulation. As biologists, our quest to understand and describe living systems is often hampered by a difficulty to consider a particular condition from a perspective outside of a tangible experience, or by technological limitations that plague experimentation. These should not deter us from seeking answers from small beginnings that could lead to great discoveries. The advancement of bio-techniques and -informatics in the recent past has accelerated the modern biologist beyond that which was conceived of a century ago, and provided opportunity for us to both think of and answer questions that were previously unattainable. This study is such a small beginning. The presence of trehalose and its synthesising enzymes and genes in higher plants has only been established in the last decade, and already significant progress has been made in both understanding and manipulating this part of metabolism. This foundation of information and understanding of trehalose metabolism established by studies like this one, have made room for further investigation of this fascinating component of plant physiology and molecular biology.

7.8. References

Alexander AG (1973) Studies on trehalase in *Saccharum* spp. leaf and storage tissues. Plant and Cell Physiology 14: 157-168

Arguelles J-C (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174: 217-224

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signalling. Plant Physiology 136: 3649-3659

Brodmann D, Schuller A, Ludwig-Muller J, Aeschbacher RA, Wiemkien A, Boller T, Wingler A (2002) Induction of Trehalase in *Arabidopsis* Plants Infected With the Trehalose-Producing Pathogen *Plasmodiophora brassicae*. Molecular Plant-Microbe Interactions 15: 693-700

Eastmond PJ, van Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. The Plant Journal 29: 225-235

Fleischmacher OL, Vattuone MA, Prado FE, Sampietro AR (1980) Specificity of Sugar Cane Trehalase. Phytochemistry 19: 37-41

Gancedo C, Flores C-L (2004) The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Research 4: 351-359

Gibon Y, Vigeoleas H, Tiessen A, Geigenberger P, Stitt M (2002) Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates and glycolytic intermediates based on a novel enzymic cycling system. The Plant Journal 30: 221-235

Jang I-C, Oh S-J, Seo J-S, Choi W-B, Song SI, Kim CH, Kim YS, Seo H-S, Choi YD, Nahm BH, Kim J-K (2003) Expression of Bifunctional Fusion of *Escherichia coli* Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in

Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. Plant Physiology 131: 516-524

Leyman B, Van Dijk P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends in Plant Science 6: 510-513

Muller J, Aeschbacher RA, Wingler A, Boller T, Wiemkien A (2001) Trehalose and Trehalase in Arabidopsis. Plant Physiology 125: 1086-1093

Rohwer JM, Botha FC (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochemical Journal 358: 437-445

Schäfer WE, Rohwer JM, Botha FC (2004) A kinetic study of sugarcane sucrose synthase. European Journal of Biochemistry 271: 3971-3977

Schluepmann H, Pellny TK, van Dijken AJH, Smeekens SC, Paul MJ (2003) Trehalose 6-Phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. PNAS 100: 6849-6854

Schluepmann H, van Dijken AJH, Aghdasi M, Wobbes B, Paul MJ, Smeekens SC (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate accumulation. Plant Physiology 135: 879-890

van Dijken AJH, Schluepmann H, Smeekens SC (2004) Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegitative Growth and Transition to Flowering. Plant Physiology 135: 9