EXPRESSION BEHAVIOUR OF PRIMARY CARBON METABOLISM GENES DURING SUGARCANE CULM DEVELOPMENT

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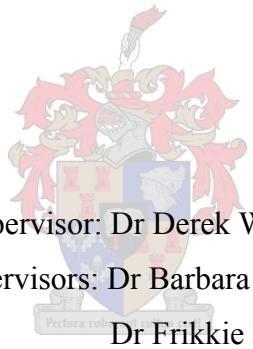
Thesis presented in fulfilment of the requirements for the degree of Master of Science at the University of Stellenbosch



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DECLARATION

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

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ABSTRACT

Despite numerous attempts involving a variety of target genes, the successful transgenic manipulation of sucrose accumulation in sugarcane remains elusive. It is becoming increasingly apparent that enhancing sucrose storage in the culm by molecular means may depend on the modification of the activity of a novel gene target. One possible approach to identify target genes playing crucial coarse regulatory roles in sucrose accumulation is to assess gene expression during the developmental transition of the culm from active growth to maturation. This study has resulted in the successful optimisation of a mRNA hybridisation technique to characterise the expression of 90 carbohydrate metabolism-related genes in three developmentally distinct regions of sugarcane culm. A further goal of this work was to extend the limited knowledge of the regulation of sucrose metabolism in sugarcane, as well as to complement existing data from physiological and biochemical studies. Three mRNA populations derived from the different culm regions were assayed and their hybridisation intensities to the immobilised gene sequences statistically evaluated. The relative mRNA transcript abundance of 74 genes from three differing regions of culm maturity was documented. Genes exhibiting high relative expression in the culm included aldolase, hexokinase, cellulase, alcohol dehydrogenase and soluble acid invertase. Several genes (15) were demonstrated to have significantly different expression levels in the culm regions assessed. These included UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase, which were down-regulated between immature and mature internodes. Conversely, sucrose phosphate synthase, sucrose synthase and neutral invertase exhibited up-regulation in maturing internodal tissue. A variety of sugar transporters were also found to be up-regulated in mature culm, indicating a possible control point of flux into mature stem sink tissues. Combined with knowledge of the levels of key metabolites and metabolic intermediates this gene expression data will contribute to identifying key control points of sucrose accumulation in sugarcane and assist in the identification of gene targets for future manipulation by transgenic approaches.

OPSOMMING

Ondanks verskeie pogings, waartydens verskeie gene geteiken is, is daar nog weinig sukses behaal om sukrose-akkumulering te verhoog. Toenemend wil dit voorkom asof suksesvolle genetiese manipulering van sukroseberging in die stingel van die verandering van ‘n nuwe geen afhanklik sal wees. Een van die moontlike benaderings wat gevolg kan word om potensiële teiken gene wat ‘n belangrike rol in die beheer van sukrose-opberging speel te identifiseer, is om geen uitdrukkingspatrone in die stingel tydens die omskakeling van aktiewe groei tot volwassenheid te karakteriseer. In hierdie studie is ‘n metode gebaseer op die hibridisering van mRNA geoptimiseer en suksesvol aangewend om die uitdrukkingspatrone van 90 verskillende geselekteerde gene, wat vir sleutelensieme in die beheer van koolhidraatmetabolisme kodeer, te bestudeer. Die doel met die ondersoek was om die beperkte kennis oor die regulering van koolhidraatmetabolisme uit te brei en om die bestaande inligting afgelei van fisiologiese en biochemiese-studies aan te vul. Drie verskillende mRNA-populasies, verkry uit verskillende dele van die stingel, is ontleed deur verskillende peilers te gebruik. Die gegewens is statisties ontleed en dit het afleidings oor die verandering in uitdrukking van hierdie gene moontlik gemaak. Die relatiewe konsentrasies van 74 verskillende gene is gedokumenteer. Gene wat sterk uitgedruk word het aldolase, heksokinase, sellulase, alkoholdehidrogenase en ongebonde suurinvertase ingesluit. Die uitdrukkingspatrone van 15 gene het tussen die verkillende weefsels gevarieer. Gene waarvan die uitdrukking tydens die oorgang na volwassenheid verlaag sluit in UDP-glukose pirofosforilase en UDP-glukose dehidrogenase en waarvan die uitdrukking verhoog sukrosefosfaatsintase, sukrosesintase en neutrale invertase in. Die uitdrukking van verskeie suikertransporter gene verhoog tydens volwassewording. Hierdie inligting te same met die huidige kennis oor heersende metabolietvlakke sal bydrae tot die identifisering van geenteikens vir toekomstige genetiese manupulering.

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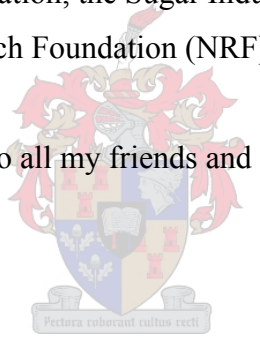

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LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromolar
^{o}C	degrees Celsius
3PGA	3-phosphoglycerate
A	adenine
A_{340}	absorbance at 340 nanometers
ADP	adenosine diphosphate
AGPase	ADP-glucose pyrophosphorylase
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAM	crassulacean acid metabolism
cDNA	complementary DNA
cm	centimeter
CV	co-efficient of variation
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dbEST	EST database
dNTP	deoxynucleotide triphosphate
dT	dioxythymidine
EDTA	ethylene diamine tetraacetic acid
EST	expressed sequence tag
EtBr	ethidium bromide
E-value	expect value
FBPase	fructose-1, 6-bisphosphatase
Fru-1,6-P_2	fructose-1, 6-bisphosphate
Fru-6-P	fructose-6-phosphate
g	gram
g	relative centrifugal force
Gluc-1-P	glucose 1-phosphate

Gluc-6-P	glucose 6-phosphate
H^+	proton
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HX/G6-PDH	hexokinase/glucose-6-phosphate dehydrogenase
HXK	hexokinase
INH	inhibitor protein
IPB	Institute of Plant Biotechnology
IPTG	isopropyl-β-D-thiogalactopyranoside
kJ	kiloJoule
LB	Luria-Bertani
M	molar
MAFF	Ministry of Agriculture, Forestry and Fisheries
malate DH	malate dehydrogenase
ml	millilitre
mM	millimolar
mmol	millimoles
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
ng	nanogram
NAD	oxidised nicotonamide-adenine dinucleotide (NAD^+)
NADH	reduced nicotonamide-adenine dinucleotide ($NAD + H^+$)
NADP	oxidised nicotonamide-adenine phosphate dinucleotide ($NADP^+$)
NADPH	reduced nicotonamide-adenine phosphate dinucleotide ($NADPH + H^+$)
NCBI	National Centre for Biotechnology Information
NI	neutral invertase
OAA	oxaloacetate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
PEP	phenol*enol*pyruvate
PFK	6-phosphofructokinase

PFP	pyrophosphate-dependent phosphofructokinase
pmol	picomoles
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
Rubisco	ribulose bisphosphate carboxylase/oxygenase
RuBP	ribulose bis-phosphate
SAGE	serial analysis of gene expression
SASEX	South African Sugar Association Experiment Station
SAI	soluble acid invertase
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SNF1	sucrose non-fermenting enzyme
SnRK1	SNF1-related kinase
SPP	sucrose-phosphate phosphatase
SPS	sucrose phosphate synthase
Suc-6-P	sucrose 6-phosphate
SuSy	sucrose synthase
TE	Tris EDTA
TPI	triose phosphate isomerase
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
Tre-6-P	trehalose-6-phosphate
triose-P	triose phosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-HCl	Tris hydrochloric acid
UDP	uridine diphosphate
UDP-Glc	uridine diphosphate-glucose
UDP-Glc DH	uridine diphosphate-glucose dehydrogenase
UGPase	uridine diphosphate-glucose pyrophosphorylase
UV	ultra-violet
V	volt
X-Gal	5’-bromo-4-chloro-3-indolyl-β-D-glactoside

CHAPTER 1

INTRODUCTION

Sugarcane (*Saccharum* L. spp. hybrids) is one of the most important sources of sucrose worldwide and accounts for more than 70% of global sucrose production (Lunn and Furbank, 1999). South Africa is ranked as the thirteenth largest sugar producer out of 121 countries, and produces an average of 2.5 million tons of sugar per season, of which approximately half is marketed nationally (Vorster, 2000). Based on 2002/2003 net estimates, the South African sugar industry generated a direct income of seven billion Rand in foreign exchange earnings (Anon., 2003). Thus, as sucrose export is a significant source of revenue for South Africa, improved yields through scientific research is an important objective of the local industry.

The introduction of new sugarcane varieties to the South African industry and improved crop husbandry began in the mid 1900's, and allowed for substantial increases in sucrose yield on a tons/hectare basis. This initial trend has been attributed to the overcoming of productivity barriers in both the source and sink tissues (Moore *et al.*, 1997). However, despite the continual introduction of new varieties sucrose yield has remained approximately constant since 1970. This phenomenon has been observed in other sugar industries and has been attributed to a number of factors, including environmental constraints and the narrow genetic base of germplasm available to breeding programmes (Watt, 2002). Consequently, much research has been focused on the development of new molecular techniques to act in concert with more conventional strategies as a possible solution to the yield plateau.

An assessment of the biophysiological capability of the sugarcane stem to accommodate a significant increase conducted by Moore *et al.* (1997) concurred with the extrapolation derived of Bull and Glasziou (1963), that the *Saccharum* complex is potentially capable of storing more than 25% sucrose on a fresh weight basis (Grof and Campbell, 2001). This estimate is almost double current commercial yields, and has thus provided a considerable incentive for several industries to utilise modern

genetic technologies as mechanisms for enhancing sucrose accumulation in sugarcane, and thus increase international competitiveness.

Sucrose is the main metabolite for long distance transport in most higher plants. The evolutionary reason for this preference could be linked to the physicochemical properties of the sucrose molecule, where even in solutions of high concentration, viscosity is still relatively low (Kühn *et al.*, 1999). An additional advantage of sucrose over reducing sugars is the chemical stability of the non-reducing disaccharide. Furthermore, sucrose creates a high osmotic potential per carbon atom in the phloem sap, which is a key parameter for translocation efficiencies within long tubes (van Bel, 1996).

As one of the main end products of CO_2 fixation, sucrose in C_3 plants is initially synthesised from triose phosphate (triose-P) primarily in the cytosol of mesophyll leaf cells. Sucrose can then either be stored temporarily in the vacuole or exported through the phloem sieve elements to the rest of the plant, thus connecting source and sink tissues such as root, stem and developing leaves (Hellman *et al.*, 2000). The reaction sequence of sucrose synthesis in C_4 plants, such as maize, sorghum and sugarcane, is essentially similar to C_3 plants, although some specialization of the process has occurred in the former (Lunn and Furbank, 1999). Unlike many plants that accumulate photoassimilated carbohydrate as starch, sugarcane stores photosynthate primarily in the form of sucrose.

Regulation and control of plant sucrose metabolism is a major field of research in agronomic and plant science. In sugarcane, sucrose accumulation is suggested to be principally regulated at the level of the sink (Whittaker and Botha, 1997), where futile cycling of carbon between sucrose and hexoses, as a result of simultaneous synthesis and degradation of sucrose, is believed to be primarily responsible for overall sucrose accumulation (Sacher *et al.*, 1963; Batta and Singh, 1986). The possible function of such energy demanding and potentially futile cycles has been discussed by Fell (1997) but not yet resolved. Despite numerous studies hence, the biochemical basis for the overall regulation of sucrose accumulation in sugarcane is still poorly comprehended and requires further study (Moore, 1995; Casu *et al.*, 2003).

Reasons for this deficiency could be due to the relative scarcity of information available concerning sucrolytic enzyme regulation in C_4 plants. Enzymological research of C_4 metabolism is currently dominated by studies on maize, yet there is a certain level of incongruity in maize being used as a model for C_4 plants. Numerous studies in maize have shown that sucrose is synthesised almost exclusively in the mesophyll cells of the source leaves (for review see Lunn and Furbank, 1999). This is not the case in many other C_4 species however, where activity of the primary sucrose synthesis enzyme, sucrose phosphate synthase (SPS) (EC 2.4.1.14), has been reported in both mesophyll and bundle sheath cells (Lunn and Furbank, 1997). Thus the assumption by Grof and Campbell (2001) that sugarcane should follow the same strict asymmetric distribution as maize has yet to be demonstrated. While the mechanisms of SPS regulation have been well characterised in several plant species (Stitt ***et al.***, 1988), SPS regulation in sugarcane is still inadequately understood.

The first paper to ascribe importance to SPS and sucrose synthase (SuSy) (EC 2.4.1.13) in managing sucrose accumulation in sugarcane (Wendler ***et al.***, 1990) was only published two decades after the early research done by Sacher and contemporaries (Sacher ***et al.***, 1963; Hatch, 1964; Hawker, 1965) who placed much emphasis on control by acid invertase (EC 3.2.1.26) alone. Using cells grown in batch culture, Wendler *et al*. (1990) demonstrated that the rates of sucrose synthesis are always in excess of storage at all stages of the cell cycle, thereby confirming the continuous turnover of sucrose. To date though, little progress has been made into further comprehending the regulation of sucrose breakdown and glycolysis in developing sugarcane internodal tissue. Whether the regulation of substrate cycling in cell suspension culture is comparable to developing sugarcane internodal tissue, as suggested by Veith and Komor (1993), is still to be confirmed.

Modern genomic studies and transgenic approaches have provided powerful tools to assess the role of particular enzymes in sucrose metabolism for a variety of plants, however sugarcane molecular physiologists are still faced with a lack of apposite data. Over the past 15 years, there has been significant success in the development of and application of transgenic tools for model dicotyledonous species, such as *Arabidopsis thaliana* (L.) and tobacco (*Nicotiana tabacum* L.), as well as important crop species such as potato, cotton and tomato (Grof and Campbell, 2001), and progress has been

facilitated by the production and analysis of large numbers of transgenic lines, in which the activities of most of the individual genes encoding carbohydrate metabolism enzymes have been modulated, alone or in combination (Fernie *et al.*, 2002). Despite these developments however, molecular tools for the transformation of sugarcane and other monocotyledons are lagging far behind. Significant advancement is being made within the Australian (Casu *et al.*, 2001), Brazilian (Burnquist, 2001) and South African (Carson and Botha, 2000; 2002) industries, where several genes associated with sucrose metabolism have thus far been identified. Nevertheless, the clarification of the role of these genes in sucrose accumulation has not yet been achieved which makes it difficult to assign them values as potential targets for transgenic manipulation (Watt, 2002).

Attempts to increase photoassimilate sink strength in the sugarcane culm have focused on the expression of single heterologous genes encoding sucrolytic enzymes, and have thus far met with mixed success. These genes include various invertases (Ma et al., 2000; Botha *et al.*, 2001) and pyrophosphate-dependent phosphofructokinase (PFP) (EC 2.7.1.90) (Groenewald and Botha, 2001). Reasons for this may be accredited to the ability of plants to physiologically compensate for small changes in their genetic environment (Halpin *et al.*, 2001). Also, a comprehensive kinetic model of sucrose metabolism in sugarcane was recently described by Rohwer and Botha (2001), which interestingly predicts a limited control on sucrose metabolism for individual genes widely regarded as having a crucial regulatory role. Thus, it is increasingly apparent that the successful manipulation of sucrose storage may depend on the modification of the activity of an alternative gene target not currently implicated as a principal regulator of sucrose metabolism. One possible approach to target genes playing crucial coarse regulatory roles in sucrose accumulation would be to assess gene expression during the developmental transition of the stalk from a non– to a sucrose accumulatory function. The way forward involves the identification of such gene assemblies via assessing gene expression in maturing sugarcane culm.

Until recently, the techniques available for gene expression analysis have been unsuitable for simultaneous investigation of large numbers of gene expression products (van Hal *et al.*, 2000). Classical northern blotting (Alwine *et al.*, 1977)

allows only a limited number of mRNAs to be studied at the same time. However, refinement and development of reverse northern hybridisation technologies, including those in array printing and hybridisation signal detection and analysis, has allowed for a substantial improvement in sensitivity and throughput of expression screening (Kurth *et al.*, 2002).

The present investigation was aimed at examining the expression of a selected group of 90 carbohydrate metabolism-related genes to extend the limited knowledge of the regulation of sucrose metabolism in sugarcane. This 'model' system was used to examine the transcriptional activity of gene expression associated with culm development in three regions of differing culm maturity. To accomplish this goal, the efficiency and reproducibility of reverse northern hybridisation technology were assessed and optimised. Array data were then subjected to statistical analysis to examine the variations in gene expression within each culm region and significant relative variations between culm regions of different maturation. As indicators of enzyme-gene expression, mRNA populations are representative of one potential level of enzymatic activity regulation and the analysis thereof account for the possible complex transcriptional events that may regulate sucrose metabolism. Combined with knowledge of the levels of key metabolites and metabolic intermediates, such gene expression analysis may indicate gene targets for future manipulation by transgenic approaches.

CHAPTER 2

LITERATURE REVIEW

2.1 Carbohydrate metabolism and accumulation in C_4 plants

The processes of photosynthesis and photosynthate transport, partitioning and accumulation are well documented for both C_3 and the specialised photosynthetic metabolism of C_4 plants (Kühn *et al.*, 1999; Lunn and Furbank, 1999). C_4 plants include a significant group of major crop species, including maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) and sugarcane, and in addition many of the world's worst weeds (Edwards and Huber, 1981). They dominate in surveys of the most productive plants and this is particularly evident under tropical and subtropical conditions (Hatch, 1992).

The characteristic high carbohydrate productivity of C_4 plants can be attributed to the co-operativity of source metabolism in the two photosynthetic cell types of the leaf that constitute Kranz-type anatomy: mesophyll and bundle sheath cells. Fixation of CO_2 initially takes place in the chloroplasts of leaf mesophyll cells via the enzyme phenol*enol*pyruvate (PEP) carboxylase (EC 4.1.1.31), where it is converted into the four-carbon organic acid oxaloacetate (OAA). OAA is then converted into malate, or aspartate, which diffuses into the bundle sheath cells. Decarboxylation of these C_4 acids results in CO_2, which is refixed via ribulose bisphosphate carboxylase/oxygenase (Rubisco) (EC 4.1.1.39) in the Calvin cycle. Three distinct decarboxylation mechanisms have evolved in C_4 bundle sheath cells: (i) a chloroplastic NADP-malic enzyme that decarboxylates malate to give pyruvate (NADP-ME-type); (ii) a mitochondrial NAD-malic enzyme (NAD-ME-type) and (iii) a cytosolic PEP carboxylase that produces PEP from OAA (PCK-type) (Hatch, 1987). The first type, which is exhibited by sugarcane, predominantly translocates the C_4 acid malate to the bundle sheath cells. The main translocation product of the other two variants is acetate, which is converted to OAA by transamination.

The three-carbon products of the Calvin cycle may return to the mesophyll in the form of triose-P (pyruvate, alanine, or PEP) that are used anaplerotically in a variety of biosynthetic pathways, e.g. starch, lipid or amino acid biosynthesis in chloroplasts, or sucrose and amino acid synthesis in the cytosol. Triose-P can alternatively be recycled for another round of carboxylation by PEP carboxylase.

The C_4 pathway acts as a biochemical 'pump' that concentrates CO_2 in the bundle sheath cells in such a way as to effectively saturate the Calvin cycle and inhibit photorespiration (Lunn and Furbank, 1999), which in C_3 plants, accounts for a substantial loss of photosynthate due to oxidation. Thus, despite the extra energy costs involved, the suppression of photorespiration in C_4 plants allows them a significantly increased potential productivity over C_3 plants, especially in environments where temperature and light intensities are high.

2.1.2 Sucrose synthesis

As the primary product of photosynthesis, sucrose plays a central role in higher plant metabolism. Sucrose synthesis in C_4 plants is fundamentally the same as in C_3 plants, and is restricted to the cytosol by strict key enzyme compartmentation (Winter, 2000). Triose-P exported from the chloroplast is converted by aldolase (EC 4.1.2.13) into fructose-1, 6-bisphosphate (Fru-1,6-P_2). Subsequent hydrolysis to fructose 6–phosphate (Fru-6-P) can be catalysed by cytosolic fructose-1, 6-bisphosphatase (FBPase) (EC 3.1.3.11) or the reversible enzyme PFP (Fig. 1). Whereas, the former enzyme is believed to be an important regulator in the pathway (Stitt *et al.*, 1987), studies on PFP down regulation in potato and tobacco suggest that it does not play an essential role in plant metabolism (Hajirezaei *et al.*, 1994; Paul *et al.*, 1995).

The importance of PFP as an additional component of metabolic regulation remains a point of contention. Certain CAM species exhibit an increased activity of PFP during gluconeogenic carbon flux (Fahrendorf *et al.*, 1987). Additionally, barley leaf Fru-1,6-P_2 is a potent allosteric activator of PFP (Nielsen, 1995), providing support for the gluconeogenic role of PFP during sucrose synthesis in young leaf tissue, where FBPase activity alone is reportedly insufficient (Nielsen, 1992). PFP is also linked to

various conditions of plant stress, such as inorganic phosphate (Pi) deficiency, anoxia, or where adenosine triphosphate (ATP) conservation is advantageous (Duff *et al.*, 1989; Mertens *et al.*, 1990; Perata and Alpi, 1993). In sugarcane tissue, PFP seems to play a crucial role at the regulatory site in plant carbohydrate metabolism (Heldt, 1997), and thus might play a key role in the process where plants adjust their growth as a function of sucrose synthesis, export, import and utilisation (Groenewald and Botha, 2001; Suzuki *et al.*, 2003).

With the conversion of Fru-6-P to glucose 6-phosphate (Gluc-6-P), glucose 1-phosphate (Gluc-1-P), and then uridine diphosphate-glucose (UDP-Glc) via UDP-glucose pyrophosphorylase (UGPase) (EC 2.7.7.9), sucrose is finally synthesised by the sequential action of SPS and sucrose-phosphate phosphatase (SPP) (EC 3.1.3.24) (Lunn and Furbank, 1999). SPS catalyses the synthesis of sucrose 6-phosphate (Suc-6-P) from UDP-Glc and Fru-6-P, and SPP irreversibly hydrolyses Suc-6-P to give sucrose and phosphate (Lunn and ap Rees, 1990). Phosphate is then returned to the chloroplast for the continued supply of triose-P to the cytosol. The activity of SPS is quite low, and is thus thought to limit the amount of sucrose that can be produced, but there is some evidence to suggest an association between SPS and SPP that might involve channelling of Suc-6-P through a multi-enzyme complex (Echeverria *et al.*, 1997). SPS is also believed to be a key regulator of sucrose accumulation, and its activity is correlated with assimilate export and plant growth, and inversely related to starch accumulation (Foyer and Galtier, 1996).

It has been found that sucrose may also be synthesised by the reverse action of SuSy, which is classically related to the sucrose degradation pathway. There is clear evidence from feeding experiments with labelled sugars that both pathways contribute to sucrose synthesis (Geigenberg and Stitt, 1991), and it is thought that the combined operation of these pathways with the degradative pathway allow the cell to respond sensitively to both variations in sucrose supply and cellular demand for carbon for biosynthetic processes (Geigenberger *et al.*, 1997). Over expression and antisense inhibition in potato plants has confirmed the crucial role of SuSy in carbon partitioning and sink strength (Zrenner *et al.*, 1995). SuSy activity has also been correlated with starch synthesis, cell wall synthesis and sucrose phloem loading and retrieval (Déjardin *et al.*, 1997; Chourey *et al.*, 1998; Hänggi and Fleming, 2001).

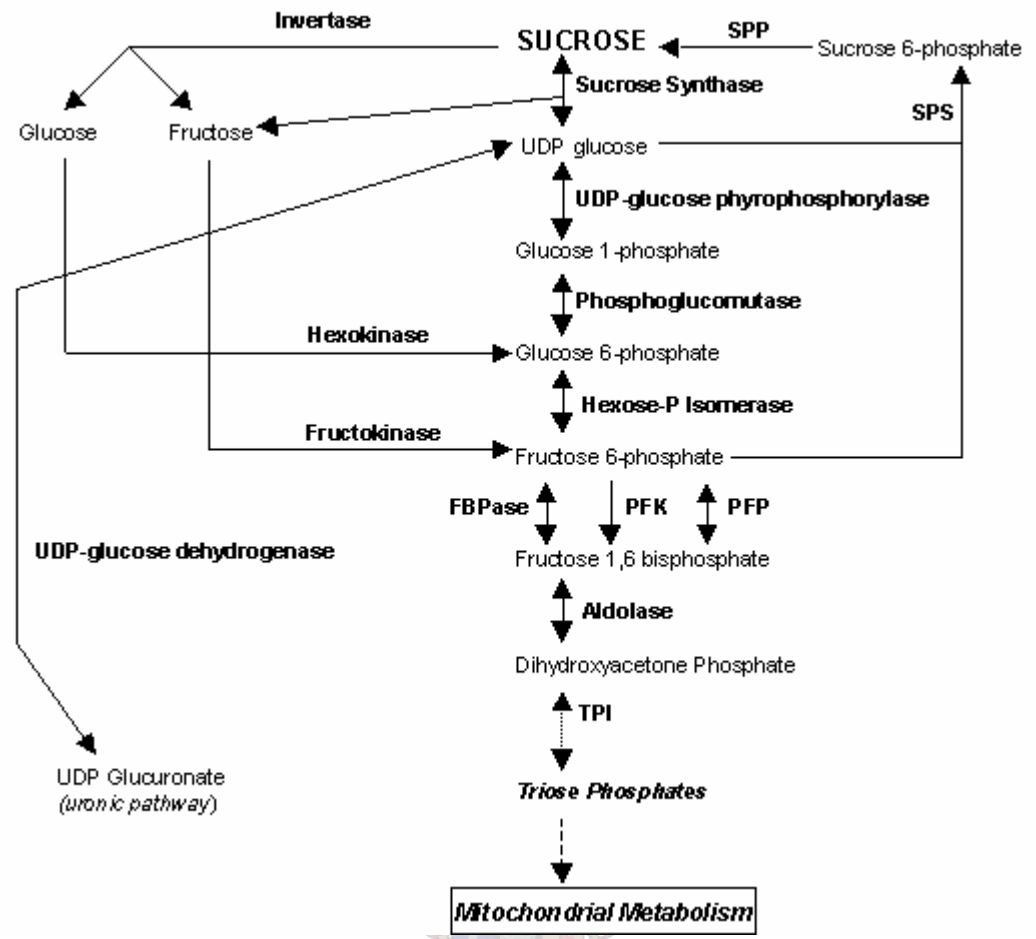


Fig. 1: *The sucrolytic sequence of reactions in the cytosol. Intermediates linked by single enzymatic (bold) reactions are represented by an unbroken line. The boxes represent compartmentalized reactions.* Modified from Fernie *et al.* (2002).

The compartmentation of sucrolytic enzymes has been investigated in a variety of C_4 plants, but by far the most studied species is maize. Maize research has concluded that the key enzymes SPS, SPP and FBPase are present almost exclusively in the mesophyll tissue of mature leaves. Such studies have shown that the incorporation of label from newly fixed $^{14}CO_2$ into sucrose occurs predominantly in these cells (Downton and Hawker, 1973, Furbank *et al.*, 1985). This may seem counterintuitive, as sucrose synthesized in the cytosol of mesophyll cells must travel through the bundle sheath cells to be loaded into the phloem. Nevertheless, to date there has been no authenticated demonstration of SPS activity in maize bundle sheath cells (Lunn and Furbank, 1999).

This strong asymmetric distribution of SPS is not found in other C_4 plants however, as activity of the enzyme has been reported in both mesophyll and bundle sheath cells for *Cyperus rotundus* (L.), *Panicum* (L.) spp. and *Digitaria pentzii* (Stent) (Chen *et al.*, 1974; Mbaku *et al.*, 1978; Ohsugi and Huber, 1987). A more recent survey on ten C_4 species has shown that the proportion of total leaf SPS activity in the mesophyll cells ranged between 65% in *S. bicolor* to 99% in *Atriplex spongiosa* (F. Meull.) (Lunn and Furbank, 1997). Thus, although C_4 plants show a tendency towards SPS localisation in mesophyll cells, maize should not be taken as a universal model for sucrose metabolism in all C_4 plants (Lunn and Furbank, 1999).

2.1.3 Starch Synthesis

Starch metabolism in higher plants is used to maintain a relatively steady supply of carbon in the form of sucrose to sink tissues throughout the day and night cycle, thus supporting growth and development (Geiger *et al.*, 2000). The two major components of starch, amylose and amylopectin, are found together as semi-crystalline granules, which may also contain small amounts of lipid and phosphate (Burrel, 2002). The exact proportions of these molecules and the size of the granule vary between species.

The majority of starch in C_4 plants is stored within the leaf bundle sheath cells (Lunn and Furbank, 1999). Labelling studies have shown that starch degradation complements or replaces sucrose synthesis from photosynthesis and maintains a supply of carbon to sinks during the night, during the day under low light, under conditions of low CO_2, water stress or a reduced capacity to transport triose-P from the chloroplast (Geiger and Batey, 1967; Fox and Geiger, 1984, 1986; Fondy *et al.*, 1989; Häusler *et al.*, 1998).

The source of carbon skeletons for starch synthesis is found in the cytosolic hexose pool, where the plastid competes for Gluc-6-P, and perhaps Gluc-1-P, originating from the sucrolytic pathway. Categorical evidence that carbon enters the plastid in the form of hexose monophosphates, rather than triose-P, was found by determining the randomisation of radiolabelled glucose units isolated from starch following

incubation of potato tuber (*Solanum tuberosum* L.) discs with glucose labelled at the C_1 and C_6 sites (Hatzfeld and Stitt, 1990). These data are in agreement with the observation that a range of higher plants which store starch also lack plastidial fructose 1,6-bisphosphates activity (Entwistle and ap Rees, 1990).

Furthermore, there is compelling evidence that Gluc-6-P is the major form in which the plastid imports carbon from the cytosol (Fernie *et al.*, 2002). The finding that a hexose monophosphate transporter cloned from potato and cauliflower (*Brassica oleracea* L.) is highly specific for glucose 6-phosphate provides strong support for the Gluc-6-P theory (Kammerer *et al.*, 1998). In addition, transgenic potato studies have shown a large reduction in starch content upon reduction of the activity of the plastidial isoform of phosphoglucomutase (EC 5.4.2.2) using antisense inhibition (Tauberger *et al.*, 2000). However as these antisense plants were not starchless, the possibility that Gluc-1-P makes some contribution to the flux of starch should not be excluded.

Following the uptake of carbon into the plastid, starch synthesis proceeds via the concerted action of plastidial phosphoglucomutase, ADP-glucose pyrophosphorylase (AGPase) (EC 2.7.7.27), and the polymerisation enzymes that are catalysed by starch synthases and branching enzymes (Fig. 2) (Smith *et al.*, 1997). Most attention to this pathway has focused on the pivotal role of AGPase, as plants exhibiting a dramatic reduction in AGPase activity also have significantly reduced starch contents (Geigenberger *et al.*, 1999). The enzyme AGPase is activated by 3-phosphoglycerate (3PGA), a triose-P originating from the sucrolytic cycle, and inhibited by Pi *in vitro*. Changes in the concentration of 3PGA are linked to changes in the rate of starch synthesis under a wide range of conditions (Geigenberger *et al.*, 1997; Geigenberger *et al.*, 1998). In addition, starch synthesis can be genetically altered via manipulation of the amyloplastidial ATP translocator, which supplies ATP for AGPase to function (Tjaden *et al.*, 1998).

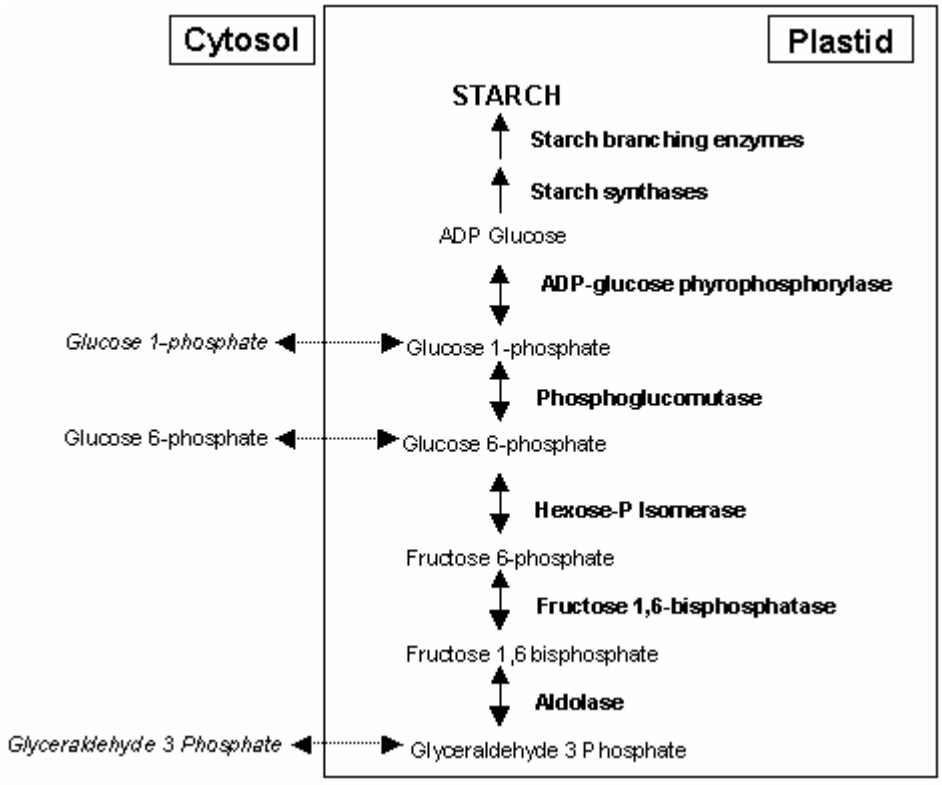


Fig. 2: *Plastid carbon metabolism. An unbroken line represents intermediates linked by a single enzymatic (bold) reaction, whereas a dotted line represents a substrate transporter. Sugar phosphates (italics) represent unlikely substrate import candidates for starch synthesis.* Modified from Fernie *et al.* (2002).

2.1.4 Phloem loading

In most plants, the main long distance transport form of carbon in the phloem is sucrose, which connects source and sink tissues, supplying the energy for growth and development. Despite the occurrence and importance of other phloem solutes such as amino acids, raffinose and stachyose sugars (Huber *et al.*, 1993), hexitols, inorganic ions and most recently found, fructans, sucrose is osmotically the dominant solute in sieve tube sap (Komor, 2000). Sucrose is thus seen not only as the main transport metabolite, but also the primary contributor to the osmotic driving force for phloem translocation (Hellman *et al.*, 2000). As the major transport metabolite sucrose is the precursor substrate to structural polysaccharide synthesis, storage polymer synthesis, respiration in recipient sinks and in several plant species, including sugarcane, the

major storage component (Avigad, 1982; Hawker, 1985; Stitt and Steup, 1985; Sung *et al.*, 1989).

Once synthesised, sucrose is either stored in the vacuole of the mesophyll cell, which represents the major diurnal storage pool (Winter, 2000), or loaded into the phloem sieve elements. Loading may either proceed symplasmically though the plasmodesmata, or apoplasmically mediated by an energy dependent active transport system (Hellman *et al.* 2000). As most plant studies have revealed that the sucrose concentration in the sieve tube sap is higher than in the mesophyll, active transport is likely to be involved at the loading site (Komor, 2000). Similarly to other active transport processes, the rate of active sucrose export should thus depend on the sucrose concentration in the leaves according to Michaelis-Menton-type kinetics (Komor, 2000).

In soybean, such a linear relationship between sucrose content in the leaf and net export rate was shown by manipulating sucrose content with varying light intensities (Fader and Koller, 1983). Interestingly, that study failed to find a rate limit for sucrose export, indicating that export capacity may indeed be so high that photosynthetic carbon assimilation could never bring it to near saturation. Alternatively, sucrose concentration may be finely tuned in the leaf to an upper limit in accordance with the export rate, so that surplus assimilate is simply diverted to starch. A similar study was done using $^{14}CO_2$ labelling of leaves, with particular emphasis on the difference between C_3 and C_4 plants (Grodzinski *et al.*, 1998). A variety of plant species were compared, and C_4 plants were clearly shown to have higher leaf solute concentrations and export rates. Increasing the ambient CO_2 concentrations also led to an increase of leaf solute level in some species, however no change in the relationship between export rate and leaf solute concentration was observed.

Symplasmic models require sucrose transport to passively overcome a concentration gradient. Plasmodesmal transport does not seem osmotically viable, yet the existence of a symplastic connection between mesophyll cells and minor veins has been demonstrated in *Ipomoea tricolor* (L.) (Madore *et al.*, 1986). Further research has revealed that plasmodesmata are not intracellular ‘holes’, but rather dynamic,

complex structures through which the transport of macromolecules is highly regulated (Lucas *et al.*, 1993). Recently, an association of cytoskeletal elements like actin or myosin-like proteins with plasmodesmal structures has been shown (White *et al.*, 1994; Radford and White, 1998) and it is hypothesised that these cytoskeletal elements could play a role in the targeting and transport of macromolecules through the plasmodesmata. Several solutions to the energy problem have been proposed (Turgeon and Beebe, 1991; Gamalei *et al.*, 1994), however the overall mechanisms of symplasmic loading are still not fully understood.

2.1.5 Phloem unloading

The deposition of exported carbon in the plant sink tissues is commonly the final fate of assimilates. However, the efficiency of carbon unloading from the phloem sieve elements depends on the sink strength of the corresponding tissue. Sink strength, depending on the plant species, is determined by the activity of enzymes involved in sucrose catabolism and/or starch synthesis (Kühn *et al.*, 1999). With regards to source-sink modelling, sink strength is often taken as the ability of sink tissues to import available assimilates from the sources (Lacionte, 1999), which is measured as net flux (g Carbon unit time^{-1}).

Although the mechanisms of phloem loading are well characterised and may be used as a means of species categorisation, albeit controversial (Kühn *et al.*, 1999), phloem unloading is less well documented *per se*. It is assumed that the path of unloading not only varies between species, but also in a tissue-dependent or even developmentally regulated manner (Turgeon, 1989). The movement of sucrose from the phloem to the vacuole of the storage parenchyma cells may proceed symplasmically or apoplasmically, however, it is feasible that unloading may incorporate a combination of the two routes (Moore, 1995), perhaps with several mechanisms co-existing within each sink organ (Turgeon, 1996).

In sink organs the assimilate concentration is higher in the phloem than in the surrounding tissue, thus unloading sucrose via symplastic diffusion seems the most plausible mechanism. Indeed, symplastic unloading has been postulated for a variety

of species as the dominant pathway for a range of sink tissues, including immature leaves, tubers and roots (Kühn *et al.*, 1999).

Nevertheless, radial transfer of sucrose through the apoplast has been reported (Patrick and Turvey, 1981) and expression analysis has also indicated that during assimilate unloading in potato tubers, sucrose is at least partially released into the apoplasmic space (Heineke *et al.*, 1992). Additionally, apoplasmic unloading must also occur in nearly all seeds, as the embryonic tissue of most species is symplasmically isolated from the maternal tissue (Thorne, 1985; Hawker *et al.*, 1991). Thus, apoplasmic unloading cannot be excluded as a viable model mechanism.

Investigations with fluorescent dyes in potato tubers have shown that metabolites can be loaded into storage parenchyma cells from the apoplasm by endocytosis (Oparka and Prior, 1988), although a more recent study in sugarcane has revealed that tracer dyes the size of sucrose remain confined to the apoplasmic space (Jacobsen, *et al.*, 1992). Sugarcane is however hypothesised to unload sucrose into the apoplast where it is cleaved by invertase to produce hexoses, which are then taken up by monosaccharide carrier sites in the plasmalemma (Hatch and Glasziou, 1963; Sacher *et al.*, 1963). Evidence for this exists from sugarcane cell suspension studies, which expressed high levels of acid invertase and took up reducing sugars but not sucrose (Komor *et al.*, 1981). Monosaccharide transporters specifically expressed in sink tissues have also been found in tobacco and *A. thaliana*, providing additional support for this hypothesis (Sauer *et al.*, 1990*a*; Sauer and Stolz, 1994).

Interestingly, monosaccharide transporters have been detected in the plasma membrane vesicles of sugar beet (*Beta vulgaris* L.) leaves (Tubbe and Buckhout, 1992). The function of these transporters may be to prevent the accumulation of hexose in the apoplast. Furthermore, under certain conditions, source tissues can switch to sink function. Typical examples are responses to wounding or pathogen infection where the reallocation of nutrients is required to sustain defence and repair responses (Paul and Foyer, 2001). Such a drastic shift in the source-sink relationship indicates the existence of complex co-ordinated regulation. Some of the regulatory mechanisms governing plant metabolism are expanded upon in the following section.

2.2 The metabolic regulation of carbohydrate accumulation and storage

The biochemical pathways of photosynthesis and carbon storage have been established for more than twenty years, while the enzymatic properties of the majority of individual enzymes involved are well understood. However, the regulation of gene expression, enzyme activity and how these regulatory mechanisms interact to determine photosynthetic leaf capacity or the fate of fixed carbon are only just beginning to be appreciated.

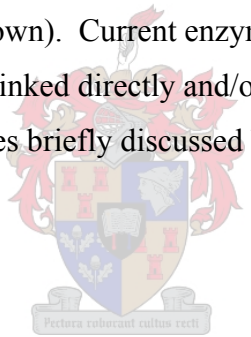
Control of any particular metabolic pathway is shared by the enzymes within it, which constitutes the basis of control theory (Kacser and Burns, 1973). Although the activity of these enzymes may be partly regulated at the translation or post-translation level ('fine control'), bulk regulation ('coarse control') seems to take place at the level of transcript accumulation (Pego *et al.*, 1999). In most cases patterns of protein accumulation reflect those of mRNA (Monroy and Schwartzbach, 1983; Nelson and Langdale, 1989; Kuhlemeier, 1992; Van Oosten and Besford, 1995), thus the measurement of mRNA expression can provide a good indication of tissue specific enzyme activity.

The most obvious candidates for the regulation of mRNA production, especially in photosynthetic studies, would be enzymes sensitive to changes in the levels of irradiance. Indeed, light-regulation of gene expression for photosynthetic proteins is a well documented process and several *A. thaliana* mutant screens have partially resolved this particular mode of regulation (Staub and Deng, 1996; Wei and Deng, 1996). Light, however, cannot account for the regulation of sucrose synthesis that must take place as the plant encounters variations in the environmental and developmental factors and processes throughout its life cycle (Pego *et al.*, 1999). Recent research has expanded the focus on metabolic regulation to studies that range from specific control at the enzymatic and genetic level (Winter, 2000; Hänggi and Flemming, 2001) to modelling of the feedback mechanisms between source and sink (Paul and Foyer, 2001). However, the mechanisms through which sink tissue regulates photosynthetic carbon fixation at the source have proved not to be simple linear pathways, but rather multifaceted networks with many points of reciprocal control that are yet to be fully understood.

2.2.1 The regulation of key steps in sucrose metabolism

Research conducted over the past two decades has contributed to an improved understanding of the modulation involved in sucrose-related enzyme activity (Stitt *et al*., 1987; Quick, 1996) and has included the isolation, cloning and transgenic manipulation of genes encoding a variety of sucrose-related enzymes, and in some cases, the elucidation of posttranslational enzyme regulatory mechanisms (Winter, 2000). Such research has not only provided clearer insight into the control of key enzyme activity, but has also provided a superior framework for the creation of hypothetical source-sink regulation models. Such models could potentially serve as powerful predictive mechanisms for the genetic manipulation of plant physiology and thus provide a dynamic tool for the improvement of crop productivity.

Sucrose accumulation in plants can be determined by the ratio of sucrose catabolism (synthesis) to anabolism (breakdown). Current enzymatic work has thus focused on a number of key enzymes that are linked directly and/or indirectly to the regulation of sucrose metabolism. The enzymes briefly discussed below represent the most documented members.



Sucrose phosphate synthase

Sucrose synthesis via SPS is relatively well defined (Geigenberger *et al.*, 1997). Although the reaction catalysed by SPS is not irreversible, the efficient conversion of its product Suc-6-P to sucrose by SPP means that SPS is the first committed reaction in this sucrose synthesis pathway (Fernie *et al.*, 2002). Rapid removal of Suc-6-P by a specific and high activity phosphatase displaces the reversible SPS reaction from equilibrium *in vivo* (Stitt *et al.*, 1987), and thus it is thought that SPS activity contributes to the control of flux into sucrose. The expression of SPS is regulated by developmental, environmental and nutritional signals and at least in some cases appears to be at the translational level (Winter, 2000). This was shown by Klein *et al.* (1993) where the transfer of spinach (*Spinacia oleracea* L.) plants from low- to high-light environments resulted in increased steady-state SPS mRNA levels, followed by a slower increase in SPS protein and activity.

Superimposed on the regulation of SPS at the gene level are several mechanisms that can rapidly regulate the catalytic activity, including allosteric control and protein phosphorylation. In spinach leaves, the activity of SPS is regulated by the allosteric effectors Gluc-6-P (activator) and inorganic phosphate (inhibitor) that allow sucrose synthesis to proceed when substrate is plentiful (Doehlert and Huber, 1983*a*; Doehlert and Huber, 1983*b*; Doehlert and Huber, 1985). This implies that sucrose synthesis through SPS is only promoted when metabolites are abundant.

The regulation of SPS activity by reversible protein phosphorylation has been shown to alter the sensitivity of SPS to metabolic effectors. The enzyme is phosphorylated on multiple seryl residues *in vivo* (Salvucci *et al*., 1995) and currently three serine residues have been implicated to have regulatory significance under a variety of environmental conditions, such as diurnal modulation and osmotic stress (Huber and Huber, 1996; Toroser and Huber, 1997). The mechanisms involved, however, are not fully understood.

Efforts to manipulate SPS expression using transgenesis have confirmed its importance in sucrose biosynthesis. Overexpression of maize SPS in tomato (*Lycopersicon esculentum* L.) has resulted in increased sucrose synthesis, increased sucrose/starch ratios in leaves, and increased photosynthetic capacity, indicating that SPS is a major point of photosynthetic control, especially under high CO_2 and saturating light (Galtier *et al.*, 1993; Galtier *et al.*, 1995; Micallef *et al.*, 1995). In some cases however, SPS overexpression did not result in increased SPS activity, and was ascribed to the likelihood of post-translational regulation of the enzyme. In contrast, antisense repression of SPS in potato leaves resulted in inhibition of sucrose synthesis and increased flow of carbon into starch and amino acids (Krause, 1994). Transgenic plants in that study produced a flux control coefficient of SPS in sucrose synthesis of 0.3 to 0.45, indicating that SPS is only one of the enzymes contributing to the overall control of sucrose production.

There is an increasing amount of data that suggest SPS regulation may also occur through discrete protein complex associations. There is support for an association between SPS and PK_{III} protein kinase (EC 2.7.1.37) (Huber and Huber, 1991), whereas both kinetic and physical evidence exists for an association between SPS and

SPP. Physical evidence for an interaction includes co-migration of SPS and SPP during native gel electrophoresis and results of isotope dilution experiments that suggested channelling of Suc-6-P from SPS to SPP (Echeverria *et al.*, 1997). Kinetic evidence includes stimulation of SPS activity and reduced phosphate inhibition in the presence of SPP (Salerno *et al.*, 1996; Echeverria *et al.*, 1997). Another plausible binding partner for SPS would be UGPase, as it forms the UDP-Glc substrate for SPS, and exhibits a putative SPS binding site (Toroser *et al.*, 1998). However, further study is required to establish the physiological significance of SPS complexes and to identify other associations (Winter, 2000).

The complexity of SPS regulation is further confounded by the presence of several SPS isoforms present in individual species. Multiple copies of SPS genes have been identified in a variety of plant genomes, including *Citrus* (L.), *Craterostigma plantagineum* (Hochst.), *A. thaliana* and *Actinidia chinesis* (Planch.) (Komatsu *et al.*, 1996; Ingram *et al.*, 1997; Langankämper *et al.*, 2002; Fung *et al.*, 2003). Plant SPS genes with a 30–40% nucleotide divergence have been shown to be transcribed differentially in specific organs and tissues, while the possibility of different functional SPS proteins has also been suggested (Reimholz *et al.*, 1997; Pagnussat *et al.*, 2000). The functional significance of these gene and protein isoforms is not well understood. Some of these genes may have evolved to confer specificity to cell types, or relate to other aspects of sugar metabolism found within the cell. Pagnussat *et al.* (2000) has proposed an SPS isoform in monocotyledon leaves that is specific to non-photosynthetic tissue, where allosteric regulation differs to that found in photosynthetic leaves. The interpretation of future studies should thus take account of the different gene products that may be present (Fung *et al.*, 2003).

Sucrose synthase

The degradation of sucrose may be catalysed by at least two separate classes of enzymes. Whereas the invertases catalyse the irreversible hydrolysis of sucrose to glucose and fructose, cleavage by SuSy to fructose and UDP-Glc may be readily reversed (Geigenberger and Stitt, 1991). The latter reaction also conserves the binding energy of the glycosidic bond in UDP-Glc. A number of important

physiological processes have been associated with SuSy, including source/sink relationships in the plant, response to anoxia, and cell wall biosynthesis (Amor *et al.*, 1995; Zrenner *et al.*, 1995; Ricard *et al.*, 1998). In most plant storage organs, there is a strong correlation between SuSy activity, the rate of growth and amount of starch accumulated (Yelle *et al.*, 1988; Nguyen-Quoc and Foyer, 2001). Nevertheless, the precise regulatory function of the reversible SuSy reaction remains debatable.

Research over the last ten years has yielded many insights into the multifaceted regulation and function of SuSy in carbohydrate metabolism. In most monocotyledons, SuSy is encoded by two differentially expressed nonallelic loci *sus1* and *sus2*, however rice (*Oryza sativa* L.) plants have recently revealed a third SuSy gene, *sus3* (Huang *et al.*, 1996). Dicotyledonous species also contain two nonallelic SuSy genes, which appear to be functional analogs of the two classes of SuSy genes from monocotyledons (Fu and Park, 1995). These isoenzymes exhibit different spatial and temporal expression, are differentially regulated at the transcriptional and translational levels and may perform different metabolic functions (Chen and Chourey, 1989; Fu *et al.*, 1995; Chourey *et al.*, 1998; Déjardin *et al.*, 1999). Expression may also vary according to tissue type and the carbohydrate metabolic state within (Winter, 2000). The maize SuSy isoform *Sh1*, for example, is maximally expressed under carbohydrate limiting conditions (0.2% compared to 2% glucose), whereas the expression *sus1* is induced by increasing glucose concentrations (Hellman *et al.*, 2000).

On the protein level, SuSy is strictly a cytosolic enzyme, which can occur as a membrane associated and as a soluble form, where the latter may interact with the actin cytoskeleton (Winter *et al.*, 1998). The enzyme has also been shown to be subject to posttranslational modification by reversible phosphorylation (Huber *et al.*, 1996), although the physiological significance of this is still unclear. In sugar beet roots, the two isoforms of sucrose synthase SuSy1 and SuSyII not only differ in subunit size and composition, but also in their differing responses to changing pH values (Klotz *et al.*, 2003). With regards to regulation, structural differences indicate the potential for differential expression of the two isoenzymes, however differences in activity to changing pH values suggest another potential method for control of SuSy activity without the need for alterations in gene expression. Such a mechanism would

allow for the rapid alteration of sucrose metabolism in response to environmental stress or metabolic changes since these are often accompanied by changes in cytoplasmic pH (Kurkdjian and Guern, 1989).

One of the most consistent observations has been a specific localisation of SuSy expression to mature phloem cells in leaf tissues, which has lead to the assumption that the enzyme may play an important role in sucrose loading/unloading in the phloem (Nolte and Koch, 1993). However, a recent report by Hänggi and Fleming (2001) has concluded that there is a specific exclusion of SuSy transcript and protein accumulation from the sink phloem tissue of young maize leaves. Thus, although SuSy may play a role in mature phloem transport, its actual physiological function in young leaves remains to be clarified (Hänggi and Fleming, 2001).

Invertases

The invertases are a group of β-fructosidases that differ in pH optimum for activity (neutral, acidic and alkaline) and solubility. The three isoforms identified thus far differ in localization and function (Quick, 1996).

Soluble acid invertase (SAI) is vacuolar and cleaves sucrose when there is high demand for sucrose hydrolysis, such as cell expansion (Richardo and ap Rees, 1970). Little is known about the regulation of SAI activity, except that many are inhibited by hexose sugars, especially fructose (Walker *et al.*, 1997). The role of SAI inhibition by fructose as a regulatory mechanism *in vivo*, however, is not known.

The suppression of SAI activity by antisense RNA has exhibited increased sucrose contents in tomato fruit and leaves, and has also been found to reduce hexose sugar concentrations in cold-stored potato tubers (Ohyama *et al.*, 1995; Scholes *et al.*, 1996; Zrenner *et al.*, 1995). This confirms the importance of SAI in the regulation of tissues in both source and sink. Northern blot analysis of various forms of SAI has also shown different developmental and tissue specific expression patterns – isoform *Ivr1* is upregulated by sugar depletion, whereas *Ivr2* exhibits increased expression levels when sugar supply is abundant (Koch, 1996; Xu *et al.*, 1996). To ascertain the full

physiological significance of such regulation however, a better understanding of the enzymatic properties of these two invertases will be required.

Interestingly, different plant species display a considerable variation of soluble acid invertase activity, particularly in fully expanded leaves, and only species with a low acid invertase activity accumulate sucrose as an end product of leaf photosynthesis (Huber, 1989). This not only confirms previous findings of a considerable variation of SAI activity between species (Huber, 1989), but also implies the existence of more than two isoforms of the enzyme.

The needs for cytosolic sucrose hydrolysis are met by soluble neutral/alkaline invertase (NI). These invertases are considered "maintenance" enzymes that are involved in sucrose degradation when the activity of SAI and SuSy are low (Winter, 2000) and have also been linked to growth metabolism (Rose and Botha, 2000). Historically, NI has been afforded much less research attention than vacuolar and cell wall invertase (Hawker, 1985). The enzyme has however, been purified and characterized for a variety species including sugarcane (Rose and Botha, 2000), while a gene encoding an enzyme with neutral/alkaline invertase activity has been cloned in *Lolium temulentum* (Lam. Kuntze.) (Gallagher and Pollock, 1998). Further cloning from other species is required, however, to elicit whether the neutral/alkaline invertases cover more than one family of enzymes.

In contrast to the other invertases, insoluble cell wall invertase is extracellular and plays a key role in phloem unloading and assimilate uptake, specifically in sink tissues (Roitsch *et al.*, 2002). Extracellular invertase is ionically bound to the cell wall, where it cleaves sucrose into its two hexose monomers, and thus ensures a steep concentration gradient of sucrose from source to sink (Escherich, 1980). It has also been suggested that the enzyme may play a pivotal role in establishing metabolic sinks (Roitsch *et al.*, 1995). A variety of genes coding for isoenzymes of the extracellular invertase family have been isolated (Roitsch *et al.*, 2000) which, even more so than SuSy, exhibit highly tissue-specific mRNA expression patterns. In tomato plants for example, Godt and Roitsch (1997) found the expression of clone *Lin7* was observed solely in anther tapetum cells and pollen grains. Anther-specific isoenzymes have recently also been reported in a number of other species including

maize, tobacco and potato (Xu *et al.*, 1996; Maddison *et al.*, l 999; Goetz *et al.*, 2001), indicating a crucial function for extracellular invertases in providing carbohydrates to the male gametophyte. Such specificity may hold promise for a route to engineering male sterility in transgenic plants as well as providing other practical genetic applications.

Sugar levels have been shown to play an important regulatory role in extracellular invertase expression and enzyme activity. Using photoautotrophic suspension cultures of *C. rubrum*, Roitsch *et al.* (1995) observed higher enzyme activity and increased levels of extracellular invertase mRNA in the presence of sucrose and glucose. Isoforms of extracellular invertase have since been upregulated by glucose in tobacco and *A. thaliana* (Krausgrill *et al.*, 1996; Tymowska-Lalanne and Kreis, 1998), and by sucrose in tomato (Godt and Roitsch, 1997), thus showing a clear specificity towards sugar modulation. Recent research using tomato suspension cultures has even observed up-regulation of an extracellular invertase isoform using non-metabolizable sucrose analogues such as palatinose, turanose and flourosucrose (Sinha *et al.*, 2002). Both turanose and palatinose are however, synthesized by plant pathogens, indicating that the invertase response may be linked to stress related stimuli, rather than a unique sugar-sensing mechanism. Nevertheless, the fact that both metabolizable sugars and stress related carbohydrate stimuli regulate extracellular invertase, makes this gene an important candidate to be used as a marker gene for the analysis of converging signalling pathways (Roitsch *et al.*, 2002).

Another recent regulatory development regarding extracellular invertase is the identification of an apoplasmic inhibitor protein (INH) that is present at certain stages of plant development. The protein has been isolated and purified to homogeneity from tobacco leaves, while the genes encoding the INH have been cloned from *A. thaliana* and tobacco (Greiner *et al.*, 1998). There have been differences among organs in expression of INH relative to extracellular invertase, but these results are difficult to interpret, as transcript levels do not necessarily reflect protein amount (Greiner *et al.*, 1998). The INH has also been found to inhibit vacuolar acid invertase activity *in vitro*, however the protein is located in the apoplasm *in vivo*, and regulation of INH action by sucrose is only found with extracellular invertase (Sander *et al.*, 1996). The physiological significance of INH is still speculative, however, one

possibility is that INH may function to modulate extracellular invertase activity under adverse conditions, such as maintenance of a minimal, critical sucrose concentration during sugar starvation (Winter, 2000).

Pyrophosphate-dependent phosphofructokinase

The reversible action of PFP catalyses the conversion of Fru-6-P and pyrophosphate to Fru-1,6-P_2 and inorganic phosphate. Unlike its counterparts FBPase and 6-phosphofructokinase (PFK) (EC 2.7.1.11), which expend ATP, PFP utilises pyrophosphate as an alternative energy source (Dancer *et al.*, 1990). The exact physiological role of the PFP enzyme in plants still needs to be determined, but it has been implicated in a number of regulatory processes, including glycolytic carbon flow, sink strength and stress response (Edwards and ap Rees, 1986; Murley *et al.*, 1998; Whittaker and Botha, 1999).

Both PFK and FBPase, are present in the cytosol and plastids, however PFP is exclusively cytosolic suggesting that it may be important in regulating carbon flow in this compartment. In addition, extractable activities of PFP are generally either comparable to or well in excess of PFK or FBPase (Carnal and Black, 1983; Botha and Small, 1987). PFP is allosterically activated by nanomolar concentrations of fructose-2, 6-bisphosphate (Stitt, 1990), which is atypical of equilibrium catalysed reactions that are generally not believed to be subject to stringent regulatory control (Stitt *et al.*, 1987). This may be significant in understanding the role of PFP in carbon metabolism, as the enzyme thus responds to cellular metabolism in a very flexible but 'regulated' manner (Whittaker, 1997).

The genes coding for PFP have been isolated from a number of species and have been investigated using transgenic technologies. Contrary to evidence from antisense research in potato and tobacco that PFP does not play an important role in overall plant metabolism (Hajirezaei *et al.*, 1994; Paul *et al.*, 1995), in sugarcane PFP levels seem to be closely related to sucrose accumulation (Whittaker and Botha, 1999). The activity of PFP activity has been shown to decrease with sugarcane culm maturity, and is inversely correlated with sucrose content in a number of different commercial

varieties. Down-regulation of the PFP in preliminary transgenic trials resulted in significantly increased levels of hexose concentrations in younger internodes, whereas sucrose levels were only significantly different in some older internodes (Groenewald and Botha, 2001). These results suggest that down-regulation of PFP activity does indeed influence sucrose metabolism, however further research is required to determine the underlying mechanisms.

Sucrose transporters

The allocation of assimilates in higher plants is mainly controlled by two transporter families. The first family of proteins are involved in the long distance transport of sucrose, and function as sucrose carriers from source to sink. Antisense inhibition of the gene coding for sucrose transporter *sut1* has been achieved in both tobacco and potato studies, resulting in comparable phenotypes (Riesmeier *et al.*, 1993; Bürkle *et al.*, 1998). A concentration increase of 5-10 fold in soluble sugars and starch was observed in transgenic source leaves, as expected for a block in the transport of sucrose into the phloem cells. The effects observed in these antisense plants, together with the localisation of *sut1* to sieve elements, demonstrate that *sut1* is essential for sucrose loading into the phloem via an apoplasmic route (Kühn *et al.*, 1997). Expression of *sut1* was also reported in the transport and delivery phloem, such as the vasculature of the potato tuber, indicating that *sut1* may also be involved in retrieval during long-distance transport and in phloem unloading (Riesmeier *et al.*, 1993).

The sucrose transporters identified so far arise from a large gene family, some of which are involved in specific functions, such as pollen nutrition (Lemoine *et al.*, 1999). Sucrose transport has been shown, in a variety of studies, to be highly regulated at the cellular and sub-cellular levels, in response to fluctuating sucrose concentrations, diurnal rhythms and phytohormones (Vreugdenhil, 1983; Daie *et al.*, 1986; Chiou and Bush, 1998; Kühn *et al.*, 1997).

The second transport group consists of the hexose transporters, which are primarily located in sink tissues. Hexose transporters have been identified in a number of species, transferring a wide range of monosaccharide substrates (Hellman *et al.*,

2000). Cloning of the first higher plant monosaccharide transporter was initially accomplished by heterologous hybridisation with hexose transporter genes from the green alga *Chlorella kessleri* (Sauer and Tanner, 1989; Sauer *et al.*, 1990*b*). A family in excess of 25 genes has since been identified in *A. thaliana*, while multiple genes have been isolated from a number of other species (Lalonde *et al.*, 1999). Both enzyme activity and gene expression have been shown to be regulated by phytohormones, abiotic stress and wounding, indicating a flexible system for the allocation of carbohydrates (Sauer and Stadler, 1993; Ehneß and Roitsch, 1997). However, a clear function has yet been demonstrated for any of the monosaccharide transporter proteins using antisense inhibition strategies.

Interestingly, the expression of hexose transporters in sink tissues also supports an apoplasmic mechanism for phloem unloading (Sauer and Stadler, 1993). The occurrence of multiple genes encoding putative hexose transporters in stem tissue of sugarcane also suggests the possibility of interaction between these transporters (Casu *et al.*, 2003). Sucrose released from the phloem into the sink apoplasm may initially be hydrolysed by acid invertase, and then taken up into sink cells via the appropriate monosaccharide transporter. A common regulation of gene expression of a plant invertase and a monosaccharide transporter has been demonstrated in *C. rubrum* cell suspension cultures, which indicates the presence of a regulatory network controlling the co-ordination of a variety of protein activities in the process of phloem unloading (Ehneß and Roitsch, 1997).

Trehalose metabolism

Until quite recently, trehalose was dismissed as a sugar of any major importance in angiosperms (Eastmond *et al.*, 2002*a*). Although its presence has been well documented in a few highly desiccation-tolerant species (Müller *et al.*, 1995), the inability to detect trehalose in the majority of higher plants had previously led to the suggestion that the capacity to produce trehalose had largely been lost (Crowe *et al.*, 1992). Nevertheless, the activity of the enzyme trehalase (EC 3.2.1.28), responsible for the breakdown of trehalose to glucose, has been shown to be present in numerous plants (Müller *et al.*, 1995). Subsequent application of trehalase inhibitor

Validamycin A established that detectable amounts of trehalose could be accumulated in tobacco and potato (Goddijn *et al.*, 1997). Further research has led to the isolation of the genes coding for trehalose-6-phosphate synthase (TPS) (EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP) (EC 3.1.3.12) in *A. thaliana* (Vogel *et al.*, 1998) and sugarcane (Carson, 2001) as well as the implication of their tentative roles in the regulation of plant sugar metabolism (Eastmond *et al.*, 2002*a*).

Over-expression of bacterial TPS genes in plants initially resulted in substantial growth defects and altered metabolism (Goddijn *et al.*, 1997; Romero *et al.*, 1997). These phenotypes were best interpreted in the context of changes in carbon allocation between source and sink tissues, which thus lead to the speculation that some aspect of trehalose metabolism might be important in sugar signalling (Goddijn and van Dun, 1999). However, more recent reports on tobacco plants expressing *E. coli* TPS have shown an increase in trehalose-6-phosphate (Tre-6-P) and improved rates of photosynthesis per unit leaf area under saturated light, whereas those expressing TPP exhibited reduced photosynthetic rates (Paul *et al.*, 2001). These data suggest a correlation with Tre-6-P rather than trehalose and imply that Tre-6-P either directly or indirectly controls carbon assimilation. Exogenous trehalose has also been shown to affect plant starch and sugar metabolism-related gene expression (Müller *et al.*, 1998; Wingler *et al.*, 2000), however it remains to be proven whether these effects are physiologically relevant.

To rationalize these effects, comparisons can be made with what is known from trehalose metabolism in yeast. Although the mechanism is not understood, TPS has been shown to play a critical role in the regulation of glycolysis in some yeasts, such as *Saccharomyces cerevisiae, w*here the predominant site of action is believed to be the initial enzymatic step, catalysed by hexokinase (HXK) (EC 2.7.1.1) (Thevelein and Hohmann, 1995). The *tps1* gene is also necessary for carbon catabolite repression of gene expression in *S. cerevisiae* (Thevelein and Hohmann, 1995). Tre-6-P has also been shown to be a potent inhibitor of *S. cerevisiae* HXKII *in vitro* (Blazquez *et al.*, 1993), thus providing a potential means of TPS/Tre-6-P glycolytic regulation. This has not been the case in higher plants, however, as studies using *A. thaliana* and spinach have both shown Tre-6-P has no affect on HXK activity (Weise *et al.*, 1999; Eastmond *et al.*, 2002*b*). Nevertheless it has been shown that TPS is essential for

growth in *A. thaliana*, as disruption of *At*TPS1 is lethal to embryo development (Eastmond *et al.*, 2002*b*). Thus, although a definitive regulatory mechanism and a specific cellular target await elucidation, it appears that Tre-6-P is in some way involved in the control of glycolytic carbon flow and is at least necessary for growth.

2.2.3 Sugar and sink-mediated regulation

There is strong evidence that sink tissues regulate the net photosynthetic rates and carbon status of leaves, even though the biochemical mechanisms involved are not yet fully understood (Farrar, 1992; Basu *et al.*, 1999, Paul and Foyer, 2001). In potatoes, a high sink demand has been associated with an increased rate of net photosynthesis and enhanced translocation of photosynthates to the extent of 90% (Moorby, 1978; Dwelle *et al.*, 1981). Alternatively, the removal of growing tuber sinks leads to a marked depression in photosynthetic rate due to an imbalance between source and sink (Nosberger and Humphries, 1965). The inhibition of photosynthesis due to source-sink imbalance, or sucrose accumulation in leaves as a result of poor carbon export from source leaves, has been reported in a number of crops (Azcon-Beito, 1983; Stitt *et al*., 1987). Current conceptions of the role of sugars in developing models of source/sink interaction have allowed for an improved appreciation of the mechanisms involved in sink regulation of photosynthetic rate (Paul and Foyer, 2001).

The studies of sugars as metabolic regulators have faced much scepticism due to the long-standing belief that signalling molecules should have a single dedicated role and only act at a very low concentration in plants (Kende and Zeevart, 1997). Recent research has shown however, that sucrose not only functions as the major transport metabolite, but also as a powerful signal to activate or repress specific genes in a variety of different tissues and pathways (Koch, 1996; Geigenberger, 2002; Rook and Bevan, 2002).

It has been known for many years that sucrose and glucose included as osmotica in protoplast cultures inhibited photosynthesis, but it was thought that this was due to osmotic stress rather than the direct effect of the sugars themselves (Fleck *et al.*,

1982). More recently Sheen *et al.* (1999) observed that sugars also repress the expression of photosynthesis-related genes. Moreover, excess sugar accumulation in transgenic tobacco plants due to the expression of a yeast invertase in the cell wall resulted in photosynthetic inhibition over several days, and progressive chlorosis in the leaves from the tip downwards as the leaves matured (van Schaewen *et al.*, 1990). Photosynthesis was clearly limited by the rate of carboxylation and ribulose bis-phosphate (RuBP) regeneration, which was consistent with a significant decrease in the activities of Calvin cycle enzymes, including Rubisco.

Sugar feeding studies have also indicated that sugar-mediated control of gene expression can occur in plants (Krapp *et al.*, 1991; Schafer *et al.*, 1992). However, due to the variety of metabolic changes encountered in *in vitro* sugar feeding experiments the interpretation of such research is complicated, and thus the extent to which this occurs under natural physiological conditions remains debatable (Paul and Foyer, 2001). Analysis of plant growth under elevated CO_2 conditions has provided a more substantial method of exploring carbohydrate control of photosynthetic gene expression as a mechanism of sink regulation of photosynthesis. When grown under elevated CO_2 conditions, photorespiration is depressed and photosynthesis in source leaves increases, providing more carbohydrate for metabolism and export to sinks. Research has shown that sink activity is stimulated due to the increase of substrate availability (Koch, 1996; Farrar, 1992). However, when sinks cannot utilize all the assimilate generated, sugar accumulation in leaves has a direct effect on the expression of source photosynthetic enzymes and variety of sugar- and starch-related enzymes (Koch, 1996; Moore *et al.*, 1997), such as AGPase (Müller-Röber *et al.*, 1991). Sucrose accumulation also represses the expression of the sugar transporter (Chiou and Bush, 1998). Overall regulation may result in adaptive changes in assimilate partitioning, such as the conversion of leaf material from source to sink. This is more obvious in instances of pathogen attack, where there is often a decrease in the export of sucrose from the infected leaves and an increase of import at infection sites (Farrar, 1992).

Sucrose has even been reported as a putative morphogen, acting to alter the developmental pathways of groups of cells. The clearest indication of this is that sucrose in combination with auxin can substitute for an excised bud in inducing

differentiation of vascular tissue within a block of undifferentiated callus tissue (Wetmore and Rier, 1963). At constant auxin concentration, increasing concentrations of sucrose increased the proportion of phloem vessels in the vascular tissue. This effect was also obtained using other α-glucosyl disaccharides, such as trehalose and maltose (Jeff and Nortcote, 1967). Thus, although there is still no mechanistic explanation for these observations, the overall effects of sucrose may include morphogenetic as well as substrate level and regulatory interactions (Farrar *et al.*, 2000).

2.2.4 Sugar sensing

Although there is an abundant amount of research data documenting the contributions of sugar as a mechanism for regulation of source by sink, only recently has research begun to focus on the primary components of sugar sensing in higher plants. For instance, in many cases the effects of sucrose regulation have been substituted by glucose and fructose, indicating that sucrose itself may not be the direct signalling molecule (Ehness *et al.*, 1997; Jang and Sheen, 1997; Jang *et al.*, 1997). Most plant tissues can readily synthesise sucrose when fed with hexoses, therefore it is impossible to attribute the effects of hexose to their direct sensing, since sucrose sensing could plausibly occur. However the direct sensing of sucrose is equally difficult to measure, as the rapid metabolism of sucrose to glucose and fructose is possibly sensed by hexose sensors. An efficient sugar-sensing mechanism should therefore theoretically include sensors able to perceive apoplastic and cytosolic sugar concentrations, and be capable of responding to the flux of carbohydrates entering and leaving the cell. In addition, a complete sugar-sensing network should be able to provide information on sucrose, hexose and other disaccharides that are present in the cytosol or are moving to the various intercellular compartments (Loreti *et al.*, 2001).

Despite anticipated difficulties in uncovering the mechanisms involved in the sugar sensing regulatory web, good progress has been made in identifying a number of candidate sensors/receptors in the past five years. Although HXK was initially characterised as a glucose sensor in yeast (Rose *et al.*, 1991), many examples now exist demonstrating the importance of HXK in glucose-dependent gene repression.

Studies using antisense repression of HXK in *A. thaliana* produced transgenic plants that were insensitive to high glucose concentrations, while plants over expressing HXK were hypersensitive to sugars (Jang *et al.*, 1997). Tomato plants overexpressing HXK show a marked reduction in growth and developed senescent leaves early on (Dai *et al.*, 1999). Antisense repression of HXK in potato, on the other hand, resulted in a leaf-specific increase of starch and soluble sugar content, while tuber metabolism appeared unaffected (Veramendi *et al.*, 1999). There is thus strong evidence for a possible role of HXK in sugar sensing, however direct proof of its sensory function or a clear understanding of its interaction with other proteins is still absent (Hellman *et al.*, 2000). This has resulted in a variety of controversial discussions involving the importance of HXK as a sugar sensor (Herbers *et al.*, 1996; Halford *et al.*, 1999).

Another component from the yeast signalling pathway now identified in plants is the sucrose non-fermenting (SNF1)-related protein kinase (Celenza and Carlson, 1986). The yeast SNF1 is activated in response to low cellular glucose levels and is required for the derepression of a variety of genes that are repressed by glucose (Gancedo, 1998). It has been suggested that changes in the AMP/ATP ratio via glucose metabolism, rather than glucose itself, are important for SNF1 signalling, due to the similarities between SNF1 and mammalian AMP-activated protein kinase (Hardie *et al.*, 1998). However, in transgenic potato tubers the expression of a SNF1-related kinase (SnRK1) in the antisense orientation has resulted in decreased expression of SuSy in tubers and loss of sucrose-dependent activation of SuSy transcripts in leaves (Purcell *et al.*, 1998). Furthermore, SnRK1 enzymes in spinach have been shown to phosphorylate nitrate reductase (EC 1.7.1.1), SPS and 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase (EC 1.1.1.34) (Douglas *et al.*, 1997; Halford and Hardie, 1998). Thus SnRK1 is not only of interest to sugar signalling research, but may prove to be potentially appealing candidates for linking carbon and nitrogen metabolism.

Despite significant progress in the understanding of how plants modulate their metabolic status by sensing sugars, the levels of complexity depicted by Loreti *et al.* (2001) suggest that a full picture of the sugar sensing mechanism is far from complete. Sugars could theoretically be sensed at a variety of levels, such as: (1) sugar concentrations in the apoplast; (2) sugar flux crossing the plasma membrane; (3) intracellular sugar levels and (4) sugar flux into glycolysis. Depending on the

specificity of sensors the scenario becomes even more elaborate, due to the probable existence of distinct hexose and disaccharide sensors. In this sense, HXK and SnRK1 can be considered as hexose sensors able to determine intracellular sugar levels and the flux of hexose into glycolysis, while sucrose transporters, possibly acting as disaccharide sensors, may sense the apoplastic sugar concentration and the flux of sugars crossing the plasma membrane (Lalonde *et al.*, 1999). However, despite the successful description of some sugar sensors, further effort is needed to fully understand sugar sensing mechanisms in higher plants.

2.3 Arrays – a tool for multiple transcript analysis

Recent attempts to understand the signalling machinery involved in plant sucrose metabolism have shed light on a complex network of inter-dependent regulatory pathways that are decisive in the regulation of carbohydrate associated gene expression. To this end, the in-depth analysis of multiple gene expression patterns could assist in the modelling of complex carbohydrate regulatory systems intrinsic to higher plants. Furthermore, large-scale gene expression analysis may be used to obtain insight into the physiological consequences of multiple gene modifications (van Hal *et al.*, 2000).

Analysis of the regulation and function of genes in the late 20th century was largely driven by step-by-step studies of individual genes and proteins. Several highly useful techniques for such research are available, including northern blotting (Alwine *et al.*, 1977), differential display (Liang and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995) and dot blot analysis (Lennon and Lehrach, 1991). However, these methods each have disadvantages that render them unsuitable if large numbers of expression products are to be studied simultaneously. In the past decade, a paradigm shift in gene research has afforded the development of a number of new tools for the effective analysis of many genes simultaneously.

2.3.1 A new approach

The first attempts to measure global levels of gene expression were based on large-scale expressed sequence tag (EST) sequencing (Adams *et al.*, 1995). This approach is particularly useful for gene discovery, however, it is far too laborious for routine analysis of gene expression. With the availability of complete genomes sequences and representative collections of gene specific ESTs, a more pragmatic tool has been the found in deoxyribonucleic acid (DNA) arrays (Brown and Bodstein, 1999). DNA array technology, or reverse northern hybridisation, was invented at Stanford University (Schena *et al.*, 1995) using a small set of *A. thaliana* express sequence tags (ESTs) and has since been applied to several model organisms. Levels of gene expression can be studied in parallel by hybridising a labelled, reverse-transcribed complex mRNA population (targets) to immobilised DNA fragments (probes) spotted on solid carriers, such as nylon membranes. After washing, the hybridisation signal at each spot on the array can be taken as a quantitative measure of the abundance of a corresponding mRNA in the sample, and thus the expression level of the particular gene (Reymond, 2000). Such DNA fragments can be cDNAs, genomic clones, or oligonucleotides, so that corresponding arrays can be generated depending on the intended application (for review see Schaffer *et al.*, 2000).

Array technology has proved to be a powerful means to substantially increase the speed at which differential gene expression can be analysed and gene functions elucidated. To date, the most advanced analyses have been performed in yeast and humans. Arrays bearing all predicted yeast open reading frames (ORFs) have been used to analyse mRNA profiles under a variety of conditions, to cluster genes into groups that coordinate expression, and to identify common themes underlying complex regulatory networks (Kao, 1999). By applying this technology to plants exposed to different growth conditions or stimuli, information on the degree to which genes respond to stresses or chemicals can be obtained. This can similarly be applied to gain information by comparing different developmental stages, mutants, or natural accessions of the same species (Kurth *et al.*, 2002). In *A. thaliana*, the most advanced arrays have covered about 11500 ESTs (Schaffer *et al.*, 2001) and 8300 genes or ESTs (Zhu *et al.*, 2001). However, monitoring of differential expression has been effectively demonstrated when comparing specific chloroplast-related gene sequence

tags (GSTs) in light- and dark-grown plants, or wild type and mutants (Kurth *et al.*, 2002). Co-ordinated plant defence responses have also been revealed using arrays tailored to examine a select group of known wound and insect-induced gene sequences (Reymond *et al.*, 2000; Schenk *et al.*, 2000).

2.3.2 Quality control

As the data format generated by an array typically consists of a large list of genes and corresponding values of that are representative of relative mRNA transcript levels, the further analysis of such huge quantities of data can often pose major bottlenecks (Ermolaeva *et al.*, 1998). To this end, good image analysis software is imperative to keep a good overview, assess the quality of the data and to help find statistical significance and relevant correlations within, and between different array experiments. Such a software package should ideally be able to identify array elements, subtract background, flag or remove artefacts, verify that controls have performed correctly and normalise the signals (Chen *et al.*, 1997).

To compare expression values directly, it is necessary to apply some sort of normalisation strategy to the data, either between paired samples or across a set of experiments. The use of control standards is thus advisable (Watson *et al.*, 1998), either as indicators of negligible hybridisation levels (an external control) or as mRNA 'spikes' contained within the target population (an internal control). The standards should be from genes that hybridise only to their complements on the array and are not present in the target sample: genes from different organisms are ideal for this (Schena *et al.*, 1996). Ideally, monitoring of an internal control standard could provide confidence that sensitivity and stringency are adequate and would allow the signals to be used as normalisation factors between different arrays (Watson *et al.*, 1998).

2.3.3 Combining arrays with other data

Although arrays have been deemed the method of choice for quantitative analysis of the multiple gene expression, there are a number of limitations to this novel

technology. If gene expression is thought of as a multi-levelled biological process beginning with mRNA transcription and proceeding to protein translation and post-translational modification, array analysis is only privy to the first level of information, that is, mRNA abundance. As transcript level does not necessarily reflect protein amount (Greiner *et al.*, 1998) array studies should ideally be accompanied by analysis at the protein and metabolite levels. Large-scale parallel analysis of the proteins that are present in a cell is developing rapidly in proteomic research (Celis *et al.*, 1998; Blackstock and Weir, 1999). Both technologies are complimentary while focussing on different steps of the same process; moreover, proteomics allows the detection of post-translational modifications (phosphorylation and glycosylation) of gene products, which are not regulated at the mRNA transcription level. Similar metabolite profiling techniques are also being investigated (Fiehn *et al.*, 2000; Roessner *et al.*, 2000), and initial efforts have already been made to combine array data with metabolite profiles in *A. thaliana* (Reymond *et al.*, 2000).

Expression profiling with cDNA arrays has only just begun to be applied to the microbiology community, yet a wealth of data has already been generated. Arrays containing a full plant genome may soon be available and will certainly contribute to an improved knowledge of the metabolic events occurring throughout the plant. Conversely, small-scale custom arrays with dedicated sets of genes could also prove to be useful for in-depth examination of specific pathways, such as sucrose metabolism. A comparison of transcript levels between source and sink materials would be the first step in unravelling the complexities of carbohydrate regulation. Combined with the simultaneous analysis of protein activity and key metabolite levels, the complex regulatory mechanisms of sucrose metabolism could be effectively modelled for individual species.

When the repertoires of transcripts, proteins and metabolites measured in a single experiment eventually increase to genome-scale levels, the challenge will be to integrate these complex databases and to extract meaningful biological information. Ultimately, these technologies may provide an interactive model of plant metabolism of complexity not previously attainable. Such a model could dramatically advance our understanding of the regulatory processes of higher plants, and our ability to successfully manipulate plant growth through genetic modification.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reverse northern hybridisation analysis

One of the chief aims of this project was to develop a reverse northern array protocol for the efficient detection of mRNA abundance of a specific group of carbohydrate metabolism-related genes expressed at various stages of culm development (Table 1). Target cDNA populations were prepared using mRNA isolated from leaf and three regions of the sugarcane stem representing different phases of sugar accumulation. These populations were then probed with an array of carbohydrate metabolism-related ESTs, thus affording a comparable representation of the change of sucrose-related gene expression during sucrose accumulation.

3.1.1 Target preparation

Precautions

As good quality ribonucleic acid (RNA) is essential for the effective analysis of gene expression, a variety of precautions were taken in this study to prevent RNase contamination and resultant RNA degradation during extraction (Sambrook and Russel, 2001). This included the use of disposable gloves at all times and regular cleaning of bench surfaces with RNase Away (Molecular BioProducts) to prevent RNase cross-contamination. Where possible, sterile disposable plastic-ware was used, including sterile aerosol-resistant disposable pipette tips. Non-disposable items, such as glassware, mortars and pestles, scalpels and spatulas, were autoclaved prior to use. Solutions were prepared with 0.1% (v/v) diethyl pyrocarbonate (DEPC)-treated water, while chemicals used for the preparation of solutions were dedicated to RNA work to avoid the risk of RNase contamination. All solutions and samples were kept chilled on ice for the duration of the extraction procedure. Electrophoresis apparatus,

including combs, gel-casting trays and tanks, were cleaned with detergent, dried with ethanol, wiped with RNase Away and rinsed with DEPC-treated water prior to use.

Plant materials

From 25 June – 7 July (2002), mature stalks were harvested at Mount Edgecombe, KwaZulu-Natal (SASEX) from field grown *Saccharum* spp. hybrid *cv.* N19 (N19). Internodes 1, 2, 5, 6, 11 and 12 were excised immediately from each individual stem. The internode directly below the youngest leaf with the uppermost visible dewlap was defined as internode 1 according to the system of Kuijper (van Dillewijn, 1952) (Fig. 3). Stalk rind was carefully removed from each internode and the underlying tissue, spanning the core to the periphery, was cut into thin slices and physically macerated. Internodal tissue was then pooled into three representative sugar accumulation phases (Whittaker and Botha, 1997) (Table 1). Thinly sliced leaf tissue representing leaf +1 and +2 (Fig. 3), and the pooled internodes were frozen in liquid nitrogen to – 196 ^{o}C and then reduced to a rough powder using an A11 Basic Analysis Mill (IKA®). The tissue powders were stored in 50ml polypropylene centrifuge tubes (Corning®) at – 80 ^{o}C prior to usage.

Table 1: *Pooling of internodes according to stage of maturity and phase of sucrose accumulation. Classification was according to Whittaker and Botha (1997). Immature (1+2), maturing (5+6) and mature (11+12) internodes were pooled to provide samples representative of various stages of sucrose accumulation.*

Stage of maturity	Phase of sucrose accumulation	Internodes
immature	'lag' phase	1 + 2
maturing	'log' phase	5 + 6
mature	'plateau' phase	11 + 12

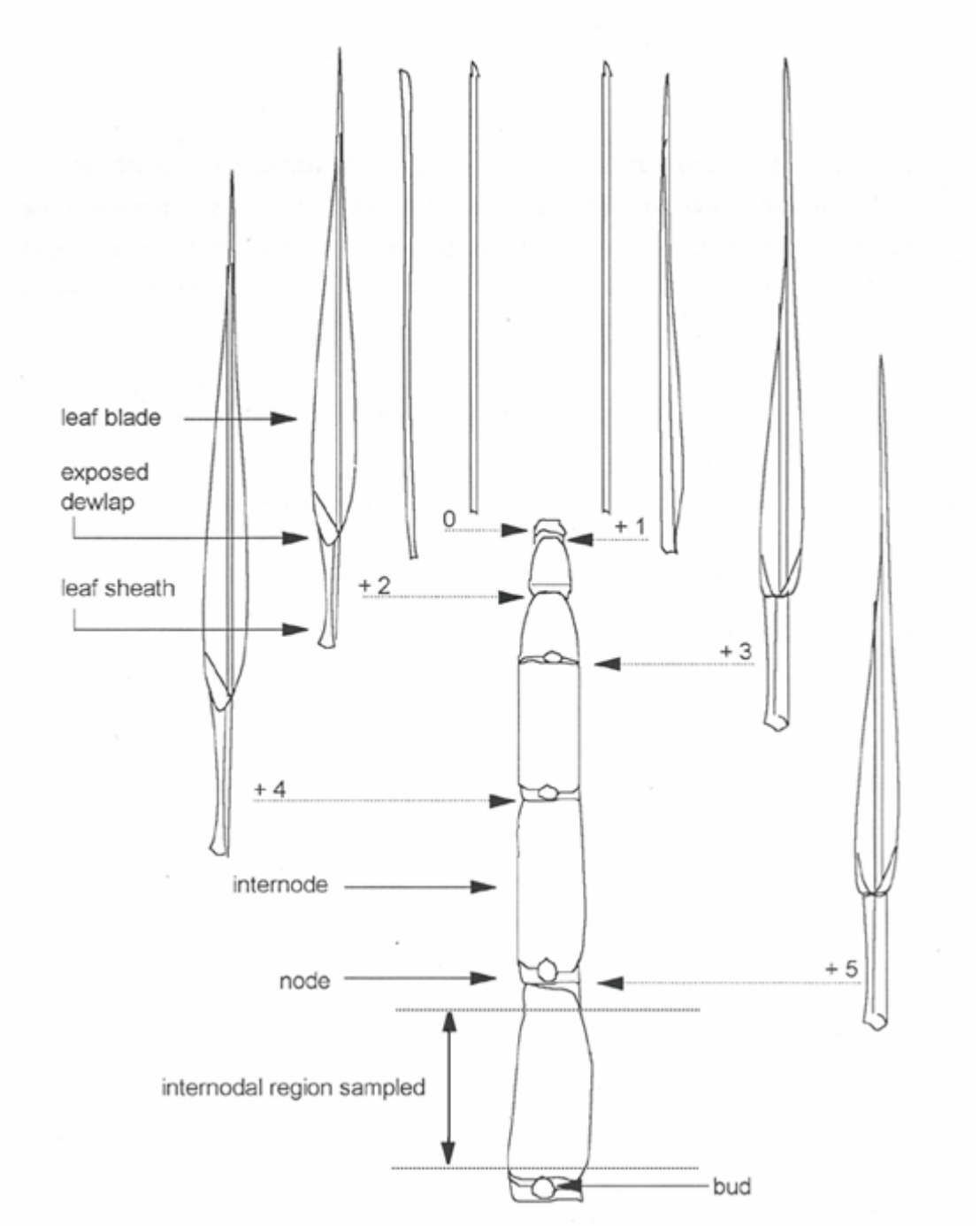


Fig. 3: *The upper section of a sugarcane stalk showing internodes +1 to +5. Leaves are numbered according to the system of Kuijper. Leaf +1 represents the first unfolded leaf with a visible dewlap. The older leaves are consecutively numbered. Internodes attached to the respective leaves carry the same number.* Adapted from van Dillewijn (1952).

Total RNA extraction and purification

Total RNA was extracted using a modified extraction protocol from Bugos *et al.*, (1995). Approximately 4g of powdered tissue was transferred into 50ml Corning tubes containing 10ml of homogenisation buffer (0.1M Tris-HCl (pH7.5), 1mM EDTA, 0.1M NaCl, 1% [w/v] SDS) and 10ml of 25:24:1 (v/v/v) phenol: chloroform: isoamyl alcohol. Samples were dispersed in the homogenisation mixture using a Vortex mixer set at high speed for 1 – 2 minutes. At room temperature (RT), sodium acetate (pH 5.2) was added to each sample to a final concentration of 0.1 M, which were further homogenised for 30 seconds followed by storage on ice for 15 minutes. After centrifugation at 12,000 x *g* for 15 minutes at 4 ^{o}C, the upper aqueous phase of each sample was transferred to a fresh 50ml Corning tube, containing an equal volume of isopropanol to precipitate the RNA. Samples were incubated at – 70 ^{o}C for 30 minutes and then centrifuged at 10,000 x *g* for 10 minutes to recover the precipitated RNA. The supernatant was discarded and RNA pellets were washed with approximately 5ml of 70% (v/v) ethanol, followed by centrifugation at 10,000 x *g* for 5 minutes and brief drying in a speed vacuum. After resuspension in 750µl DEPC-treated water, any remaining insoluble material was removed by centrifugation at 10,000 x *g* for 5 minutes at 4 ^{o}C, and the supernatant transferred to a fresh microfuge tube.

The RNA was precipitated overnight at 4 ^{o}C by the addition of 250µl of lithium chloride to a final volume of 2M. Precipitated RNA was recovered by centrifugation at 12,000 x *g* for 15 minutes at 4 ^{o}C, the supernatant decanted, and the pellet again washed with 70% (v/v) ethanol (approximately 1 ml), followed by centrifugation at 10,000 x *g* for 5 minutes and brief drying in a centrifugal sample evaporator. RNA pellets were resuspended in DEPC-treated water (150 – 300µl) and stored at – 80 ^{o} C.

RNA quantification and quality assessment

Concentrations of RNA were calculated from ultra-violet (UV) spectrophotometric absorbance measurements at 260nm using standard photometric equations (Beckman

DU-7500 spectrophotometer). RNA quality was expressed as the ratio of absorbance measurements (260:280nm) (Sambrook and Russel, 2001) and confirmed via gel electrophoresis, where a 1.2% (w/v) RNA denaturing agarose gel was used to size fractionate the RNA (Ingelbrecht *et al.*, 1998). Samples (5µg total RNA) were suspended in RNA denaturing buffer (20mM MOPS (pH 7.0), 2.2 M formaldehyde, 50% [v/v] formamide) to a final total RNA concentration of $0.5\mu g.\mu l^{-1}$, heated to 65 ^{o} C for 5 minutes to denature secondary and tertiary structures in the RNA and immediately quenched on ice. RNA loading buffer (0.9% [w/v] bromophenol blue, 0.09% [w/v] xylene cyanol, 25% [w/v] Ficoll) (4µl) was added to each sample, mixed briefly and centrifuged to collect the tubes contents. Samples were run at $5V.cm^{-1}$ for 1.5 hours, stained with $1\mu g.ml^{-1}$ ethidium bromide (EtBr) for 30 minutes, de-stained for 30 minutes in DEPC-treated water and visualised by means of trans-illumination with short-wavelength UV light (302nm).

Poly A^+ RNA isolation

According to yield predictions given by the manufacturer of the poly A^+ RNA isolation kit Dynal® Oligo $(dT)_{25}$ mRNA Purification kit), an estimated 2µg poly A^+ RNA (mRNA) was isolated from 100µg portions of each RNA sample. Final elution from the magnetic beads was in a 10µl volume. Samples were stored at – 80 ^{o} C.

cDNA synthesis and labelling

A LabelStar™ Array Kit cDNA Labeling Module (Qiagen) was utilised to generate single stranded (ss) cDNA fragments from mRNA populations. For cDNA synthesis, 1µg mRNA isolates (5µl) were adjusted to 18µl with RNase–free water. Denaturation Solution Plus (2µl) was added to the solution, which was then incubated at 65 ^{o} C for 5 minutes. After immediate quenching on ice, the following components were added to a final volume of 50µl: 5µl reverse transcriptase buffer (10x), 5µl dNTP mix, 5µl oligo-dT primers (20µM), 0.5µl RNase inhibitor (40 units/µl), 12µl RNase-free water,

2.5µl LabelStar Reverse Transcriptase and 5µl [α-^{33}P] labelled dCTP. The reaction mixture was incubated at 37 ^{o}C for 120 minutes, after which 2µl Stop Solution was added.

cDNA purification and labelling efficiency

Labelled cDNA was purified from the reaction mixture using the Cleanup Module of the LabelStar Array Kit (Qiagen) and eluted in 10µl Tris-HCl (10mM, pH 8.5). The efficiency of target cDNA synthesis and labelling was tested via paper chromatography. A small aliquot of labelled mixture (1 – 2µl) was spotted onto a 5 x 15cm portion of filter paper (Whatman No. 3), the end of which was placed in 0.75M Na_2PO_4. The mobile phase was allowed to migrate 10 – 12cm, after which the chromatogram was exposed to a similarly sized strip of Hyperfilm^{TM}-MP (Amersham) for 60 minutes. The film was developed in 250ml stocks of Ilford Phenisol (developer), acetic acid (stop), and Ilford Hypam (fixative) diluted to manufacture specifications for approximately 5 minutes in each solution.

The consistency of target labelling and the distribution of the cDNA fragment size were further tested by a polyacrylamide gel electrophoresis (PAGE) microgel technique modified from Lage *et al.* (2002). Radiolabelled cDNA (1µl) was added to polished water to a final volume of 6µl. RNA loading buffer (0.9% [w/v] bromophenol blue, 0.09% [w/v] xylene cyanol, 25% [w/v] Ficoll) (4µl) was then added to each sample, mixed briefly and centrifuged to collect the tubes contents. Samples (10µl) were size fractionated by means of electrophoresis on a 15% polyacrylamide gel prepared using a Mighty Small^{TM} SE245 Gel Caster kit (Hoefer), and run at 12V.cm^{-1} for 1 – 2 hours. The polyacrylamide gels were then dried, wrapped in Versafilm (Kohler) and exposed to a high-resolution phosphor screen (Cylcone^{TM}) for 1 hour. The phosphor screen images were captured and viewed by means of a Cyclone^{TM} Storage Phosphor Screen imaging system (Packard).

3.1.2 Probe preparation

Source and format of ESTs

Seventy rice expressed sequence tages (ESTs) identified as homologous to genes playing key roles in carbohydrate metabolism were received as lypholised recombined Bluescript plasmid DNA from the MAFF DNA Bank in Tsukuba, Japan (Table 2). A further eleven ESTs were obtained from an in-house SASEX collection (Carson and Botha, 2000), while nine gene constructs from the Institute of Plant Biotechnology (IPB) in Stellenbosch, South Africa, were also used in expression analysis (Table 2). The clone numbers of the ESTs provided by MAFF and SASEX were cross-referenced to their accession numbers and putative identities by comparison with records in the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov).

Table 2: *Carbohydrate metabolism related genes selected for expression analysis. EST identity was established by sequence homology searches with known gene sequences in the NCBI GenBank database. The Expect (E) value is the statistical indicator of the significance of the match between query and database sequence.*

Clone ID	Ref No.	Identity	Genbank Accession No.	E-value	Size (kb)	Notes
MAFF DNA Bank						
C1808	8	Enolase	U09450	0	0.8	(*Oryza sativa* enolase, complete cds)
C2992	7	Sugar transporter	D87819	e-46	0.5	(*Oryza sativa* mRNA for sucrose transporter, complete cds)
C10027	6	Aldolase	P46563	0	1.4	(Fructose-bisphosphate aldolase 2)
C10545	5	Alcohol dehydrogenase	P4174	0	1.8	
C10785	4	Invertase	AF276704	e-15	1.3	(*Oryza sativa* vacuolar acid invertase (*inv 2*) gene, complete cds)
C11743	3	Trehalose-6-P phosphatase	P31688	0	1.6	
C12836	2	PFP	Q41140	0	1.4	(alpha subunit)
C30275	1	Malate dehydrogenase	S17781	0	1.6	((NADP+) II *Sorghum*)
C40059	16	Citrate lyase	P53396	0	1.7	(ATP-citrate (pro-S)-lyase)

Clone ID	Ref No.	Identity	Genbank Accession No.	E-value	Size (kb)	Notes
C51580	15	Aldolase	D50307	e-127	1.6	(C-1)
C52296	14	Pyruvate carboxylase	P78992	0	0.9	
C52369	13	Trehalose-6-P synthase	Q00217	0	0.9	(56kD subunit)
C52371	12	Pyruvate kinase	D17768	0	0.6	(Rice mRNA, partial sequence)
C53217	11	Pyruvate kinase	P31865	0	1.0	
C60517	10	Enolase	D17767	0	0.8	(Rice mRNA, partial sequence)
C60519	9	Alcohol dehydrogenase	X16297	0	1.6	(*Oryza sativa* mRNA alcohol dehydrogenase 2)
C61017	24	PFP	Q41141	0	1.5	(beta subunit)
C61816	23	Alcohol dehydrogenase	X16296	e-100	1.5	(Oryza sativa mRNA alcohol dehydrogenase 1)
E335	21	Sugar transporter	D87819	0	1.5	(Oryza sativa mRNA sucrose transporter, complete cds)
E587	20	Malate dehydrogenase	P17606	0	1.4	([NADP] 1, chloroplast precursor)
E667	19	Invertase	P26792	0	1.4	(beta-fructofuranosidase, insoluble isoenzyme 1 precursor (invertase 1)
E714	18	Malate dehydrogenase	P37229	0	1.4	([NADP] 2, chloroplast precursor)
E742	17	Invertase	U31451	0	1.2	(*Zea mays* invertase mRNA, partial cds)
E1149	32	Cellulase	S20026	0	1.5	(beta-glycanase - rice)
E1171	31	G-3-P dehydrogenase	P81406	0	1.3	(NADP-dependent G-3-PD)
E1890	30	Sucrose synthase	Q41607	0	1.6	
E2102	29	Sucrose synthase	AJ001117	0	1.6	(*Triticum aestivum* mRNA type 1)
E2214	28	Phosphoglucomutase	U89342	0	1.8	(*Zea mays* phosphoglucomutase 1 mRNA, complete cds)
E2256	27	Cellulase	E11341	e-87	1.9	
E3926	26	Sucrose phosphate synthase	E08233	0	0.8	(DNA encoding)
E4237	25	Cellulose synthase	E26621	0	0.9	
E10248	40	ADP-glucose pyrophosphorylase	P52417	0	1.8	(Glucose-1-phosphate-6-adenyltransferase large subunit 2)
E12049	39	Sugar transporter	P53631	0	0.6	(Hexose transporter *hxt17*)
E20893	38	UDP glucosyltransferase	AF199453	e-169	0.8	(*Sorghum* bicolor UDP-glucose glucosyltransferase)
E21058	37	Hexokinase	P04807	0	2.1	(PII) (B)
E31987	36	Sugar transporter	P39004	0	2.5	(High-affinity hexose transporter *hxt6*)
E40404	35	Invertase	P93761	0	1.5	(acid beta-fructofuranosidase AIV-18)

Clone ID	Ref No.	Identity	Genbank Accession No.	E-value	Size (kb)	Notes
E50295	34	Invertase	Q39693	0	0.9	(beta-fructofuranosidase, insoluble isoenzyme 3 precursor (invertase 3)
E50614	33	Sugar transporter	AF280050	e-133	0.9	(*Oryza sativa* subsp sucrose transporter (*sut 1*) gene, complete cds)
E60080	48	ADP-glucose pyrophosphorylase	S24984	e-170	1.9	(Glucose-1-phosphate adenyltransferase – barley)
E60710	47	Sucrose synthase	AJ000153	0	1.7	(*Triticum aestivum* mRNA type 2)
E61128	46	PEP carboxylase	AF158091	0	1.1	(*Mes.crys.* phosphoenolpyruvate carboxylase-kinase (SNIK) mRNA)
E70473	45	ADP-glucose pyrophosphorylase	P30521	0	1.2	(glucose-1-phosphate adenyltransferase)
F23	43	PEP carboxylase	AB018744	0	1.4	(*Zea mays* mRNA for phosphoenolpyruvate carboxylase, complete cds)
F10388	42	Hexose-P isomerase	P50309	0	1.0	(glucose-6-phosphate isomerase)
r801	56	Triose-phosphate isomerase	A25501	0	0.9	(maize)
r1002	55	Sucrose-phosphate phosphatase	AF283566	e-126	2.0	(*Medicago truncatula* sucrose-phosphatase)
r3339	54	UDP-glucose-6 dehydrogenase	O70475	0	0.7	
r3844	53	Fructokinase	P40713	e-176	1.8	
r10484	52	Sugar transporter	P53387	0	0.6	(Hexose transporter 2)
r10509	51	Sugar transporter	AJ272309	0	0.6	(*Hordeum vulgare* mRNA for sucrose transporter (*sut 1* gene))
S44	49	Phosphoglucomutase	U89341	0	2.3	(*Zea mays* phosphoglucomutase 2 mRNA, complete cds)
S1776	64	Trehalose phosphatase	P31678	0	1.8	
S2869	63	PFK	O08333	0	1.6	(6-phosphofructokinase)
S4330	62	Aconitate hydratase	P70920	0	1.2	
S4718	61	Phosphoglucokinase	P46880	0	2.5	(glucokinase (glucose kinase)
S5237	60	Succinate dehydrogenase	P35721	0	0.7	(succinate dehydrogenase cytochrome B560 subunit (subunit III))
S6405	59	Citrate synthase	P51042	0	1.0	
S10457	58	Invertase	AF062734	e-97	1.7	*Saccharum robustum* soluble acid invertase mRNA, partial cds)

Clone ID	Ref No.	Identity	Genbank Accession No.	E-value	Size (kb)	Notes
S10917	57	Sugar transporter	AJ272308	0	1.4	(*Hordeum vulgare* mRNA for sucrose transporter 2
S11329	72	Invertase	AF276703	e-175	1.0	(*Oryza sativa* vacuolar acid invertase (*inv 2*)
S12782	71	PEP carboxylase	Y15897	e-166	2.7	(*Triticum aestivum* mRNA for phosphoenol pyruvate carboxylase, partial cds)
S13076	70	UDP-glucose pyrophosphorylase	Q9SDX3	0	1.8	(UTP-glucose-1-phosphate uridylyltransferase)
S13722	69	Trehalose phosphate synthase	P38426	e-152	1.3	(alpha, alpha-trehalose phosphate synthase – 115kD subunit)
S20255	68	Invertase	P49175	0	1.8	(beta-fructofuranodase 1 precursor (*inv 1*)
S20761	67	Fructose-1,6-bisphosphatase	P00637	0	1.7	
S21015	66	Sugar transporter	P54862	0	2.0	(Hexose transporter *hxt11* (low-affinity glucose transporter 1gt3))
S21246	65	Sugar transporter	O74849	0	1.8	(high-affinity fructose transporter *ght6* (hexose transporter 6)
S21474	80	PFK	O53257	0	1.3	(6-phosphofructokinase)
S21560	79	Hexokinase	P93834	0	1.2	(_2_)
SASEX						
A45	77	Enolase	AA080586	0	0.9	Zea mays
C52	76	Alcohol dehydrogenase	AA269289	e-158	0.9	Zea mays
E37	75	UDP-glucose dehydrogenase	AA525658	0	1.0	Glycine max
G5	74	Triose phosphate isomerase	AA577653	0	1.0	Zea mays
G41	73	Cellulose synthase	AI216932	0	0.8	Gossypium hirsutum
C48	88	Pyruvate dehydrogenase	AA269174	e-156	0.9	Synechocystis spp.
A84	87	Sucrose synthase	AA080610	0	0.5	Beta vulgaris
B63	86	Sucrose synthase	AA080634	0	0.9	Triticum aestivum
I2-245	85	Trehalose-6-P synthase	[1]	-	0.6	Arabidopsis thaliana
I7-154	84	Trehalose-6-P phosphatase	-	-	1.0	Arabidopsis thaliana
I2-166	83	G-3-P dehydrogenase	AI216915	0	0.5	Zea mays
IPB						
pANSI 510	81	Neutral invertase	-	-	1.8	Sugarcane
pASE 510	96	Enolase	-	-	0.8	Sugarcane EST
pASS 510	95	Sucrose synthase	-	-	0.8	Sugarcane EST
pAUGDf 510	94	UDP-glucose dehydrogenase	-	-	1.6	Sugarcane
pEF2K 510	93	6PF2K	-	-	1.3	Rat liver
pEGP 510	92	PFP	-	-	1.7	Giardia
pHPPase	91	VPPase	-	-	2.7	Hordeum
pUBiASSCA	90	Aldolase	-	-	1.0	Sugarcane
pUSPc 510	89	PFP	-	-	1.1	Sugarcane

[1] Data not available

Cloning of selected ESTs and cDNA-containing plasmids into bacterial host cells

Lypholised MAFF and IPB plasmid preparations were resuspended in 20µl TE buffer and incubated for 30 minutes at 37 ^{o}C. Each cDNA-containing plasmid (2µl) was then introduced into bacterial host cells by the addition of 50µl *E. coli* (strain JM109) in a 10ml Falcon tube. The cells and plasmid DNA were incubated on ice for 20 minutes, heated to 42 ^{o}C for precisely 50 sec, and then heat-shocked by returning them to ice for 2 minutes. Nutrient SOC medium (20mg.ml^{-1} Bacto® -tryptone, 5mg.ml^{-1} Bacto®-yeast extract, 10mM NaCl, 250µM KCl, 20mM Mg^{2+}, 20mM glucose) (950µl) was added to the tubes which were then shaken at 150rpm for 1.5 hours at 37 ^{o}C. The tubes were centrifuged (2000 x *g* at 4 ^{o}C for 10 minutes), the supernatant decanted, and the cells resuspended in 200µl SOC medium. The transformation mixture (100µl) was spread evenly over the surface of 1.5% agarose plates, each containing 30 ml Luria-Bertani (LB) medium and 100µg.ml^{-1}ampicillin, and incubated overnight at 37 ^{o}C. Sterile toothpicks were used to transfer single, distinct colonies from each plate to fresh 15ml Corning tubes containing 10 ml of RT LB medium, and shaken at 150rpm overnight at 37 ^{o}C.

The eleven ESTs from the SASEX collection (Carson and Botha, 2000) were amplified via the polymerase chain reaction (PCR) using a (reverse/forward) primer set. Amplified products were inserted into pGEM plasmids using a pGEM® T Easy Vector System II kit (Promega). The plasmid stocks were then cloned into *E. coli* (strain JM109) competent cells, plated onto 1.5% agarose plates containing 80µg.ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-GAL), 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100µg.ml^{-1}ampicillin and grown overnight at 37 ^{o}C. Cell colonies were either blue or clear in colour, due to the absence or presence of an EST gene insert respectively. Clear cell colonies were then selectively grown in 10ml LB medium overnight in a shaker (150rpm) at 37 ^{o}C.

Preparation of external standards

Five standards were prepared as external controls for expression analysis. These included unrestricted pGEM®–T Easy Vector and pBlueScript II KS (-) plasmid stocks, and four pGEM plasmids stocks each containing heterologous bacterial genes encoding a δ-endotoxin crystal protein (*cry1A(b)*), phosphothricin acetyltransferase (*bar*), S-enolpyruvylshikimate-3-phosphate synthase (*CP4*) and β-glucoronidase (*gus*). cDNA inserts (*cry1A(b), bar, CP4 and gus*) were amplified via PCR using previously prepared, gene-specific primer sets for each plasmid stock respectively. The PCR products (2µl) were fractionated on a 1.2% (w/v) agarose gel at $6V.cm^{-1}$ for 2 – 3 hours and visualised by staining with $1\mu g.ml^{-1}$ EtBr for 30 minutes, de-staining for 30 minutes in distilled water, and exposure to short-wavelength UV light (302nm).

Amplified products were then ligated into pGEM plasmids using a pGEM® T Easy Vector System II kit (Promega) in accordance to manufacturer's instructions. The plasmid stocks were then cloned into *E. coli* (strain JM109), plated onto 1.5% agarose plates containing $80\mu g.ml^{-1}$ X-GAL/IPTG and $100\mu g.ml^{-1}$ampicillin and grown overnight at 37 °C. Cell colonies containing plasmids with an EST gene insert were distinguishable due to their clear colour, and were selectively grown in 10ml LB medium overnight with shaking (150rpm) at 37 °C.

Storage of bacterial cells

Glycerol stocks were prepared from the overnight LB bacterial cultures. Aliquots (1.5ml) of each bacterial culture were added to 0.5ml of sterile 60% glycerol in a 2ml Corning cryotube, vortexed, frozen in liquid nitrogen, and then transferred to – 70 °C for long-term storage. Stab cultures were also prepared from agarose plates using a sterile inoculating needle, and stored in the dark at RT.

Isolation of recombinant plasmid DNA

The remaining 8.5ml of each LB culture was centrifuged at 2000 x *g* to collect cells. Plasmid extraction was conducted using a QIAprep ® Spin Miniprep Kit (Qiagen) as per manufacturer's instructions. Isolated plasmid stocks were stored at – 20 ^{o} C for later use.

Quantification of plasmid DNA

Following quantification using a DyNA Quant 200 flourometer (Hoefer), 300ng of each plasmid stock was added to 5µl gel loading buffer and visualised on a 1.2% (w/v) agarose gel to assess plasmid size, accuracy and consistency of plasmid isolation. The plasmids were size fractionated at 6V.cm^{-1} for 2 – 3 hours, stained with a 0.01% (v/v) EtBr solution for 30 minutes and then visualised by exposure to short-wavelength UV light (302nm).

Confirmation of insert identity

Plasmid stocks were prepared for single run partial sequencing reactions as described in the protocol supplied with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Each reaction contained 8µl Terminator Ready Reaction mix, 3.2pmol T7 primer, 400-500ng recombinant plasmid DNA and de-ionised water to a final volume of 20µl. After amplification, unincorporated dye terminators were removed via an ethanol precipitation step, as recommended by the manufacturer (PE Applied Biosystems), and resuspended in 18µl Template Suppression Reagent. Samples were then heat denatured at 95 ^{o} C for 2 minutes, chilled on ice for 1 minute, and analysed via capillary electrophoresis on an ABI Prism® 310 Genetic Analyzer.

Electropherograms obtained were edited to remove vector and ambiguous sequences using Sequencing Analysis 3.7 (ABI Prism®, PE Applied Biosystems). The edited

sequences were submitted to the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al.*, 1990) for comparative sequence analysis against the NCBI non-redundant protein and dbEST databases, using the BLASTx and BLASTn algorithms.

The Expect (E) value decreases exponentially with the bit score obtained for each sequencing hit, with higher bit scores resulting in lower E-values. Thus, a lower E-value is indicative of a more statistically significant hit. As the E-value also takes into account the length of the sequence being compared, E-values are considered as more reliable indicators of sequence homology than bit scores. The E-value can be related to the p-value, thus indicating statistical significance (Equation 1).

$$\underline{\text{Equation 1}}\text{: p-value} = 1 - e^{-\text{E-value}}$$

As the E-value tends towards zero, the E-value and p-value may converge. Thus in cases where E-values of less than 0.01 were obtained for a particular sequence, these were considered homologuous proteins for the sequence submitted (Altschul *et al.*, 1990). Where several protein homologies were assigned to a sequence, the insert was assigned the identity of the most likely protein (i.e. a sucrose related enzyme) showing the lowest possible p-value. Samples with alignment scores greater than 0.01 were discarded.

3.1.3 Array querying

Array printing

Prior to array printing, all probes in the form of recombinant plasmid DNA were denatured by the addition of NaOH to a final concentration of 0.2M. Either a 0.2 – 2µl pipette (Labsystems) or a 96-pin manual gridding device (V&P Scientific Inc., San Diego) was used to transfer 2µl aliquots of denatured plasmids containing either 0.05pmol insert DNA or 20ng control DNA onto a positively charged nylon Hybond-N+ membrane® (Amersham). For replication, aliquots of each probe were delivered

to either two or three addresses on the array (Fig. 4). Membranes were air-dried and the probe DNA cross-linked by means of short-wavelength UV-radiation (120 kJ.cm^{-1}) (Hoefer UV-Crosslinker). The arrays were then wrapped in filter paper, sealed in polyethylene film and stored at RT until required.

Hybridisation conditions

The array membranes were incubated for 8 – 18 hours in 20ml Church and Gilbert buffer (0.5M sodium phosphate (pH 7.2); 7% (w/v) SDS; 0.94mM EDTA) (Church and Gilbert, 1984) containing 10μg.ml^{-1} denatured fragmented salmon or herring sperm DNA. Incubation was performed at 65 ^{o}C in 300ml volume hybridisation bottles within a rotary hybridisation oven (Hybaid Micro-4). After prehybridisation the original solution was discarded and an aliquot of fresh, pre-warmed (65 ^{o}C) hybridisation buffer containing the cDNA target population was added. Following overnight hybridisation at 65 ^{o}C, the membranes were washed with a 1X SSC (155mM tri-sodium citrate; 150mM NaCl), 0.1% (w/v) SDS solution for 10 – 20 minutes to remove traces of unbound target.

Array column

Array row

	H	G	F	E	D	C	B	A
1	C30275	C12836	C11743	C10785	C10545	C10027	C2992	C1808
2	C60519	C60517	C53217	C52371	C52369	C52296	C51580	C40059
3	E742	E714	E667	E587	E335	pGEM	C61816	C61017
4	E4237	E3926	E2256	E2214	E2102	E1890	E1171	E1149
5	E50614	E50295	E40404	E31987	E21058	E20893	E12049	E10248
6	*gus*	F10388	F23	pBScript	E70473	E61128	E60710	E60080
7	S44	*bar*	R10509	R10484	R3844	R3339	R1002	R801
8	S10917	S10457	S6405	S5237	S4718	S4330	S2869	S1776
9	S21246	S21015	S20761	S20255	S13722	S13076	S12782	S11329
10	G41	G5	E37	C52	A45	*CP4*	S21560	S21474
11	pANSI 510	*cry1A(b)*	I2-166	I7-154	I2-245	B63	A84	C48
12	pUSPc 510	pUBiASSCA	pHPPase	pEGP 510	pEF2K 510	pAUGDf 510	pASS 510	pASE 510

Fig. 4: *Probe location on arrays. The entire array was printed in duplicate or triplicate to specific addresses on each membrane. Control probes were distributed accordingly to allow for image orientation inference. Probe identities are given in Table 2.*

Array calibration

The *gus* gene insert was excised from a previously constructed pBScr-GUS000 plasmid stock, supplied by the IPB. Approximately 1.5µg pBScr-GUS000 was incubated in 2µl SuRE/Cut Buffer B (Roche), 4U BamH1 (Roche) and filter-sterilised water to a final volume of 20µl for 90 minutes at 37 ^{o} C. The reaction was stopped by incubation at 65 ^{o} C for 10 minutes. Linearised pBScr-GUS000 was quantified using a DyNA Quant 200 flourometer (Hoefer) and visually assessed by agarose gel electrophoresis. Approximately 100ng linearised pBScr-GUS000 was added to 5µl gel loading buffer and fractionated according to size using a 1.2% (w/v) agarose gel at 6V.cm^{-1} for 2 – 3 hours. The linearised plasmid DNA was visualised by staining with a 0.01% (v/v) EtBr solution for 30 minutes, destaining in distilled water for 30 minutes and then exposure to short-wavelength UV light (302nm). *Gus* RNA was generated by reverse transcription of linearised pBScr-GUS000 construct using a T7 RiboMAX^{TM} Express Large Scale RNA Production System kit (Promega). The reverse transcribed *gus* RNA quality was then assessed (as per section 3.1.1) and stored at – 80 ^{o} C.

Initial array calibration was undertaken using *gus* RNA (target) and pGEM-GUS plasmid (probe). The *gus* probes at amounts of 1, 10, 20, 50 and 100ng (6x10^{-4} – 0.061ρmol *gus* insert) were queried with *gus* target cDNA ranging from 2ρg – 1µg (3x10^{-6} – 1.51ρmol). As a sufficient hybridisation signal intensity was accomplished in the cDNA target amounts spanning 10 – 500ng, three cDNA amounts (10, 100 and 500ng) were further examined for adequate signal quality when spiked into 1µg target populations of sugarcane mRNA. Three arrays bearing immobilized pGEM-GUS plasmid at amounts of 0.02, 0.01 and 0.0001pmol were constructed to probe the spiked mRNA populations.

EST calibration

Five randomly selected ESTs were queried at three incremental amounts (0.005, 0.01 and 0.05 pmol insert) with a 1µg target cDNA population derived from pooled

internodes 1+2. Aliquots of each amount of EST were delivered to the array membrane in duplicate, whereas both unrestricted pGEM® -T Easy Vector and pBlueScript II KS (-) plasmid stocks were included as standards in triplicate at 20ng.

Signal detection/imaging

Membranes were sealed in polyethylene film and exposed to high-resolution phosphor screens (Cylcone™). After 8 – 24 hours exposure, the images on the phosphor screens were captured and viewed by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard).

Signal analysis

Array images were analysed using QuantArray® MicroArray Analysis Software (version 3.0, Packard Bioscience). This software was used to quantify the hybridisation intensity to each of the probes contained on the array membranes in response to each querying event. Data generated included spot diameters, intensities, uniformity and circularity, background uniformity, and signal to noise ratios. Moreover, the software permitted the calculation of proportional hybridisation intensities to each probe between different target cDNA populations.

3.2 Sugar and protein determination

3.2.1 Enzyme-linked sugar assays

Sucrose, fructose and glucose concentrations of leaf and each culm region under investigation were estimated on a Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc.) by means of a modified enzymatic coupling technique (Jones *et al.*, 1977), whereby the phosphorylation of sugar in solution by an enzyme specific reaction can be quantified by following the co-reduction of NADP to NADPH at 340nm (A_{340}). Data analysis was performed using KC4 software

(Biotek® Instrument, Inc.). Plant tissue (100mg) was ground for 1 – 2 minutes in an A11 Basic Analysis Mill (IKA®) and incubated overnight in 1ml of freshly prepared sugar extraction buffer (70mM HEPES [pH 7. 8], 14mM $MgCl_2$, 30% [v/v] ethanol 70% [v/v]) at 70 ^{o}C. Extracts were centrifuged for 10 minutes at 23,200 x *g* and the supernatants transferred to clean tubes.

In a 96-well microtitre plate, 5µl of sample was added to 200µl assay cocktail (150mM Tris (pH 8.1), 5mM $MgCl_2$, 0.06 NADP, 0.27mmol ATP) and 45µl polished water. Samples were diluted 1:1, 1:2, 1:10 and 1:100, and absorbance readings were taken at A_{340} (a). Sugar standards (5µl) (0.5 – 10mM glucose, fructose and sucrose) were run concurrently with each sample plate to negate any variations in substrate or enzyme preparation. The assay was performed in triplicate and three blank wells were also prepared using 5µl 70% (v/v) ethanol.

Glucose was measured by initiating the reaction with 5µl (0.5U) of hexokinase/glucose-6-phosphate dehydrogenase (HX/G6-PDH) (EC 1.1.1.49) (Roche). The change in absorbance due to the reduction of NADP to $NADPH^{+}$ (b) was measured following the complete phosphorylation of glucose present in solution after approximately 30 minutes. Completion of the reaction could be visually assessed via a peak plateau in absorbance readings. Glucose concentrations were calculated according to Equation 2.

To ascertain fructose concentrations, 5µl (0.7U) of phosphoglucose isomerase (EC 5.3.1.9) (Roche) was further added to each sample. The A_{340} (c) was taken subsequent to the completion of the reaction after approximately 45 minutes and was used to determine the concentration of the sugar (Equation 3).

Sucrose levels were determined by reacting 5µl sample in 40µl citrate buffer (100mM Citrate [pH 5.0], 5mM $MgCl_2$) with 5µl invertase (10U) (Roche). After incubating solutions at RT for 15 minutes, 200µl of assay cocktail was added to each well. The absorbance (d) of each sample was calculated, whereupon 5µl of HX/G6-PDH was added and the mixture incubated at RT. Absorbance stabilised (e) after approximately

20 -30 minutes, whereupon sucrose levels in the samples were calculated according to Equation 4.

Equation 2: glucose $dA_{340} = b - a$

Equation 3: fructose $dA_{340} = c - b$

Equation 4: sucrose $dA_{340} = (e - d) - (b - a)$

The sugar concentrations (mM) of each sample were calculated using their absorbance average and the sugar standard absorbance curve.

3.2.2 Protein determinations

Total protein concentrations were determined by the Bradford method (Bradford, 1976). Samples were crushed in liquid nitrogen using a mortar and pestle to which five volumes of cold (– 20 ^{o}C) 80% (v/v) acetone was added. The resulting slurry was filtered through Whatman No. 2 filter paper under vacuum. Precipitates were washed three times with 100% (v/v) acetone at RT, dried under vacuum, and transferred to 1.5 ml Eppendorf tubes. Powdered samples (10mg) were resuspended in 1ml of 0.1M phosphate buffer (pH 7.5) containing 1mM EDTA, and incubated on ice for 45 minutes. Samples were clarified by centrifugation (10,322 x *g*) for 10 minutes at 4 ^{o}C and the supernatant transferred to fresh tubes.

In a 96-well microtitre plate, 50µl of sample was added to 150µl Bradford reagent (Sigma), mixed and the absorbance read on a Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc.) after exactly two minutes. The assay was performed in triplicate and blanks were prepared using 50µl 0.1M phosphate buffer. A protein standard prepared using bovine serum albumin (6 – 0.5µg) was run concurrently with each sample plate to negate absorbance variation. The protein standard was performed in triplicate.

CHAPTER 4

RESULTS

4.1 Internodal sugar content during culm development

One of the main hypotheses of this study is that sucrose accumulation may be regulated, or perhaps regulate, at the transcriptional level (Moore, 1995; Farrar *et al.*, 2000). To test this hypothesis the expression levels of sucrose-related genes were examined in internodes of different developmental status. To substantiate the validity of this supposition, the concentration of sucrose needed to be measured in immature, maturing and mature internodes used in this study, and equated to previous data on sugar levels in regions of differing culm maturity (Whittaker, 1997; Botha *et al.*, 2001).

Previously, sugar concentrations in sugarcane stalks have been reported in a variety of units, ranging from $mmol.L^{-1}$ (Moore, 1995) to percent fresh mass (Botha *et al.*, 2001). However, the comparison of sugar content using fresh mass is not always reliable due to the variation of water content between tissue samples. In this study the results were compiled as sugar percentage to fresh mass (Fig. 5a), and sugar molarity per gram protein (Fig. 5b). The reasoning for the latter measurement is twofold – firstly to equate sugar levels to the variation in protein content in changing developmental regions of the culm, and secondly, to provide comparable data to previous work on sugarcane where sugar was expressed in terms of protein concentration (Whittaker, 1997).

In immature internodal tissue (internodes 1 and 2), all three sugars were found at low levels, with high quantities of protein reflecting even lower relative concentration figures. Internodes 5 and 6 displayed an increase in all sugar concentrations, however hexose levels dropped further down the stalk (internodes 11 and 12) whilst sucrose levels increased at an apparent uniform rate. This apparent accumulation of sucrose as the stalk matures has been suggested to be principally regulated at the level of the

sink (Whittaker and Botha, 1997). Protein content decreased down the stalk, from 3.6 ±0.33mg gram fresh mass^{-1} (internodes 1+2) and 2.3 ±0.5mg gram fresh mass^{-1} (internodes 5+6) to 1.2 ±0.1mg gram fresh mass^{-1} (internodes 11+12).

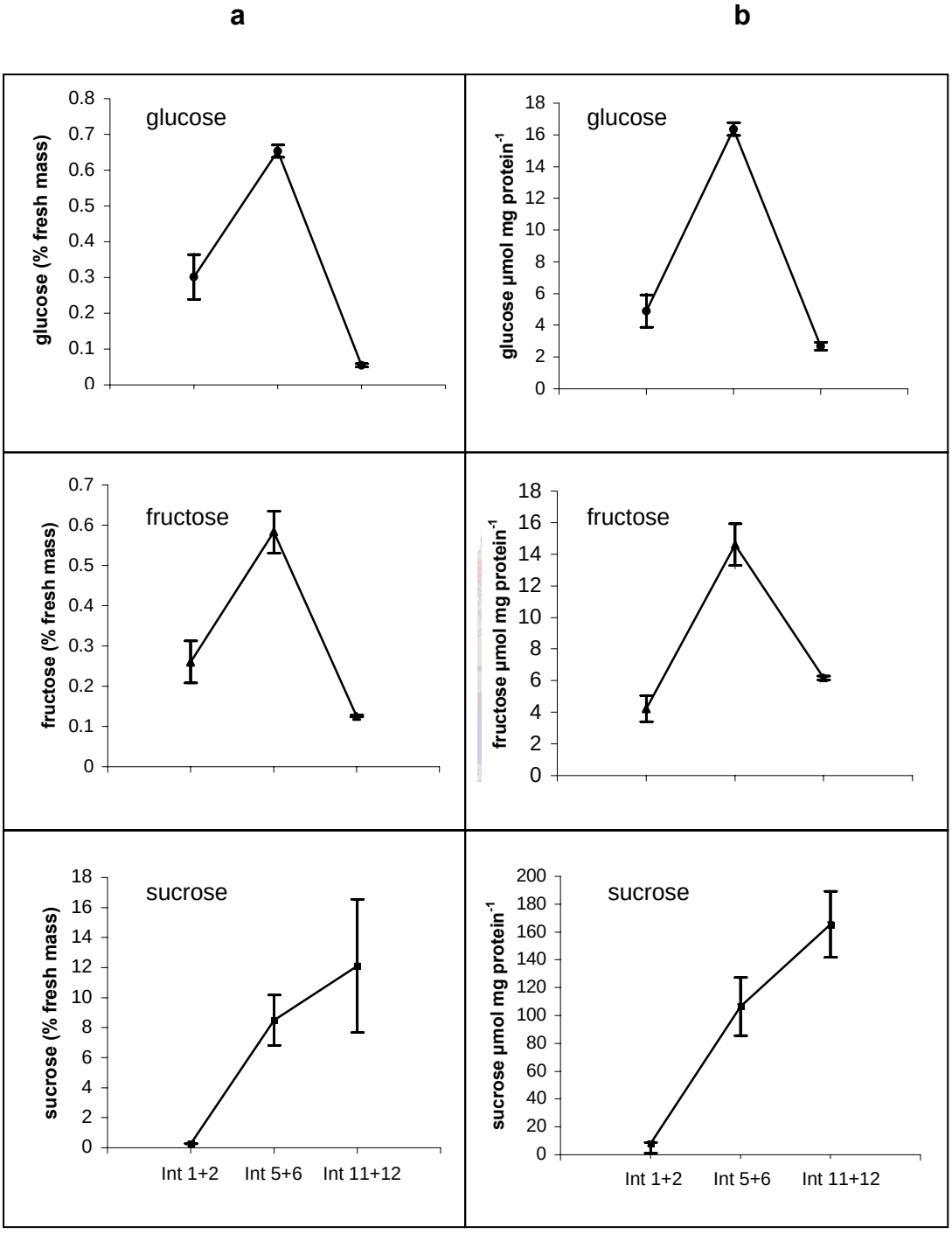
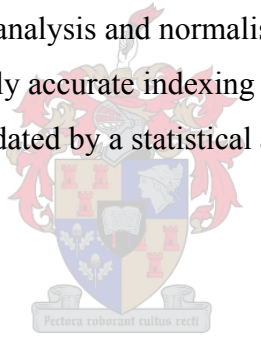


Fig. 5: *Levels of sugars (glucose, fructose and sucrose) in three pooled culm regions (internodes 1+2, 5+6 and 11+12) in mature stalk of field grown Saccharum spp. hybrid cv. N19. Figures are presented as percentages of total fresh mass (a) and per milligram protein (b). Mean values are representative of 6 sample replicates.*

4.2 Technology establishment: reverse northern analysis

Plant gene expression profiling using reverse northern technology has only just begun to be applied to a range of plant species, with the majority of current available data being produced from *A. thaliana* (Seki *et al.*, 2001; Schaffer, *et al.*, 2001; Kurth *et al.*, 2002). Although the technique promises to improve our understanding of gene expression, there are still a number of technical issues that remain unresolved. At the forefront of these is the standardisation of data analysis (Harrington *et al.*, 2000). To efficiently evaluate array data a number of factors need to be addressed, including reproducibility, the linearity of hybridisation intensity and the significance of intensity variation. These issues are highly pertinent with regard to data comparison between different arrays, as without an efficient normalisation procedure the contrasting between hybridisation events becomes inconsistent and ultimately unreliable. To avoid such imprecision, target-probe hybridisation data generated in this study were subjected to rigorous replication analysis and normalised for intra- and inter-target comparison according to a suitably accurate indexing system. Furthermore, comparative array data were validated by a statistical analysis of control variation between different arrays.



4.2.1 Optimisation of target cDNA and probe DNA preparation

Template RNA isolation from target material

Plant tissue is commonly considered one of the more difficult tissues from which to extract RNA. This can be attributed to the ubiquitous presence of ribonucleases (RNases), which are particularly prolific in meristematic regions. Problems are also encountered due to the co-purification of plant polysaccharides and phenolic compounds (Sambrook and Russel, 2001). High concentration and quality RNA yields thus rely on sterile working environments to minimise possible RNA degradation by RNases, and an adequate number of precipitations steps.

RNA was successfully extracted from leaf and three pooled internodal regions using a modified RNA extraction protocol optimised for sugarcane (Bugos *et al.*, 1995).

Yields varied from between 20 to 200µg RNA per gram of fresh mass. Quality and quantity of RNA were determined by spectrophotometry as well as agarose gel electrophoresis with EtBr staining (Fig. 6). As the bulk of RNA within a cell consists of ribosomal RNAs (rRNAs), mostly in the form of 18S and 28S RNA, the integrity and concentration of the sample was assessed by the discreteness of these two bands (Wilkinson, 1991).

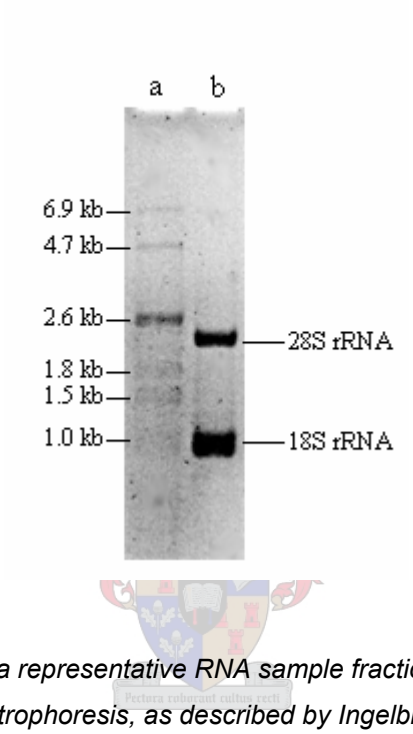


Fig. 6: *Electropherogram of a representative RNA sample fractionated via denaturing agarose gel (1.2% [w/v]) electrophoresis, as described by Ingelbrecht et al. (1998). RNA was visualised via EtBr staining and short-wavelength UV radiation. Lanes represent RNA marker 1 (a) (Roche) and RNA (5µg) extracted from a pooled sample of internode 1 and 2 (b).*

mRNA isolation and quality

As the poly-A^+ messenger RNA (mRNA) portion of total RNA comprises only up to 5%, rRNA sequences were removed from the RNA pool to increase the sensitivity of detection of less abundant transcripts and thus allow for an improved comparison of differential gene expression.

Most mRNA sequences contain long portions of adenylic acid residues located at the 3'-end of intact transcripts (Davis *et al.*, 1986). The presence of this poly A^+ tail

(typically 50 – 100 bases long) allows for the purification of mRNA from the total pool by means of affinity chromatography (Wilkinson, 1991). Isolation of mRNA from total RNA preparations was based on a method in which oligo(dT) chains, coated on to magnetic beads (Dynal® Oligo $(dT)_{25}$ Dynabeads mRNA Purification kit), bound to the poly A^+ RNA in the presence of a high concentration salt, while non-poly A^+ species were removed along with the supernatant. Subsequent washes with low salt buffers then allowed for the elution of the poly A^+ mRNA. This magnetic procedure for the isolation of mRNA was much faster and efficient that other methods that require precipitations steps or the use of organic solvents. Additionally, this method is reportedly one of few which does not carry over oligo(dT) into the mRNA extract (Martins-Wess and Leeb, 2003). The presence of oligo(dT) contamination can cause a variety difficulties, including background problems and possibly cross- hybridisation.

Typically, yields of mRNA were expected to be in the region of 2% of the total RNA used, which corresponds to the concentration of mRNA in the total RNA pool. Sample concentrations of approximately $0.2\mu g.\mu l^{-1}$ were not readily detectable via agarose gel electrophoresis unless multiple samples (10µl each) were pooled, while fluorometric and spectrophotometric methods of quantification were also not suitable. Consequently, due to these limitations, the concentration of $0.2\mu g.\mu l^{-1}$ was presumed for mRNA isolated from 100µg total RNA.

Target cDNA synthesis and labelling

To visually verify the efficiency of target synthesis and labelling as well as the consistency of the reverse transcription labelling reaction between different querying events, radiolabelled cDNA target populations were evaluated via paper chromatography and PAGE. All targets assessed chromatographically conformed to a single uniform spot, demonstrating efficient label incorporation in the target cDNA population (Fig. 7a). As PAGE is more efficient than chromatography for fragment size assessment, it was used to assess the efficacy of cDNA synthesis. Probe samples did not produce a single band, but rather an expected smear representative of a range of fragment lengths (Fig. 7b). Although the PAGE system was modified for

radiolabelled cDNA analysis from a previously described technique (Lage *et al.*, 2002), this quality control step provided a useful verification of the consistency of labelling efficiency between different representative cDNA populations. Good incorporation across a range of fragment sizes was observable, such that cDNA synthesis was within the acceptable limits proposed by Lage *et al.* (2002).

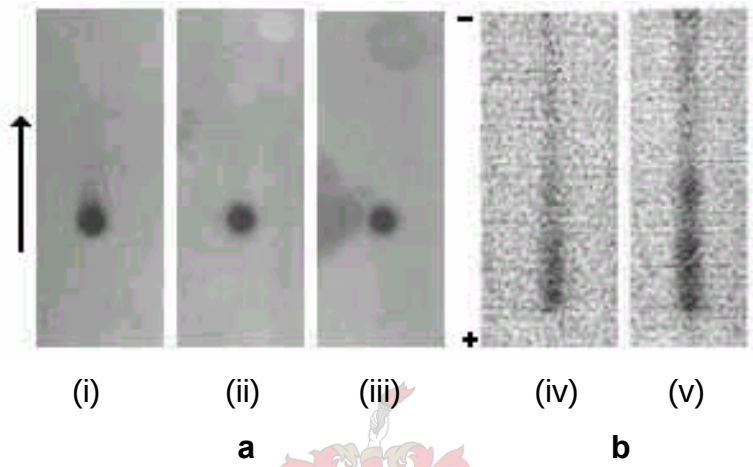


Fig. 7: *Chromatographs (a) of three $\propto^{33}$P dCTP-labelled total ss cDNA target populations synthesised from poly A^{+} RNA isolated from internodes 1+2 (i) and 5+6 (ii) and 11+12 (iii). Electropherograms (b) derived from PAGE of $\propto^{33}$P dCTP-labelled populations of cDNA targets reverse transcribed from internodes 1+2 (iv) and leaf (v). The arrow indicates the direction of the mobile phase(a), whereas the cathode and anode are represented by (-) and (+) respectively (b). Discreteness of hybridisation signal in (a) is an indicator of efficacy of target labelling, whereas (b) demonstrates the range of fragment sizes found within a representative total target cDNA population.*

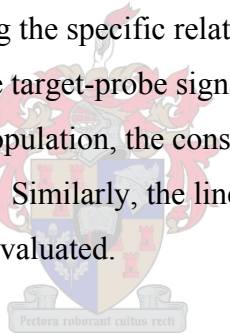
Preparation of probe DNA

Within the plasmid EST suite, the size of the DNA inserts differed quite substantially between plasmid stocks (*c*. 2200bp). Due to this size discrepancy, it was not desirable to spot equivalent concentrations of probe onto the array in terms of overall concentration (i.e. ng.µl^{-1}), as the hybridisation of probe to target would be influenced by the difference in molar concentrations of gene insert. Probes were thus diluted accordingly and spotted at equimolar amounts.

4.2.2 Characterisation of target-probe interactions

DNA array technology is currently at the forefront of gene expression research, surpassing differential display and serial analysis of gene expression (SAGE) as the techniques of choice for the isolation of differentially expressed genes (Kuhn, 2001). DNA array technology holds a significant advantage over other methods with regard to improvements in sensitivity and throughput of expression screening (Watson et al., 1998). Differential display does enable simultaneous detection of multiple differences in gene expression, however only a limited number of different conditions can be compared, while screening is based in differences in mRNA length and not identity. Conversely, SAGE is laborious and involves complex sample preparation and extensive DNA sequencing (van Hal *et al.*, 2000).

As the study of gene expression by DNA array technology is still a recent development, information regarding the specific relationship between target-probe interactions is limited. To calibrate target-probe signal for efficient hybridisation sensitivity to a 1µg target cDNA population, the consistency of target hybridisation to replicate probes was thus assessed. Similarly, the linearity of target-probe hybridisation signal intensity was evaluated.



Preliminary calibration of target-probe hybridisation

To estimate the sensitivity of probe-target hybridisation, 1µg target cDNA derived from young internodal tissue (1+2) was probed with five randomly selected ESTs at three amounts of probe DNA (0.05, 0.01 and 0.005pmol insert). With decreasing DNA probe amounts, the difference between target-probe hybridisation signals was reduced as expected, thus lowering the overall capacity for detection of differential expression between query events (Fig. 8). At 0.005 pmol, probe C48 produced a signal of similar intensity to that of the pBlueScript II KS (-) external standard (pBS). At a tenfold decrease, C48 thus had no quantifiable hybridisation sensitivity to suggest the presence of that gene in the target cDNA population. Therefore, to ensure the highest efficiency of target cDNA detection, all probes were included on arrays at an EST insert amounts of 0.05pmol.

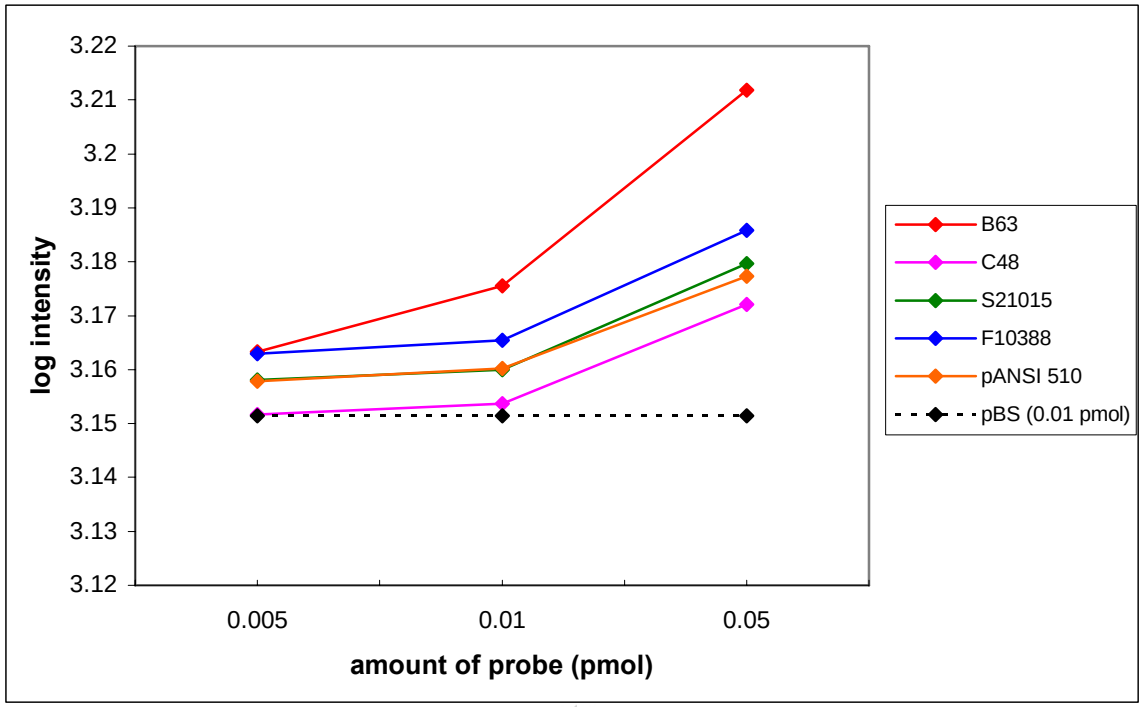


Fig. 8: *Target-probe hybridisation signal intensities at decreasing probe amounts (0.05, 0.01 and 0.005pmol insert) produced by five ESTs. pBlueScript II KS (-) (0.01pmol) was included as an external standard (pBS).*

Consistency of target-probe hybridisation

The *gus* gene insert was successfully transcribed *in vitro* from linearised pBScr-GUS000 using a T7 RiboMAX™ Express Large Scale RNA Production System kit (Promega) (Fig. 9). The consistency of hybridisation intensity between target (*gus* cDNA) and probe querying events (a pGEM-GUS plasmid stock) was then quantitatively measured.

Gus probe DNA was immobilised onto three array membranes at amounts of 1, 10, 20ng (each in triplicate). Each array was then queried with 1µg sugarcane cDNA populations derived from leaf tissue, containing 500, 100 or 10ng *gus* cDNA as internal standards. The percent relative standard deviation, often called the coefficient of variation (CV) (Blalock, 1979), where *s* is the standard deviation

between samples and $\overline{x}$ is the sample mean, was then calculated to determine the percentage relative difference between replicate hybridisation events (Equation 5). In all cases, the CV between replicated events was found to be below 10% (Table 3). This was taken as confirmation of the lack of variation between replicate target-probe hybridisation intensities, regardless of probe or target amounts in that range, and clear indication of the consistency between replicate querying events.

Equation 5: coefficient of variation (CV) = ($s / \overline{x}$) X 100%

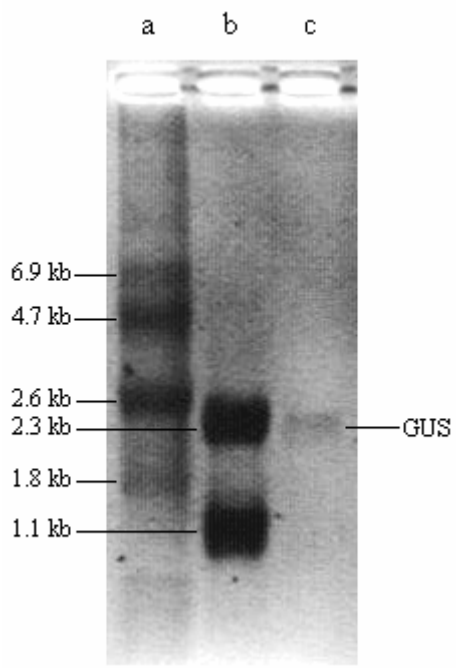


Fig. 9: *Electropherogram of RNA derived from an in vitro transcribed gus gene and size fractionated by denaturing agarose gel (1.2% [w/v]) electrophoresis (Ingelbrecht et al., 1998). Lanes represent RNA marker 1 (Roche) (a); 2µg pGEM® Express Positive Control Template (Promega) (b) and approximately 0.5µg in vitro transcribed gus RNA synthesized from the pBScr-GUS000 construct (IPB) (c).*

Table 3: *Comparison matrix of coefficient of variation (CV) values for gus target-probe hybridisation signal levels. Each CV represents an assessment of the variation between three replicates.*

		Standard target amounts (ng)		
		500	100	1
Amount of probe (pmol)	0.02	3.319	6.602	3.558
	0.01	1.764	8.273	1.964
	0.001	6.926	2.874	1.999

Linearity of target-probe interactions

To confirm variation between hybridisation signals produced by cDNAs of differing amount, the linearity of target-probe hybridisation data produced by the *gus* arrays was graphically and statistically evaluated. An assessment of the known amounts of *gus* target cDNA (10, 100, 500ng) produced a sigmoidal curve for all query events (Fig. 10). A linear intensification of target-probe signal is expected with increased probe amount, whereas the levelling off of signal intensity may be indicative of the propensity towards eventual target-probe signal saturation. However, since all amounts of target produced a sigmoidal curve, it can be assumed that the point of signal saturation was not reached in these ranges of target and probe.

The change of signal intensity between different querying events was further assessed via an analysis of variance (ANOVA). An ANOVA is a statistical test which explores the hypothesis that the means from two or more samples are equal (i.e. drawn from populations with the same mean). Single factor ANOVAs were performed to test the null hypothesis that intensity signals produced at three target strengths (500, 100 and 10ng) were statistically similar for a standard probe query event. This was done for each of the three probe amounts (0.001, 0.01 and 0.02pmol). The three comparisons of signal intensity between target samples all produced p-values below the range generally attributed to significantly differential populations (< 0.05), with a highest p-value of 2.52×10^{-7}. These results thus provided strong evidence against the null

hypothesis and demonstrated that, in this range, differing target amounts produce dissimilar hybridisation signals for the same query event. Thus, it can be assumed that the intensity signal produced by a query event is proportional to the amount of targeted cDNA in the cDNA population being probed.

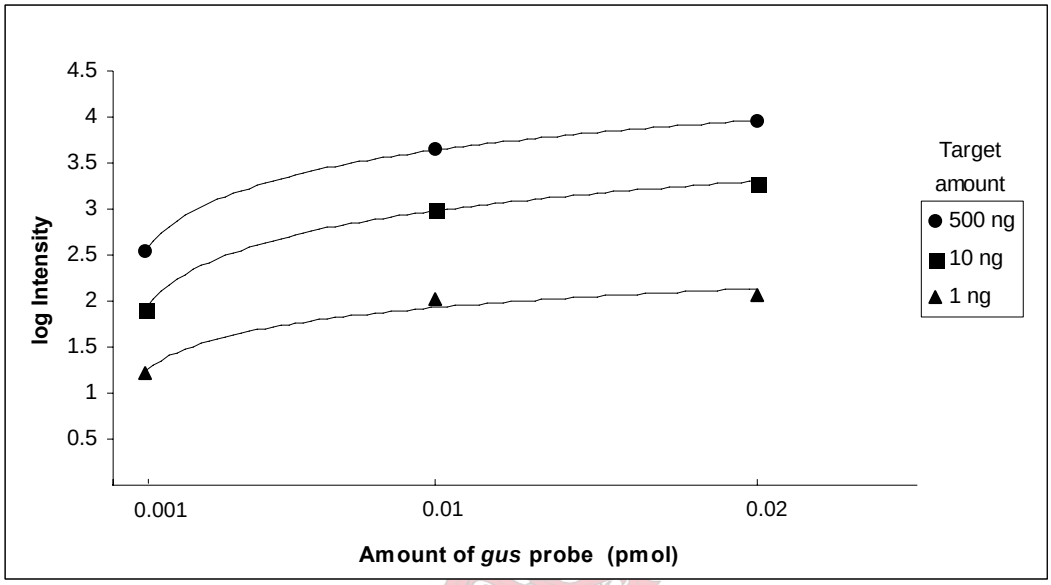


Fig 10: *Comparison of the average intensity indices of three amounts of gus target standard (500, 100 and 1ng) contained within a 1µg sugarcane leaf cDNA population hybridised to three gus probe amounts of 0.001, 0.01, and 0.02pmol.*

4.2.3 Verification of replication and sensitivity of technology

cDNA populations from the three internodal regions of differing culm maturity (1+2, 5+6 and 11+12) were each queried twice with an array of 90 EST probes, spotted in duplicate (Fig. 11). This produced a total of four replicates for each query event. It was visually apparent that a number of genes were differentially expressed amongst the three culm regions under analysis. The signal intensities of each query event were quantified using QuantArray® Microarray Analysis Software (Version 3.0, Packard Bioscience). This software generated hybridisation intensity values for each event as well as background intensity readings. This data was then exported in the form of a spreadsheet, whereupon background intensity was scrutinised for variation.

The mean background intensity of each array was found to produce a CV of less than 5% in all cases, whereas the mean standard deviation in background intensity was found to account for only 1.4 – 4.5% of the mean deviation in probe intensity. Based on these data, it was evident that background subtraction would only serve to hamper the sensitivity of differentiation between probe-probe analysis and was thus unwarranted.

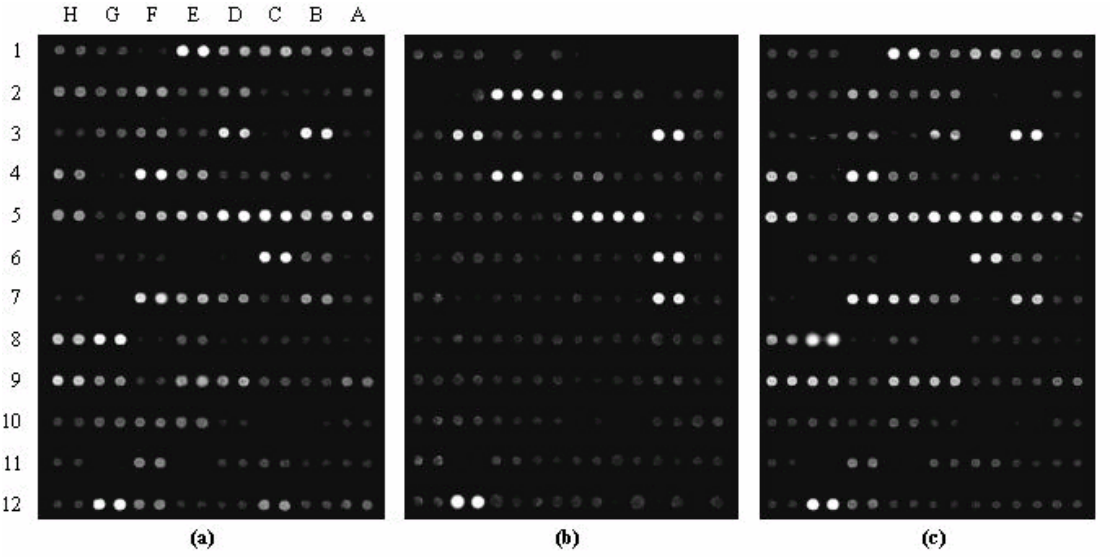


Fig. 11: *DNA arrays produced from probing three target cDNA populations (1µg) derived from pooled internode regions 1+2 (a), 5+6 (b), and 11+12 (c). The arrays carry a total of 90 probe ESTs (0.05pmol) and 6 controls (20ng) arranged in horizontal duplicates. Columns (letters) and rows (numbers) correlate to probe locations in Fig. 4.*

Reproducibility of target-probe hybridisation amongst query events

On each array, duplicate probe signals were quantitatively compared, and excluded from further analysis if their CV yielded a value of greater than 10%. On average, each array reproduced a total of 87 valid query events (90.6 %) with the lowest valid number of query events being 77 (80.2%).

In order to standardise signal levels for further array comparison between duplicate arrays, a relative intensity index value was calculated for each valid probe (Equation

6). The equation takes into account the number of valid query events, thus normalising the intensity index values between arrays with differing quantities of valid query events.

Equation 6:

$$\text{intensity index value} = \frac{\text{(average valid query event intensity)}}{\text{(sum of total average valid query event intensities)}} \times \text{(number of valid query events)}$$

The intensity indices of matching probes were compared between duplicate arrays (arrays targeted with the cDNA populations derived from similar culm regions) and excluded if their CV values exceeded 10%. Internodal culm region 1+2 comprised 70 valid query events (72.9%), 5+6 produced 83 (86.5%), and 11+12 had 62 (64.6%). Query events with just one valid intensity index value (i.e. only one array provided a valid CV from replicate spots) were included for comparitive purposes, but flagged for exclusion from statistical analysis. Valid probe intensity indices from the three target groups were then compiled and subjected to comparative evaluation.

Significance of variation in target-probe hybridisation events

To validate further comparison of transcriptional activity between arrays targeted with different mRNA populations (inter-array querying), six control intensities from three duplicate arrays were indexed and subjected to six separate ANOVAs (Fig. 12, Table 4). The resultant p-value produced by control *gus* was 0.95, whereas no other control indices generated a p-value below 0.05, providing strong evidence that all controls displayed significantly equal intensity indices across duplicate arrays and arrays targeted with cDNA populations derived from different culm regions. Based on data generated from the controls, a significant comparison of the intensity indices of differentially expressed probes was thus considered possible.

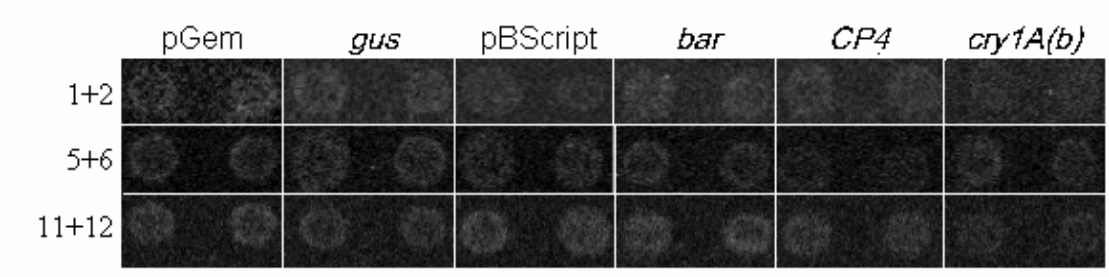


Fig. 12: *Query events (in duplicate) produced from six control standards (pGEM, gus, pBScript, bar, CP4 and cry1A(b)) hybridised to target cDNA populations isolated from three pooled internodal regions of differing culm maturity (1+2, 5+6, and 11+12).*

Table 4: *Control intensity index values produces by three different culm internodal regions (1+2, 5+6 and 11+12). p-values were produced following an analysis of variants (ANOVA) on each control group.*

Control	Intensity Index Value			p-value
	1+2	5+6	11+12	
pGEM	1.002008	0.993534	0.984589	
	1.036824	0.952435	0.996671	0.262034
gus	1.006294	0.908296	0.993791	
	0.976987	1.038766	0.968822	0.948769
pBScript	0.987178	1.036008	1.015222	
	1.006779	0.9739	1.014824	0.806443
bar	1.02001	0.967865	1.033422	
	0.985325	1.015417	1.01284	0.530804
CP4	0.996628	0.936925	1.009299	
	1.011547	0.971017	1.022315	0.05962
cry1A(b)	0.987882	1.157372	0.963677	
	0.982538	1.048465	0.984527	0.111853

4.3 Variation in gene expression associated with culm development

4.3.1 Expression patterns at three stages of culm maturity

Initial examination of gene expression focussed on analysis of intra-array querying and comparison between duplicated array data. An assessment of intensity indices representing relative expression levels of the 90 ESTs at each stage of culm development produced a range of index values (Fig. 13). The majority of genes were located at an index level of approximately 1, indicating a similar level of expression, however, at least ten index values in each culm region exhibited an index higher than 1.5, conceivably demonstrating increased relative levels of mRNA expression for these genes (Table 5). Five out of the ten most highly expressed ESTs were common to all three culm regions under investigation, indicating that such genes may have a higher overall mRNA expression level throughout the sugarcane stem. These included ESTs encoding aldolase (pUBiASSCA), SAI (C10785), hexokinase (E21058), cellulase (E2256) (EC 3.2.1.4), and alcohol dehydrogenase (C61816) (EC 1.1.1.1).

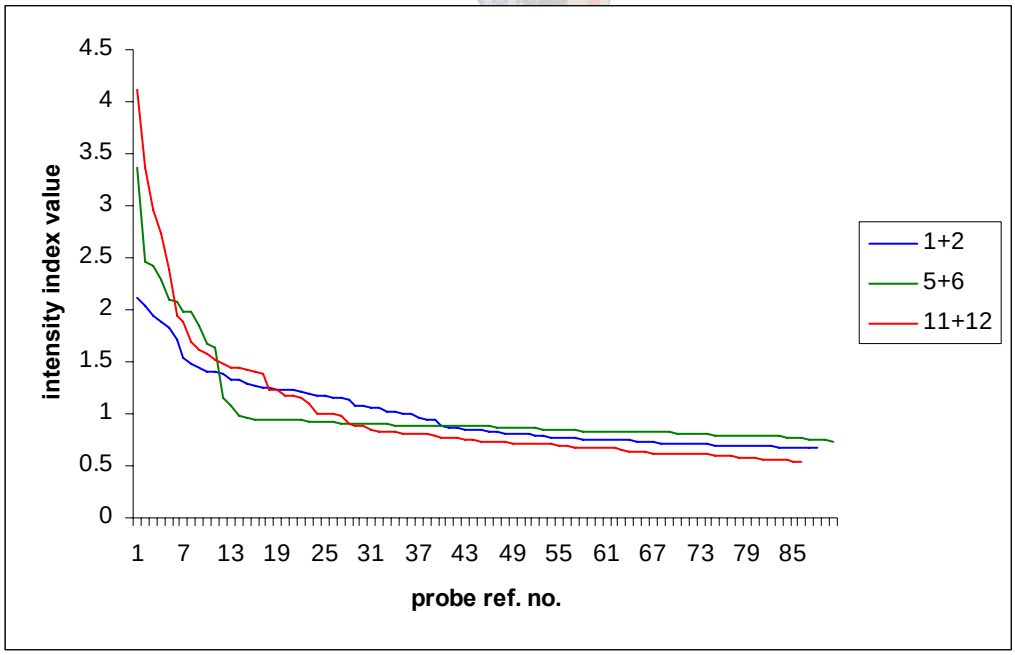


Fig. 13: *Representation of gene expression levels for three developmentally unique culm regions (internodes 1+2, 5+6 and 11+12). Each line represents the totality of valid query events for each region expressed as an intensity index value (Equation 6).*

Table 5: *Ranking list of 10 ESTs with the highest relative expression pattern for each internodal culm region under investigation (1+2, 5+6 and 11+12).*

	1+2		5+6		11+12	
No.	Clone ID	Identity	Clone ID	Identity	Clone ID	Identity
1	pUBiASSCA	Aldolase	pUBiASSCA	Aldolase	E20893	UDP glucosyltransferase
2	C10785	Invertase	C61816	Alcohol dehydrogenase	E21058	Hexokinase
3	E21058	Hexokinase	E20893	UDP glucosyltransferase	pUBiASSCA	Aldolase
4	E2256	Cellulase	C53217	Pyruvate kinase	C10785	Invertase
5	S10457	Invertase	E60710	Sucrose synthase	E2256	Cellulase
6	E61128	PEP carboxylase	R1002	SPP	C61816	Alcohol dehydrogenase
7	C61816	Alcohol dehydrogenase	E21058	Hexokinase	E61128	PEP carboxylase
8	R10509	Sugar transporter	E2256	Cellulase	S10457	Invertase
9	S10917	Sugar transporter	C52371	Pyruvate kinase	R10509	Sugar transporter
10	S21246	Sugar transporter	C10785	Invertase	E50614	Sugar transporter

4.3.2 Comparative expression between culm regions

Array data was further assessed by the comparison of expression signals across different regions of culm maturity. A number of target-probe indices were omitted from inter-array analysis, as they did not generate sufficiently low CV values (<10%) between duplicate query events. This may have been due to experimental error in array spotting, or a miscalculation of amounts between replicates. Thus, out of the total of 90 ESTs, 16 did not provide index values for all three culm regions (Fig. 14).

Although a large number of genes provided evidence of relative differential expression, statistical analysis was limited to query events which had produced two valid intensity indices and were thus not previously flagged (see section 4.2.3). To statistically evaluate the differential expression patterns of genes across the three different sections of culm maturity, ESTs with valid data for all three pooled internodal regions were each subjected to an ANOVA. The inter-analysis of signal intensity between dissimilarly targeted array data provided a means of determining if a significant level of differential expression existed amongst validated ESTs. A total of fifteen ANOVA produced p-values below 0.05 (Table 6), providing strong evidence of differential expression for those genes.

Pectora roborant cultus recti

Table 6: *ESTs with putative differential expression patterns in different regions of the sugarcane culm. Duplicate expression indices produced from cDNA populations extracted from each pooled internode zone (1+2, 5+6 and 11+12) were subjected to statistical analysis to determine the likelihood of a difference in expression level. Statistical significance of differential expression is indicated by ANOVA-derived p-values.*

No.	Clone ID	EST ID	Ref. No.	Rep.	Intensity Index Value			p-value
					1+2	5+6	11+12	
1	C30275	Malate dehydrogenase	1	1	0.8714701	0.9140196	0.8497993	
				2	0.9040519	0.9281343	0.8598742	0.0492588
2	C11743	Trehalose phosphatase	3	1	0.7053665	0.7289962	0.5692103	
				2	0.6919312	0.7851126	0.6157706	0.0269353
3	C2992	Sugar transporter	7	1	0.9796233	0.8138854	0.9639895	
				2	1.0225607	0.7688386	1.0367998	0.0196275
4	C51580	Aldolase	15	1	0.6797531	0.6734883	0.5426927	
				2	0.6975325	0.6532403	0.5830496	0.015571
5	E587	Malate dehydrogenase	20	1	0.8240999	0.8407016	0.6097464	
				2	0.7814189	0.8275569	0.6827167	0.0238345
6	E3926	Sucrose phosphate synthase	26	1	0.7093752	0.8994212	0.5866246	
				2	0.6830342	0.9086581	0.5811713	0.0002359
7	E70473	ADP-glucose pyrophosphorylase	45	1	0.7181546	0.7557255	0.5410221	
				2	0.673814	0.8028669	0.6059397	0.0266637
8	E60710	Sucrose synthase	47	1	1.0747537	2.1306329	0.9497646	
				2	0.9798623	2.1142498	1.0180861	0.0002692
9	R3339	UDP-glucose dehydrogenase	54	1	0.7638481	0.7367377	0.6005795	
				2	0.7824156	0.7649605	0.6440937	0.0126033
10	R1002	Sucrose phosphate phosphatase	55	1	1.2509124	2.1449167	1.5977006	
				2	1.1933129	2.1047316	1.6401615	0.0002621
11	S21015	Sugar transporter	66	1	1.1887691	0.8550033	1.4733318	
				2	1.0791119	0.9300983	1.4270282	0.0053762
12	S13076	UDP-glucose pyrophosphorylase	70	1	0.8719464	0.7751852	0.7607387	
				2	0.8680203	0.8138899	0.7834265	0.0260685
13	G5	Triose phosphate isomerase	74	1	1.0775278	0.8890298	0.8105207	
				2	0.9780736	0.9516529	0.8030295	0.0438104
14	pANSI 510	Neutral invertase	81	1	0.7827549	0.944074	0.6267791	
				2	0.7404157	0.9577624	0.6237774	0.000884
15	pAUGDf 510	UDP-glucose dehydrogenase	94	1	1.2429936	1.1872307	0.6985829	
				2	1.2434657	1.0545387	0.7251947	0.004867

4.4 Expression profile categorisation

A variety of different gene expression patterns were found in the three regions of culm maturity under investigation. To distinguish between the differing expression profiles, query events generated by 15 EST probes were compiled into three expression profile categories (Fig. 15). The expression profile produced for each probe was previously subjected to statistical analysis (ANOVA) to test the significance of the differential expression levels observed (Table 6).

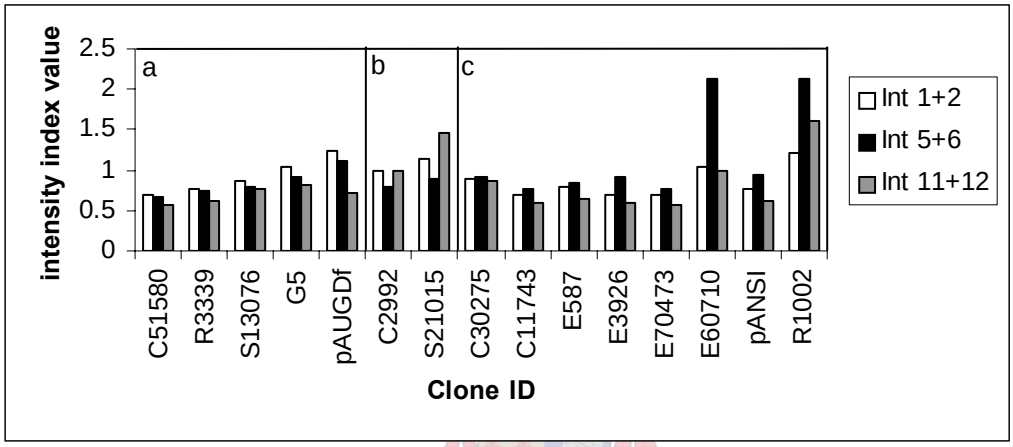


Fig. 15: *Comparison of intensity index values for 15 probes across three regions of culm maturity. Expression profiles have been divided into three categories viz.: (a) genes that were down-regulated from immature to mature tissue, (b) genes that were up-regulated in mature tissue (internodes 11+12) and (c) genes that were up-regulated in maturing tissue (internodes 5+6).*

CHAPTER 5

DISCUSSION

Large-scale gene expression profiling using reverse northern hybridisation technology is a powerful tool for investigating transcriptional activity, effectively bridging the gap between sequence information and functional genomics (Kehoe *et al.*, 1999). Application of this technology in plants can yield information on the degree to which genes respond to different growth stimuli; furthermore, gene expression levels can be compared between different developmental stages (Kurth *et al.*, 2002).

Sucrose metabolism-related genes have been successfully manipulated in a variety of crop species, including tomato, potato and tobacco (Galtier *et al.*, 1993; Krause, 1994; Paul *et al.*, 2001). Several attempts have been made to increase sucrose accumulation in sugarcane by the manipulation of putative key sucrolytic enzymes (Ma *et al.*, 2000; Botha *et al.*, 2001; Groenewald and Botha, 2001). However, those approaches have met with mixed success, indicating the importance of improving our current understanding of sucrose regulation. The goal of this study was to evaluate the hypothesis that sucrose-accumulating potential during sugarcane culm maturation is associated with coarse modulation of the expression of genes associated with primary carbon metabolism. Consequently, a reverse northern hybridisation technique was developed and optimised to assess the transcriptional activity of a suite of 90 carbohydrate metabolism-related ESTs. This 'model' system facilitated the extraction of information regarding gene expression associated with culm development in three regions of differing culm maturity.

5.1 Efficiency of reverse northern technology

5.1.1 Isolation and identification of genetic materials

One of the primary requirements of this study was to optimise a reverse northern protocol for the efficient detection of differential gene expression. This primarily required efficient isolation of mRNA from each pooled internode region (targets) and the identification of sequence homology of a suite of EST-containing plasmids (probes). Scrutinising the putative identity of each EST was crucial for assessing the biological implications of the intensity data produced. Similarly, without the successful isolation of non-degraded RNA, the performance of the technology would have become considerably less informative.

5.1.2 Assessing the significance of array data

The intensity signals produced by each array are unlikely to be directly comparable, due to inevitable variations in hybridisation conditions. There are a number of factors which might cause discrepancy in overall signal intensity, including the decay state of [α-^{33}P] labelled dCTP, the efficiency of cDNA synthesis and labelling, the effectiveness of membrane washing and unavoidable variations during the performance of the experiment (Bowtell and Sambrook, 2003). Individual query events might also differ due to the quality of the spotting tool used, as the inadvertent aging of pins can influence pin geometry, thus effecting volume transfer (Kotschote *et al.*, 2003).

The signals produced on each array by duplicate query events were thus subjected to stringent statistical analysis to confirm intensity replication. Valid duplicate intensity values were then averaged and their relative intensity indices (Equation 6) calculated. Consequently, the intensity index system allowed for a relative inter-array comparison between query events.

Although the indexing system used in this study was suitable for an approximation of relative gene activity, the quantitative comparison of transcription levels between

arrays was not possible – only a relative signal level could be determined. The benefits however of using the indexing analytical technique in this study were twofold, *viz*.: (1) the examination of relative differential levels of gene expression in the same culm tissue and (2) a comparative evaluation of gene expression levels across different culm regions.

5.2 Variations in gene expression during culm development

A multitude of gene ESTs examined in this study exhibited expression levels that fluctuated down the developing sugarcane stalk (Table 7), indicating that numerous enzymatic pathways, either closely or directly related to sucrose synthesis, are controlled by transcriptional regulation. Although the measurement of mRNA expression can often provide a good indication of tissue specific enzyme activity (Kuhlemeier, 1992), the regulation of gene expression, enzyme activity and how these regulatory mechanisms interact is only just beginning to be appreciated. To this end, the putative regulatory control of expression patterns exhibited by several carbohydrate metabolism-related genes is discussed in the following three sections.

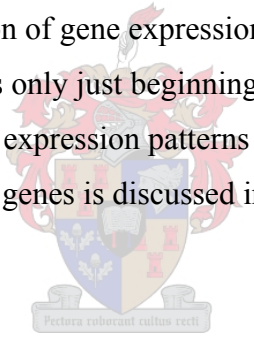


Table 7: *Gene categorisation of coarse regulatory events observed in the sugarcane culm. Genes in italics represent query events that were not statistically evaluated by ANOVA for significance of differential expression.*

Down-regulated	Up-regulated	
Immature to mature internodes	in mature internodes	in maturing internodes
aldolase	glucose transporter (hxt11)	malate DH
TPI	*glucose transporter (hxt6)*	TPP
UGPase	*glucose transporter (hxt17)*	AGPase
UDP-Glc DH	*fructose transporter (ght6)*	SPP
	sucrose transporter (sut1)	SPS
	sucrose transporter (sut2)	SuSy
		NI
		citrate synthase
		aconitate hydratase

5.2.1 Down-regulation between immature to mature internodes

A relative decrease in expression from immature to mature culm was observed for a variety of genes involved directly and indirectly in the regulation of sucrose synthesis. Aldolase (C51580) and TPI (G5) (EC 5.3.1.1) function as the key links between the triose and hexose phosphate pools in the glycolytic pathway, participating in the interconversion of Fru-1,6-P_2, dihydroxyacetone and glyceraldehyde 3-phosphate (Dennis and Blakeley, 2000). Both enzymatic reactions are readily reversible, such that the equilibrium position is determined by the concentrations of the three compounds involved. The aldolase reaction, in particular, is an important regulator of FBPase activity. As FBPase is an important affecter of sucrose accumulation (Stitt *et al.*, 1987), the activity of aldolase can be used to relate the rate of sucrose synthesis to the amount of triose-P exported from the chloroplast (Dennis and Blakeley, 2000). The relative decrease in transcript abundance of aldolase and TPI as the sugarcane stalk is perhaps indicative of glycolytic regulation, and the transition of culm status from source to sink. Furthermore a decline in the levels of triose-P is also evident as the culm matures (Whitaker, 1997). A lowering in transcription for these two enzymes in the developing stalk would thus complement the fine metabolite regulation observed.

UGPase (S13076) catalyses the synthesis of UDP-Glc, the chief substrate for several metabolic pathways, including sucrose synthesis, glycolysis and cell wall synthesis (Dennis and Blakeley, 2000). In non-photosynthetic tissues and developing leaves of *A. thaliana*, UGPase has also been linked to sucrose degradation, providing carbon skeletons for starch synthesis (Ciereszko *et al.*, 2001). The reaction catalysed by UGPase is readily reversible, such that the equilibrium *in vivo* is dependent on the concentration of cytosolic inorganic pyrophosphate (PPi). As PPi decreases in molarity down the culm (Whittaker, 1997), the enzyme is thought to further operate in the direction of UGP-Glc synthesis. Thus, in young internodes UGPase may preferentially synthesise Gluc-1-P, the substrate for glycolysis. In older internodes however, UGPase might favour the formation of UGP-Glc that is then used in the reaction catalysed by SPS to form sucrose (Kleczkowski, 1994). This phenomenon is supported by the flux in sugar levels observed as the stalk matures (Whittaker, 1997; Botha *et al.*, 2001). A general decline in total metabolic activity has been previously

observed along the length of the sugarcane culm (Botha *et al.*, 1996; Bindon and Botha, 2000). The expression levels of UGPase observed in this study are thus indicative of the regulation of carbon flux according to the metabolic requirements and developmental status of the plant (Robertson *et al.*, 1996).

UDP-glucose dehydrogenase (UDP-Glc DH) (R3339, pAUGDf 510) (EC 1.1.1.22) is the first step in the uronic acid pathway, oxidising UDP-Glc to produce UDP-glucuronate. The products of this pathway are primarily involved in cell wall synthesis, such that UDP-glucuronate is reportedly either directly or indirectly responsible for the total polysaccharide content of cell walls (Johansson *et al.*, 2002). UDP-Glc DH has been shown to be highly active in young tissues where there is a demand for the synthesis of structural polysaccharides (Dalessandro and Northcote, 1977).

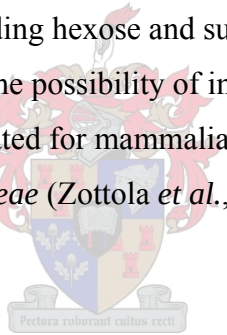
The relatively higher expression levels of UGP-DH observed in immature sugarcane internodes may reflect a correlative relationship with plant growth processes, especially primary cell wall synthesis (Kleczkowski, 1994). The demand for UDP-glucuronate derived sugars, however, has been observed to markedly decline in mature, differentiated cells (Tenhaken and Thulke, 1996). As the culm thickens towards maturity, a relative decrease in UGP-DH transcript abundance is thus suggestive of a reduced requirement for polysaccharide synthesis. Recently, this phenomenon has been independently confirmed in UGP-DH expression and promoter studies (Casu *et al.*, 2003; van der Merwe *et al.*, 2003) by conventional northern analysis.

5.2.2 Up-regulation in mature internodes

Low affinity glucose transporter *hxt11* (S21015) and an *O. sativa* sucrose transporter (C2992) were the only ESTs in this category of gene expression to be statistically assessed for significance regarding the relative levels of differential transcript abundance observed. Transcription of the *hxt* gene family has been studied in detail in *S. cervesiae* (Diderich *et al.*, 1999), however to date only limited data is available on the mechanisms of glucose transporters in higher plants (Hellmann *et al.*, 2000).

A putative sugar transporter (PST-2a) has recently been identified in sugarcane (Casu *et al.*, 2003) exhibiting similar expression patterns to S21015 and C2992. The transcript distribution suggests that these genes may play a role in maintaining sugar fluxes into mature stem sink tissues, where sucrose concentrations are at their highest, but has little or no involvement in transport in mature leaves or roots. Attempts to demonstrate a hexose or sucrose transport function for PST-2a however, have not yet been successful.

Interestingly, several sugar transporter ESTs in the present study showed similar relative transcript patterns, including *O. sativa sut1* (E50614), *hxt6* (E31987), *hxt17* (E12049), *Hordeum vulgare sut1* and *sut2* (R10509, S10917), and high affinity fructose transporter gh*t6* (Heiland *et al.*, 2000) (S21246). Further research is required to elucidate the significance of these expression profiles, however the similar occurrence of multiple genes encoding hexose and sucrose transporters in the mature stem tissue of sugarcane suggests the possibility of interaction between these transporters, as has been demonstrated for mammalian glucose transporters and sucrose transporters in the *Solanaceae* (Zottola *et al.*, 1995; Reinders *et al.*, 2002).



5.2.3 Up-regulation in maturing internodes

Numerous gene ESTs in this study produced an up-regulated expression pattern in maturing tissue. Malate dehydrogenase (malate DH) (C30275, E587) (EC 1.1.1.38) is a mitochondrial enzyme involved in the last step of the citric acid cycle, which has incidentally been linked to aluminium tolerance in sugarcane (Drummond *et al.*, 2001). The enzymatic conversion of malate to OAA is NAD-dependent and produces NADH that is then fed into the electron transport chain to yield three ATP molecules. Relative up-regulation of malate DH suggests a higher citric acid cycle activity in maturing tissue. Although not statistically assessed, the inclusion of mitochondrial metabolism-related ESTs citrate synthase (S6405) (EC 2.3.3.1), aconitate hydratase (S4330) (EC 4.2.1.3), and ATP-citrate lyase (C40059) (EC 4.1.3.6) in this category of up-regulated expression provides further support for this hypothesis.

TPP (C11743) catalyses the synthesis of trehalose from Tre-6-P, the latter of which is implicated in the control of glycolytic carbon flow (Müller *et al.*, 1995) (previously discussed in section 2.2.1). Increased levels of Tre-6-P have been correlated with improved rates of photosynthesis per unit leaf area under saturated light (Paul *et al.*, 2001), which suggests that the increase in TPP expression during culm maturation observed in this study is perhaps a product of coarse sucrose regulation. TPP up-regulation during culm maturation implies a lowering of Tre-6-P, and thus a decrease in sucrose production (Paul *et al.*, 2001). This tentatively implicates TPP as a putative regulator of sucrose metabolism in the maturing stalk.

Similar expression profiles were found for tagged TPP ESTs from rice and sugarcane (s1776, I7-154), however an ANOVA of sugarcane TPS (I2-245) produced a p-value of 0.61, indicating a relatively constant level of TPS transcript abundance throughout the stalk. Little is known concerning trehalose metabolism in sugarcane, however based on these results, coarse control by TPP expression modulation may play a more significant role than previously thought.

Unlike many plants that accumulate photoassimilated carbohydrate as starch, sugarcane stores photosynthate primarily in the form of sucrose. Starch metabolism in sugarcane is therefore not well reviewed, albeit this pathway may play an important role in sucrose accumulation. In this study, significant up-regulation of AGPase (E70473) was found during culm maturation. AGPase is credited as the pivotal regulator of starch accumulation (Geigenberger *et al.*, 1999; Nguyen-Quoc and Foyer, 2001), however the flux of carbon between sucrose and starch pathways is still poorly understood, especially in non-photosynthetic tissues (Stitt, 1995). As starch accumulation has been shown to replace sucrose synthesis under a variety of conditions (see section 2.1.3), an appreciation of the role played by starch metabolism in sugarcane could contribute to a better understanding of overall carbon flux between source and sink. Antisense repression of AGPase in sugarcane may even provide a novel means of further favouring sucrose production during culm maturation.

Several genes involved directly in sucrose synthesis and degradation also demonstrated a significant increase in relative expression levels in maturing internodal tissue. These include SPS (E3926), SPP (R1002), SuSy (E60710) and NI

(pANSI 510). Although NI is generally considered less central to glycolysis than SAI (Winter, 2000), the relative increase in NI expression in maturing tissue (Dendsay *et al.*, 1995) may indicate a more important role involving cytosolic carbon flux during sugarcane culm development. The relative increase in transcription of SPS and SPP can be attributed to the increase in sucrose content as the stalk matures (Whittaker, 1997; Botha *et al.*, 2001), however the inclusion of NI in this expression category may be indicative of a well characterised phenomenon of futile cycling between hexose and sucrose found in sugarcane tissue (Rose and Botha, 2000). Further evidence in support of this hypothesis was demonstrated by Vorster and Botha (1999), where similar levels of NI enzyme activity were observed along the sugarcane stalk. Cycling of carbon between sucrose and hexoses, as a result of simultaneous synthesis and degradation of sucrose, is believed to be primarily responsible for overall sucrose accumulation in sugarcane (Sacher *et al.*, 1963; Batta and Singh, 1986), however, the possible regulatory function of such energy demanding and potentially futile cycles has yet to be resolved (Fell, 1997).

The trends in transcript abundance for some of these enzymes correspond to reported changes in enzyme activity. For example, SPS activity is highest in internodes of maturing culm where sucrose accumulation is highest (Botha and Black, 2000). This suggests that the down regulation of transcripts encoding enzymes involved in sucrose synthesis as the turnover rate of sucrose in fully mature internodes is reduced may operate as a coarse control mechanism (Casu *et al.*, 2003). However, in contrast to the up regulation of SuSy expression observed in this study, the enzymatic activity of SuSy has been reported to be relatively constant and then decrease as sucrose accumulation plateaus in mature culm (Botha and Black, 2000). This illustrates that the mechanisms of fine control, including post-transcriptional modification and phosphorylation, likely play an important role in the overall regulation of carbon metabolism (Winter, 2000).

5.2 Future work

A variety of reviews have previously suggested that sugarcane would most likely serve as a simplistic model for the manipulation of sucrose accumulation in C_4 plants (Grof *et al.*, 1998; Lunn and Furbank 1999). The accumulation of sucrose in sugarcane stem has been reported to be largely determined, at the biochemical level, by the activities of SPS and SAI (Zhu *et al.*, 1997), thus the antisense repression of SAI in the stem, or overexpression of SPS in stem or leaf was presumed the straightforward approach towards increasing sucrose accumulation.

Attempts to manipulate SAI and other invertases in sugarcane however have not proved as promising as expected (Ma *et al.*, 2000; Botha *et al.*, 2001), while efforts to manipulate SPS in species other than tomato (Galtier *et al.*, 1993) have met with only limited success (Signora *et al.*, 1998; Sparks *et al.*, 2001). As plants are adept at compensating for small changes in their genetic or external environment (Halpin *et al.*, 2001), future attempts to influence coarse regulation of sucrose accumulation in sugarcane may depend on manipulation of an alternative gene target not currently implicated in the regulation of sucrose accumulation, or perhaps the co-ordinated manipulation of the activity of multiple gene targets (Sonnewald and Herbers, 1999; Watt, 2002). In order to meet these objectives, a better understanding of the regulatory events governing the source-sink relationship is required.

The current project examined the relative expression levels of carbohydrate-related genes for three culm regions of differing maturity in sugarcane culm, and identified a variety of trends in transcript abundance of closely related enzymes. Results from this study have yielded information on the relative change in gene expression for fifteen ESTs involved in various aspects of sucrose metabolism, including transcripts that code for enzymes functioning in triose/hexose phosphate pool transitions, sugar transport and sucrose synthesis and degradation. These results indicate that assessment of the expression of carbohydrate metabolism-related genes during culm development has the capacity to provide insight into variations in gene activity associated with sucrose accumulation.

This work demonstrated the value of the reverse northern array hybridisation technique for the detection of variations in transcript abundance in the sugarcane culm and as such has provided a technical basis for further work into investigating the regulation of sucrose accumulation. The current system however, is limited, as transcript abundance is measured purely on a relative basis. This restricts analysis considerably, such that conventional northerns are required for conclusive quantitative assessment of gene expression levels. Transcript levels are also merely representative of coarse metabolic management, such that a role in regulation cannot be conclusively determined without knowledge of the fine control exhibited in the regulation of protein activity.

Future analysis will thus need to fulfill a number of additional requirements. Quantitative analysis of reverse northern data will require the use of an internal standard of known amount included in the cDNA target population. The inclusion of such a standard will not only provide information on levels of gene transcript abundance but will also greatly aid in normalisation between different array data. The analytical potential of such investigations will likewise be intensified by the simultaneous analysis of the levels of numerous key metabolites and metabolic intermediates, thus gaining data on both coarse and fine regulatory mechanisms. Furthermore, the inclusion of such data could dramatically increase the predictive power of current kinetic models of sucrose metabolism in sugarcane (Botha and Rohwer, 2001).

A further adjustment to improve the likelihood of uncovering novel differential transcript expression would be the inclusion of more probes on the array membrane. cDNA-querying using a wider selection of EST probes, such as the recently released carbohydrate metabolism-related sugarcane EST libraries developed by Casu *et al.* (2003) and the Brazilian SUCEST programme (Vettore *et al.*, 2001), may significantly improve the likelihood of uncovering novel regulatory transcriptional events. Future carbohydrate expression profiling should also be carried out in a system that permits experimental manipulation of sugar levels and examination of potential feedback between source and sink. Such artificial modification may further assist in pinpointing transcriptional events that regulate sucrose storage.

5.5 Conclusions

This investigation resulted in the development of a sensitive and reliable methodology for the analysis of variations in the expression behaviour of carbohydrate metabolism-related genes associated with culm development. This was facilitated by the use of an array of selected genes, the products of which have a known involvement in carbohydrate metabolism. A focussed approach was advantageous as information in general arrays containing a multitude of ESTs is often lost due to 'noise' generated by irrelevant hybridisation data. Rigorous replication and statistical analysis further enhanced the comparative quality of these results.

The array data have presented a substantial number of new and potentially important insights into sucrose metabolism-related gene expression during culm development. The relative mRNA transcript abundance of 74 genes from three differing regions of culm maturity was documented. A number of genes were found to be highly expressed throughout the three culm regions under investigation, including aldolase, hexokinase, cellulase, SAI and alcohol dehydrogenase. Both UGPase and UDP-Glc DH were down-regulated between immature to mature internodes, whereas SPS, SPP, AGPase, SuSy and NI exhibited up-regulation in maturing internodal tissue. Sucrose, hexose and fructose transporters were also found to be up-regulated in mature culm. This suggests a possible control point of flux into mature stem sink tissues and is perhaps indicative of a putative set of targets for future transgenic manipulation.

This study has yielded novel and supportive information on the relative change in gene expression for a variety of genes involved in carbohydrate metabolism. In addition, these expression data will guide gene selection for future array work. The technology described in this study will be used to examine carbohydrate metabolism gene expression patterns associated with source/sink transitions, such that future studies on sugarcane sucrose metabolism may provide clarity on coarse regulation that was previously unattainable.

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