Sink Regulation of Photosynthesis in Sugarcane

By

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Abstract

The C_4 plant, sugarcane (*Saccharum* spp. hybrids), accumulates sucrose to high concentrations and, as a result, has been the focus of extensive research into the biochemistry and physiology of sucrose accumulation. Despite this, the relationship between source leaf photosynthetic rates and sucrose accumulation in the culm has not been well documented. The observations that photosynthetic activity declines during culm maturation in commercial cultivars and that high-sucrose accumulating ancestral genoptypes photosynthesize at rates two-thirds of those of low-sucrose ancestral *Saccharum* species indicate that source-sink communication may play a pivotal role in determining sucrose yield.

The relationship between source and sink tissues in sugarcane was investigated using a supply-demand paradigm, an approach novel in the study of the crop. The demand for photosynthate from the primary culm growth sink was shown to be closely linked to photosynthetic rates, sucrose export and the eventual physiological decline of source leaves. Results from initial field experiments revealed that leaf assimilation rates were negatively correlated with leaf hexose concentrations, but not those of sucrose. Further manipulation of leaf sugar status, through sugar-feeding and cold-girdling techniques, demonstrated the regulatory role of leaf sugar concentrations on photosynthetic activity, thus revealing sucrose, and particularly hexose, as key signal molecules in the modulation of the amount of photosynthate available for export to the sink. Gene expression profiling, by means of array technologies, indicated that changes in leaf sugar status and photosynthetic rates result in concurrent modifications in the expression of several genes involved in fundamental metabolic pathways, including photosynthesis, carbohydrate metabolism, stress response and sugar-signaling. Notable amongst these, was the identification of a potential trehalose 6-phosphate (T6P) sugar-signaling mechanism, thus implicating the trehalose pathway as a central regulatory system in the communication of sink carbon requirements to the source leaf.

This study demonstrated that maturation of the culm results in a decreased demand for sucrose, which invokes a sugar-mediated feedback signal to decrease leaf photosynthetic supply processes. However, sugarcane leaves appear to retain the

capacity to increase the supply of assimilate to culm tissues under conditions of increased assimilate demand. Uncoupling of the signaling pathways that mediate negative feedback between source and sink tissues may result in improved leaf assimilation rates and, consequently, lead to increased sugarcane sucrose yields.

Declaration

The research that forms the foundation of this thesis was conducted at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, from January 2003 to April 2007 under the supervision of Dr Derek Watt (SASRI and University of KwaZulu-Natal) and Prof. Michael Cramer (University of Cape Town).

These studies represent original work by the author and have not otherwise been submitted in any form for any other degree or diploma to any other tertiary institution.

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Alistair J. McCormick Date

Foreword

This thesis is presented as a compilation of eight chapters. Research work is described in Chapters 3, 4, 5, 6 and 7. Each of these chapters is written according to the style of the journal to which the manuscript was submitted for publication.

Chapter 1: General Introduction.

Chapter 2: Literature Review.

Chapter 3: Sink strength regulates photosynthesis in sugarcane (McCormick AJ, Cramer MD, Watt DA. 2006. *New Phytologist* **171**: 759-770).

Chapter 4: Changes in leaf gene expression during a source-sink perturbation in sugarcane (McCormick AJ, Cramer MD, Watt DA. 2007. *Annals of Botany* doi:10.1093/aob/mcm258).

Chapter 5: Regulation of photosynthesis by sugars in sugarcane leaves (McCormick AJ, Cramer MD, Watt DA. *Journal of Plant Physiology*; under review, submitted August 2007).

Chapter 6: Sugar accumulation induces differential expression of genes related to carbohydrate metabolism, photosynthesis and sugar-sensing: evidence for a trehalose-related signaling mechanism in sugarcane leaves (McCormick AJ, Cramer MD, Watt DA. *Physiologia Plantarum*; under review, submitted November 2007).

Chapter 7: Culm sucrose accumulation promotes physiological decline of mature leaves in sugarcane (McCormick AJ, Cramer MD, Watt DA. Field Crops Research; in preparation for submission).

Chapter 8: General Discussion.

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Contents

List of Abbreviations

α_{leaf}	leaf absorptance
^{o}C	degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
1,3PGA	1,3-bisphosphoglycerate
3PGA	3-phosphoglycerate
A	photosynthetic assimilation
A_{340}	absorbance at 340 nanometers
A_a	assimilation in the absence of stomatal limitation
A_i	assimilation in the presence stomatal limitation
AD; ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AGPase	ADP-glucose pyrophosphorylase
Ald; ALD	aldolase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
C_a	ambient CO_2
CA	carbonic anhydrase
CAM	crassulacean acid metabolism
CE	carboxylation efficiency
cDNA	complementary DNA
C_i	intercellular CO_2
CO_2	carbon dioxide
CS	chalcone synthase
CV	co-efficient of variation
CWI	cell wall invertase
d	day
DEPC	diethylpyrocarbonate
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DST	disaccharide transporter
dT	dioxythymidine

E	transpiration rate
E-value	expect value
E4P	erythrose-4-phosphate
EDTA	ethylene diamine tetraacetic acid
EST	expressed sequence tag
ETR	electron transport rate
f	fraction of absorbed quanta used by photosystem II
F1,6P	fructose-1,6-bisphosphate
F2,6P	fructose-2,6-bisphosphate
F6P	fructose-6-phosphate
FBPase	fructose bisphosphatase
Fm'	maximal fluorescence during a saturating light flash
Fs	"steady-state" fluorescence
FW	fresh weight
g	gram
g	relative centrifugal force
G1P	glucose-1-phosphate
G3P	glyceraldehyde-3-phosphate
G6P	glucose-6-phosphate
G6PT	glucose 6-phosphate/phosphate translocator
GPD; GPDH	glyceraldehyde phosphate dehydrogenase
G_s	stomatal conductance
h	hour
H^+	proton
HCO_3^-	bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hexose-P	hexose phosphate
HPI	hexose phosphate isomerase
HXK	hexokinase
I	incident photon flux density
INH	inhibitor protein
IPB	Institute of Plant Biotechnology
J_{max}	CO_2 and light saturated photosynthesis
kJ	kiloJoule
m	meter
M	molar
MAFF	Ministry of Agriculture, Forestry and Fisheries

MAPK	mitogen-activated protein kinase
min	minute
ml	millilitre
mM	millimolar
mmol	millimoles
mRNA	messenger RNA
MST	monosaccharide transporter
MUP	mitochondrial uncoupling protein
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^+)
NADH-MD	NADH-dependent malate dehydrogenase
NADP-MDH	NADP-dependent malate dehydrogenase
NADP-ME	NADP-malic enzyme
NCBI	National Centre for Biotechnology Information
ng	nanogram
NI	neutral invertase
OAA	oxaloacetate
PCR	photosynthetic reduction cycle
PPase	inorganic pyrophosphatase
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
PEP	phenol*enol*pyruvate
PEPc; PEPC	phospho*enol*pyruvate carboxylase
PFK	6-phosphofructokinase
PFP	pyrophosphate-dependent phosphofructokinase
PGK; PGKase	phosphoglycerate kinase
PGMase	phosphoglucomutase
PPdK	pyruvate orthophosphate dikinase
PRKase	phosphoribulokinase
PSII	photosystem II
R5P	ribose-5-phosphate
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction

R5P	ribose-5-phosphate
R_d	dark respiration
RPE	ribulose phosphate epimerase
RPI	ribose phosphate isomerase
Rubisco	ribulose-1.5-bisphosphate carboxylase/oxygenase
Ru5P	ribulose-5-phosphate
RuBP	ribulose-1,5-bisphosphate
S1,7P	sedoheptulose-1,7-bisphosphate
S6P	sucrose-6-phosphate
S7P	sedoheptulose-7-phosphate
SASRI	South African Sugarcane Research Institute
SBPase	sedoheptulose bisphosphatase
SDS	sodium dodecyl sulphate
SE-CCC	sieve element-companion cell complex
SNF1	sucrose non-fermenting enzyme
SnRK1	SNF1-related kinase
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
SuSy	sucrose synthase
T6P	trehalose-6-phosphate
TCA	tricarboxylic acid cycle
TPI	triose phosphate isomerase
TPP	trehalose phosphate phosphatase
TPS	trehalose phosphate synthase
TKase	transketolase
Trase	trehalase
triose-P	triose phosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine diphosphate
UDPGlc	uridine diphosphate-glucose
UTP	uridine triphosphate
UTP-GD	uridine triphosphate glucose dehydrogenase
UGPase	uridine diphosphate-glucose pyrophosphorylase
UV	ultra-violet
VAI	soluble vacuolar acid invertase
X5P	xylose-5-phosphate

Chapter 1:
General Introduction

1.1 Introduction

Sugarcane (*Saccharum* L. spp. hybrids) is a major crop plant grown in both tropical and subtropical regions throughout the world. The crop occupies an estimated area of 19.6 million hectares worldwide, with a total average annual production of 130 million metric tons of sugar (Clay, 2004). Sugarcane accounts for more than 70% of sucrose produced worldwide (Lunn & Furbank, 1999) and is also a source of molasses, fibre, fertilizer and ethanol for fuel. The South African sugar industry is currently ranked 11th in the world, producing approximately 2.5 million tons of sugar per season and generating an annual estimated direct income of R6 billion (Anon, 2006). Sucrose production and export in South Africa is thus a significant source of revenue, and improved yields through scientific research is an important objective of the local industry.

Sugarcane has several unique characteristics that have resulted in the agricultural success of the crop. The C_4 *Saccharum* genus is classified as a member of the 'tall grasses' family (*Poaceae*). This family includes genera that are amongst the most photosynthetically efficient in the plant kingdom, in that some members are capable of converting up to two percent of incident solar energy into biomass. Previous assessments of the biophysiological capability of the sugarcane stem to accumulate sucrose have estimated that the *Saccharum* complex is potentially capable of storing approximately 30% sucrose on a fresh weight basis (Bull & Glasziou 1963, Moore, 1995; Jackson, 2005). This limit has recently been challenged (Wu & Birch, 2007), indicating that sugarcane culm may be able to attain even higher sugar contents.

During the past century, successful breeding strategies in South Africa have resulted in 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 yields have remained approximately constant since the 1970's. 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 (Grof & Campbell, 2001; Moore, 2005).

In recent decades, improvements in sugarcane agriculture have been achieved almost entirely through increases in cane yield, despite higher heritability of sucrose content (Jackson, 2005). Jeanneau *et al.* (2002) has attributed the yield increase phenomenon observed in C_4 crops to an increase in leaf surface area and not an improved performance of the source photosynthetic apparatus.

Importantly, current yields are still only 65% of the predicted capacity of sugarcane culm tissue (Moore *et al.*, 1997; Jackson, 2005). Therefore, there is considerable incentive for sugar industries to develop and utilise new molecular techniques to act in concert with conventional breeding programs to enhance sucrose accumulation in sugarcane. However, when compared to C_3 plants, only a relatively low number of molecular biologists work on C_4 species, with the view to improving crop performance. In 'model' plants such as *Arabidopsis thaliana* (L.) the wide availability of whole genome, metabolome and proteome data (Gibon *et al.*, 2006; Kolbe *et al.*, 2006) has allowed C_3 research to make enormous progress towards a better understanding of metabolic regulation. Multifaceted online databases, such as genevestigator (https://www.genevestigator.ethz.ch) (Zimmerman *et al.*, 2004), will soon move *Arabidopsis* studies into the advanced realms of systems biology (Trewavas, 2006). In contrast, C_4 species lag behind, with the majority of C_4 research focusing on maize (*Zea mays* L.) (Lunn & Furbank, 1999).

Numerous EST sequencing projects targeting sugarcane have provided the basis for gene discovery and expression profiling studies (Carson & Botha 2002; Casu *et al*. 2004; Ma *et al*. 2004; Vettore *et al*. 2003). Suites of genes, whose expression is correlated with various conditions, have been identified using cDNAs derived from these collections, including sucrose accumulation in the culm (Carson *et al*. 2002; Casu *et al*., 2004; Casu *et al*., 2007; Watt *et al*. 2005), cold stress (Nogueira *et al*. 2003) and methyl jasmonate treatment (Bower *et al*. 2005). A commercial array system consisting of the entire world collection of sugarcane ESTs has recently been compilled by Affymetrix (Casu *et al*., 2007). Furthermore, at least two studies have thus far examined the sugarcane metabolome (Rowher *et al*., 2003; Glassop *et al*., 2006). Thus, good progress is being made towards a better understanding of the regulatory mechanisms involved in sucrose metabolism and accumulation in sugarcane (Moore *et al*., 1997; Casu *et al*., 2004).

1.2 The source-sink relationship

In order to draw level with C_3 plant research, sugarcane researchers will have to work hard to augment their current knowledge base. One of the crucial advances will be a better appreciation of the role of sugarcane leaves in the accumulation of sucrose in the culm. Plant biomass accumulation is a complex feedback process, controlled by both the photosynthetic apparatus (source) and the carbon storage tissues (sinks) (Ho, 1988). The coordinate regulation of both source and sink activity is crucially important for plant growth and may involve fine (substrate and allosteric) and coarse (gene expression) regulation, mediated by specific sugar-signaling mechanisms (Rolland *et al*., 2006). Increasing evidence from several species has highlighted the significant roles of the major transport sugar, sucrose, and hexoses (glucose and fructose) in regulating the interaction between source and sink systems (Pego *et al*., 2000; Rolland *et al*., 2002; Franck *et al*., 2006; Rolland *et al*., 2006).

Sugarcane has evolved as a unique source-to-sink system, in that the major storage tissue is also the growing stalk. Thus accumulated carbon must be partitioned in the culm amongst growth, respiration and storage requirements. Regardless of leaf age or environmental conditions, research has shown that the photosynthetic rate of sugarcane leaves declines as the stalk matures (Amaya *et al*., 1995). However, partial defoliation has been shown to not affect culm sucrose concentrations (Pammenter & Allison, 2002; Gutiérrez-Miceli *et al*., 2004), suggesting that sugarcane leaves are capable of adapting the supply of carbon based on the demand from sink tissues. Furthermore, under conditions of reduced source leaf material (either through shading or defoliation), current commercial sugarcane cultivars exhibit a preference towards sucrose accumulation, at the expense of structural growth (Pammenter & Allison, 2002). The regulatory pathways and physiological feedback limitations involved in these studies have not been examined. In addition, the existence of a carbon-dependent relationship between source and sink tissue in sugarcane is not yet resolved.

1.3 Project aims

i. To determine the relationship between photosynthesis and sugar biosynthesis in the leaf, and the storage of sucrose in the maturing sugarcane culm.

ii. To clarify the regulatory roles of the primary metabolites, sucrose and hexose (glucose and fructose) in mediating the source-sink relationship and ascertain the involvement of putative sugar signaling/sensing mechanisms related to carbohydrate metabolism and photosynthesis in the leaf.

1.4 Broad project objectives

i. To determine if source leaf photosynthetic activity responds to changing demand for carbon from culm sink tissues.

ii. To examine the putative roles of primary sugars, sucrose and hexoses in the regulation of leaf photosynthetic activity.

iii. To analyse changes in expression of genes linked to carbohydrate metabolism and photosynthesis in the leaf with the aim of identifying putative sugar signaling mechanisms.

This novel study has combined the traditional strengths of physiology with the precision of modern molecular biology techniques. The parallel analysis of phenotype and gene transcript expression has provided a powerful methodology for examining the sugar-mediated processes that link leaf photosynthetic activity to sucrose accumulation in sugarcane. The knowledge generated from this project, together with that contained in the international literature, will ultimately be used to devise a strategy to uncouple the signaling pathway that mediates negative feedback between source and sink tissues.

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Chapter 2:
Literature Review

Most higher plants, including sugarcane, fix carbon through photosynthesis and capture it as carbohydrates in the form of sugars and starch. Assimilated carbon is then translocated from source leaves, most often in the form of sucrose, through sieve tubes or vascular bundles to sink organs for growth, development and storage. The metabolic pathways involved are well known; however, a thorough understanding of the mechanisms that regulate metabolic pathways, such as glycolysis and photosynthesis, remains elusive (Benning & Stitt, 2004). Research into both local and long distance control of carbon metabolism, and the integration thereof at the gene, protein and metabolite levels, is ongoing, and a more integrated perception of how plants regulate growth is gradually becoming apparent. The current review will examine present knowledge of the regulation of carbon metabolism using a three-level hierarchical approach. In this scheme, the metabolic pathways of photosynthesis can be considered as the foundation, while carbohydrate metabolism and the numerous associated sensing and signaling pathways act as the regulatory link between source and sink tissues (Fig. 2.1). Using this simplified schematic, the review will present a comprehensive examination of the regulatory mechanisms involved in carbon-related plant growth.

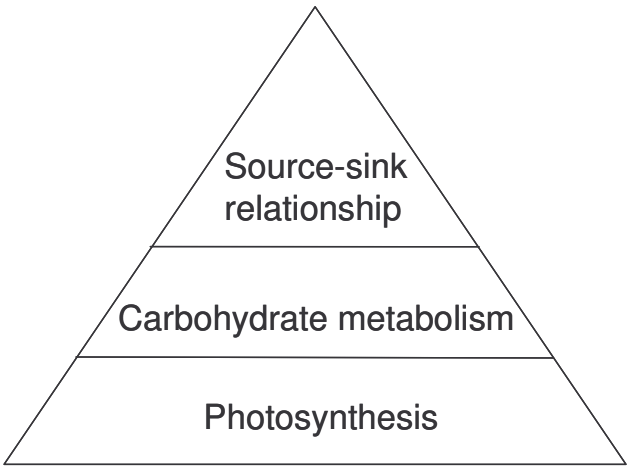

Fig. 2.1 Schematic diagram illustrating the three levels of complexity involved in carbon-related plant growth and which serve to structure Chapter 2.

2.1 Photosynthesis

2.1.1 The C_3 and C_4 pathways

In the vast majority of higher plant species, photosynthetic assimilation of CO_2 occurs by the photosynthetic carbon reduction (PCR) cycle, otherwise known as the 'dark reactions' of photosynthesis (Calvin, 1962). In the initial step of this process, CO_2 is fixed in the chloroplast of photosynthetically-active cells via ribulose-1.5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), and converted into the three-carbon compound 3-phosphoglycerate (3PGA). It is due to these three-carbon compounds that this process is referred to as the C_3 pathway (Fig. 2.2A). In total, the PCR cycle involves eleven enzymes that catalyze a total of 13 reactions, which ultimately return carbon back to regenerate the CO_2-acceptor molecule ribulose-1,5-bisphosphate (RuBP). The PCR cycle is responsible for the supply of triose phosphates (triose-P) derived from 3PGA to the sucrose and starch pathways for basic growth and development (Raines, 2003). In addition, the PCR cycle also supplies numerous pathways in the chloroplast, including the shikimate pathway for biosynthesis of amino acids and lignin, isoprenoid biosynthesis and the precursors for nucleotide metabolism and cell wall synthesis (Lichtenthaler, 1999). Plants that utilize the C_3 pathway are characterised by a single chloroplast type that performs all the reactions that convert light energy into the chemical energy that is used to fix $C0_2$. However, due to the inherent physiological limitations of the C_3 pathway, Rubisco may also catalyze the fixation of O_2 in an energetically wasteful process known as photorespiration, which competes directly with the fixation of CO_2. It is this balance between photorespiration and photosynthesis that largely determines the efficiency with which C_3 species accumulate carbon (Woodrow & Berry, 1988).

In comparison, the C_4 pathway can best be described as a complex adaptation of the C_3 pathway, whereby CO_2 is initially fixed in the leaf by exterior mesophyll cells and then transported to internalised bundle sheath cells. This cyclical reaction effectively suppresses photorespiration by concentrating CO_2 in the bundle sheath cells where photosynthesis occurs exclusively, via the PCR cycle (Hatch & Slack, 1966) (Fig. 2.2B). The key feature of C_4 photosynthesis is the compartmentalisation of activities, typically into these two specialised cells and chloroplast types. In all C_4 plants, CO_2 is initially fixed by phospho*enol*pyruvate carboxylase (PEPc; EC 4.1.1.31) in the cytosol of mesophyll cells to form the 4-carbon dicarboxylic acid oxaloacetate (OAA) (hence the

name, C_4). 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 then refixed via Rubisco in the PCR 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 (NADPME-type); (ii) a mitochondrial NAD-malic enzyme (NADME-type) and (iii) a cytosolic PEPc that produces phospho*enol*pyruvate (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 aspartate, which is converted to OAA by transamination.

Plants that photosynthesize via the C_4 pathway include a variety of important crop plants, including maize, sorghum (*Sorghum bicolor* L.) and sugarcane, a large portion of the grass family (*Poaceae*) (David & Soreng, 1993), and, in addition, many species of problematic weeds (Edwards & Huber, 1981). Leaves of C_4 plants are typically distinguished by extensive vascularisation (Kranz anatomy), where a ring of bundle sheath cells surrounds each vein and an outer ring of mesophyll cells surrounds the bundle sheath. By virtue of this arrangement, the mesophyll cells have been described as 'biochemical pumps' which concentrate CO_2 in the bundle sheath, creating an estimated ten-fold increase over atmospheric concentrations (± 3.7 mmol mol^{-1}) (Jenkins *et al.*, 1989). Kranz anatomy is not only credited with suppressing the oxygenase activity of Rubisco, but it also permits the PCR cycle to function more effectively under conditions of increased light and temperature (Hatch 1987). However, some photorespiration does still occur in C_4 species, albeit at a suppressed level, specifically under conditions of high O_2, low CO_2, and high temperature (Dai *et al.*, 1993; Maroco *et al.*, 1998).

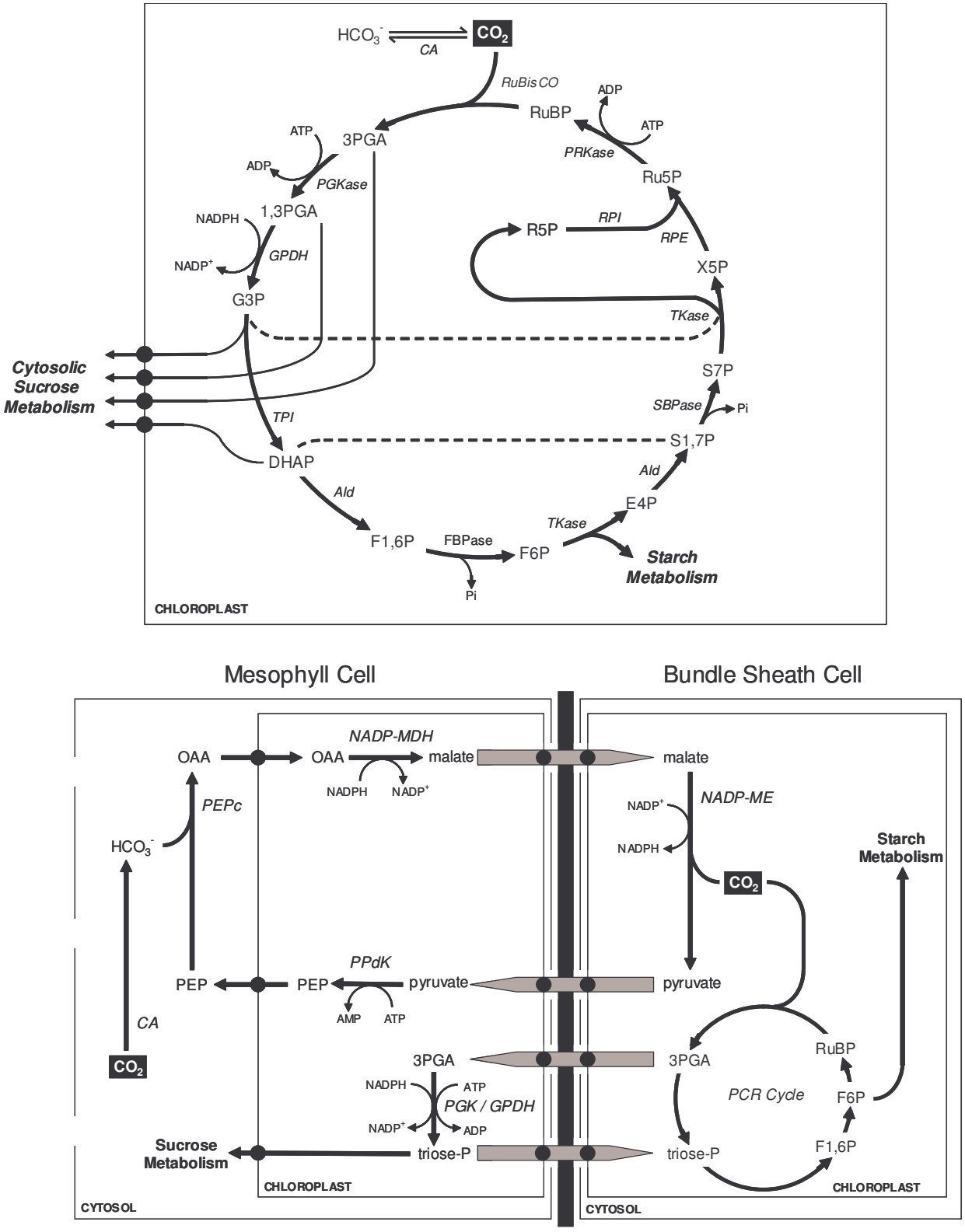

Fig. 2.2. Representations of the photosynthetic reduction (PCR) cycle in C_3 plants (A) and the C_4 photosynthetic pathway in NADPME-types (B). Filled circles represent membrane transporters (Further legend details provided overleaf).

Fig. 2.2. (*Legend continuation from previous page*)

(A) The PCR cycle, highlighting the metabolites and enzymes (italics) involved in sucrose and starch metabolism, and ribulose-1,5-bisphosphate (RuBP) regeneration. Carbon dioxide (CO_2) released by carbonic anhydrase (CA; EC 4.2.1.1) is initially fixed by Rubisco (EC 4.1.1.39) to produce 3-phosphoglycerate (3PGA), which is phosphorylated to 1,3-bisphosphoglycerate (1,3PGA) and reduced to glyceraldehyde-3-phosphate (G3P) by phosphoglycerate kinase (PGKase; EC 2.7.2.3) and glyceraldehyde phosphate dehydrogenase (GPDH; EC 1.2.1.13) respectively. Triose phosphate isomerase (TPI; EC 5.3.1.1) then catalyzes the conversion of G3P to dihydroxyacetone phosphate (DHAP). These four triose-phosphate (triose-P) compounds (3PGA, 1,3PGA, G3P and DHAP) can be exported from the chloroplast (in exchange for inorganic phosphate) for sucrose synthesis in the cytosol. Alternatively, aldolase (Ald; EC 4.1.2.13) catalyzes DHAP to fructose-1,6-bisphosphate (F1,6P) and fructose bisphosphatase (FBPase; EC 3.1.3.11) catalyzes F1,6P to fructose-6-phosphate (F6P). F6P can be retained in the chloroplast for starch synthesis, or used for RuBP regeneration. Transketolase (TKase; EC 2.2.1.1) catalyzes F6P to erythrose 4-phosphate (E4P), Ald catalyzes E4P to sedoheptulose 1,7-bisphosphate (S1,7P) and sedoheptulose bisphosphatase (SBPase; EC 3.1.3.37) catalyzes S1,7P to sedoheptulose 7-phosphate (S7P). TKase then splits S7P (seven carbons) to form ribose-5-phosphate (R5P) (5 carbons) and xylose-5-phosphate (X5P) (2 carbons + G3P). R5P is catalyzed to ribulose-5-phosphate (Ru5P) by ribose phosphate isomerase (RPI; EC 5.3.1.6), while X5P is catalyzed to Ru5P by ribulose phosphate epimerase (RPE; EC 5.1.3.1). Ru5P is then phosphorylated by phosphoribulokinase (PRKase; EC 2.7.1.19) to give RuBP.

(B) The CO_2-concentrating mechanism of C_4 plants using the NADPME-type pathway, emphasizing the compartmentation of enzymes between mesophyll and bundle sheath cell types. In the outer mesophyll cytosol, CO_2 is converted by CA to HCO_3^- that is then fixed in the form of OAA by phosphoenolpyruvate carboxylase (PEPc; EC 4.1.1.31). OAA is reduced in the mesophyll chloroplast to form malate by NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82) and transported to chloroplast of the inner bundle sheath. Malate is then decarboxylated by NADP-malic enzyme (NADP-ME; EC 1.1.1.40) to produce CO_2 and pyruvate. Phosphoenolpyruvate (PEP) is regenerated from pyruvate by pyruvate orthophosphate dikinase (PPdK; EC 2.7.9.1) in the mesophyll chloroplast, while CO_2 is fixed by Rubisco in the PCR cycle within the bundle sheath chloroplast. In addition, 3PGA may be shuttled back to the mesophyll for reduction to triose-P, which can be used for sucrose synthesis (in the mesophyll cytosol) or recycled to the bundle sheath chloroplast.

The significance of the characteristic C_4 Kranz anatomy has recently been brought into question, as evidence suggests that C_4 photosynthesis can operate within a single photosynthetic cell in certain aquatic and terrestrial plants (Voznesenskaya *et al.*, 2001; Leegood, 2002). Such cellular systems seem to be unique for each species, yet are still expected to consist of highly organised internal compartmentations with low CO_2 permeability to prevent leakage from the site of CO_2 release (Leegood, 2002). In aquatic *Orcuttia* species, this is achieved through the centripetal arrangement of mesophyll chloroplasts, where the chloroplasts are placed at the greatest distance from the outside environment (Keeley, 1998). In contrast, the CO_2-concentrating mechanism in the terrestrial plant *Borszczowia aralocaspica* has distinct cellular di-morphism, with NAD-malic enzyme located in the mitochondria at one end of the cell while PEP is distributed throughout the cytosol (Voznesenskaya *et al.*, 2001). Many questions remain to be answered concerning single cell C_4 species and their evolution compared to the Kranz anatomical structure, including whether they may provide useful models for engineering C_4 characteristics into C_3 plants (Leegood, 2002).

Fossil records date the explosion of C_4 plant biomass at some six to eight million years ago, when CO_2 concentrations fell to about 200 μmol mol^{-1} (Edwards *et al.*, 2001). Under the low CO_2 and the warmer periods of the Palaezoic, the penalty for photorespiration was exacerbated and provided C_4 species a considerable competitive edge over their C_3 counterparts (Sage & Monson, 1999). The subsequent evolutionary success of the C_4 photosynthetic pathway can be accredited to improved water use efficiency (WUE), nutrient efficiency and an increased photosynthetic capacity at high temperature (Leegood, 2002). The four basic requirements for a typical C_4 plant leaf can be summarized as follows: (i) cell-specific location of enzymes required for C_4 photosynthesis (i.e. PEPc in the mesophyll, and Rubisco in bundle sheath cells) with complementary adjustments of photosystem and electron transport activities; (ii) barriers to resist the diffusion of CO_2 between the site of fixation by PEPc and sites of CO_2 release and fixation by Rubisco; (iii) a novel set of cell-specific organelle metabolite translocators; and (iv) a system of symplastic connectors between the spatially separated sources and sinks of the 4-carbon acid transport metabolites (Edwards *et al.*, 2001).

2.1.2 Regulation of C_3 photosynthesis

The sequence of reactions in the PCR cycle of C_3 plants has been known for some time, although the mechanisms that regulate and co-ordinate enzyme activity are still not fully understood. However, research over the past 15 years using transgenic tools, such as "antisense" RNA technology, has brought to light a variety of novel regulatory aspects regarding this important pathway (for detailed review see Raines, 2003). The primary advantage of the RNA antisense technique is the precision with which the activity of a single enzyme can be manipulated *in vivo*, without significantly altering the levels of other cellular components (Furbank *et al.*, 1996).

The highly regulated Rubisco enzyme has been previously hypothesized to catalyze the rate-limiting step in photosynthesis (Farquhar & von Caemmerer, 1982) and various transgenic studies have shown that Rubisco activity exerts high control over photosynthetic carbon flux (Quick *et al.*, 1991; Hudson *et al.*, 1992). Under conditions of high light and ambient CO_2, Rubisco is a limiting enzyme in C_3 plants (Furbank *et al.*, 1996). However, under varying and controlled light conditions, the high flux control co-efficient of sedoheptulose bisphosphatase (SBPase; EC 3.1.3.37) has more recently provided convincing evidence that SBPase *in vivo* is also a major determinant of photosynthetic capacity in C_3 systems (Harrison *et al.*, 1998; Olcer *et al.*, 2001; Raines *et al.*, 2000). Consequently, Rubisco and SBPase appear to be the dominant factors influencing overall carbon assimilation rates in the PCR cycle.

In addition, transketolase (TKase; EC 2.2.1.1) and aldolase (Ald; EC 4.1.2.13), have both been shown to potentially contribute to regulating carbon flux through the PCR cycle (Haake *et al.*, 1998, 1999; Henkes *et al.*, 2001), suggesting that these enzymes may fall into a 'co-limiting' category. Other enzymes previously thought to be 'co-limiting' include glyceraldehyde phosphate dehydrogenase (GPDH; EC 1.2.1.13), fructose bisphophatase (FBPase; EC 3.1.3.11) and phosphoribulokinase (PRKase; EC 2.7.1.19) (Furbank and Taylor, 1995); however, more recent data have discounted any significant regulatory roles of these enzymes based on their low flux control co-efficients (Raines, 2003). Similarly, the chloroplastic enzyme carbonic anhydrase (CA; EC 4.2.1.1) also falls into the 'non-limiting' category (Price *et al.*, 1994).

Transgenic efforts to manipulate the rate-limiting PCR cycle enzymes *in vivo* have yielded exciting prospects for future metabolic modifications in C_3 plants. For example, expression of a bi-functional cyanobacterial Ald/SBPase enzyme in transgenic tobacco (*Nicotiana tabacum* L.) has recently resulted in plants with a higher photosynthetic capacity and increased height and dry weight (Miyagawa *et al.*, 2001). Furthermore, Raines (2003) has reported tobacco expressing an *Arabidopsis* SBPase with increased photosynthetic capacity and plant biomass. Such results are promising and indicate that increased yields in C_3 plants through a single manipulation in the PCR cycle may soon be realised.

2.1.3 Regulation of C_4 photosynthesis

Beyond the complexity of the PCR cycle, determining which enzymes regulate flux in C_4 photosystems is complicated by the cellular localisation of particular enzymes (Furbank & Taylor, 1995). Thus, although the pathways of C_4 photosynthesis have been resolved (Hatch, 1987), much less progress has been made in unraveling the regulatory events that govern C_4 photosynthetic rates (Brown *et al.*, 2005). Very little is known about how C_4 photosynthetic cell differentiation occurs, however, the position of cells relative to the developing vein appears to determine their fate as either bundle sheath (closely associated to the vein) or mesophyll (at least one cell away from the vein) cells (Nelson & Langdale, 1992). The key regulatory enzymes of the NADPME-type C_4 pathway are shown in Fig. 1B. PEPc, NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82) and pyruvate orthophosphate dikinase (PPdK; EC 2.7.9.1) are located in the mesophyll cells with PEPc in the cytosol and NADP-MDH and PPDK in the chloroplast, while NADP-malic enzyme (NADP-ME; EC 1.1.1.40) is found in the chloroplast of bundle sheath cells (Jeanneau *et al.*, 2002). Furthermore, the gene expression and enzyme activities of the three mesophyll enzymes *in vivo* have been shown to increase markedly under illumination (Hatch, 1987), which suggests that these enzymes are potentially rate limiting.

NADP-MDH has since been demonstrated to be a light-regulated enzyme that undergoes reductive activation in the light via photosynthetic electron transfer and the thioredoxin system (Buchanan, 1991). The enzyme is activated through thioredoxin by the reduction of a disulphide bridge between two cysteine residues, and using site-directed mutanogenesis, this pair of cysteine residues has been identified in the

sorghum NADP-MDH enzyme (Issakidis, 1994). To assess the role of light activation of NADP-MDH, mutant forms of NADP-MDH that are not inactivated through oxidation, and thus are not inactivated in the dark, have been transformed into the C_4 dicot *Flaveria bidentis* (L.) and tobacco (Furbank & Taylor, 1995). Furthermore, suppression of NADP-MDH activity using genetic co-suppression in transgenic *Flaveria* was investigated to determine its effect on C_4 photosynthetic flux (Trevanion *et al*., 1997). Under high light, NADP-MDH activity could be reduced to 10% with little effect on carbon assimilation. At low light even the most severely suppressed lines showed no change, however, the activation state of NADP-MDH did increase accordingly to compensate for the reduced amounts of available enzyme. Thus although NADP-MDH is far from limiting regarding photosynthetic flux, its activity seems to correlate closely with photosynthesis at different irradiances, confirming that the enzyme is subject to sophisticated control of its activation state (Edwards *et al*., 2001).

PEPc is the primary CO_2-fixing enzyme of the C_4 pathway and its activity is regulated by numerous metabolic effectors, including malate (feedback inhibition) glucose-6-phoshate (G6P; activation), and a highly complex, light-dependent reversible phosphorylation process (Bakrim *et al.*, 1993). The latter reversible phosphorylation process is performed by a protein-Ser kinase (PEPcK) (Bakrim *et al.*, 1993), which alters the functional and regulatory properties of PEPc. PEPcK effectively confers PEPc with decreased sensitivity to malate, and increased activity under suboptimal levels of pH (7.3) and substrate (PEP; 2.5 mM), while also increasing V_m and K_a for G6P (Chollet *et al*., 1996). In *Amaranthus edulis* (L.), transgenically reduced PEPc transcription resulted in an increase in PEPc phosphorylation status (Dever *et al*., 1997), suggesting that *Amaranthus* has the capacity to adapt to a reduction in PEPc availability by modulating the activity of the enzyme. Furthermore, in both sorghum and maize, blocking PEPcK produced a marked inhibition of CO_2 assimilation, indicating that phosphorylation is a crucial event governing the mechanism of the C_4 pathway (Bakrim *et al*., 1993). Thus, although a low control coefficient is hypothesized for PEPc (Furbank *et al*., 1997), this measurement may not represent a realistic indication of its overall role in photosynthetic flux control.

Various attempts have been made to manipulate the level of PEPc in C_4 plants (Jeanneau et al., 2002), and furthermore, to introduce C_4 PEPc into several C_3 species, including rice (*Oryza sativa* cv. Kitaake), potato (*Solanum tuberosum* L.) and tobacco

(Kogami *et al.*, 1994; Gehlen *et al.*, 1996; Agarie *et al.*, 2002). For C_4 plants, PEPc activity has been linked to the control of carbon flux in maize under water stressed conditions (Rodriguez-Penagos & Munoz-Clares, 1999). Based on this observation, transgenic studies either under- and over-expressing a sorghum PEPc in maize have showed a significant improvement in CO_2 fixation rates and a 30% improvement in WUE under moderate drought conditions (Jeanneau *et al.*, 2002). Elevated values of WUE were accounted for by the capacity of the plants to better fix CO_2 when the stomatal gas conductance was reduced by water-limiting conditions (Jeanneau *et al.*, 2002).

In C_3 plants, PEPc is not involved in photosynthesis, but, instead, participates in the anaplerotic pathway to replenish the citric acid cycle with C_4 acids during amino acid synthesis (via the Go/GOGAT cycle) and protein synthesis (Stitt, 1999). However, PEPc is also considered to be involved in the refixation of CO_2 evolved by mitochondrial respiration (Wirth *et al.*, 1977), such that increased PEPc activity in leaves might contribute to a decrease in respiratory CO_2 loss (Agarie *et al.*, 2002). Attempts to transgenically confer C_4 charcteristics to improve yields in the C_3 plants, through the addition of C_4 PEPc, have thus far provided the most tantalizing results in rice, where a 20% reduction in photosynthetic O_2-inhibition was observed in plants expressing the C_4-specific maize PEPc (Ku *et al.*, 1999). It was concluded that C_3 plants possess the necessary genetic machinery to express high levels of C_4 specific genes, and that the observed reduction in O_2-inhibition was in part due to direct fixation of atmospheric CO_2 by the maize PEPc. However, further analyses by Agarie *et al.* (2002) suggested that the latter conclusion was not the case, as the observed reductions in O_2-inhibition were linked to lower carbon assimilation rates that were likely due to reduced levels of available inorganic phosphate (P_i), owing to a lower expression of sucrose synthesis-related enzymes in the PEPc transformants. Furthermore, increased PEPc activity was correlated with increased respiration, due to an enhanced flux of malate into the citric acid cycle (Agarie *et al.*, 2001); a phenomenon that has also been observed in transgenic potatoes (Gehlen *et al.*, 1996).

PPdK is responsible for regenerating PEP for PEPc, and similar to PEPc and NADP-MDH, it has also long been recognized as a light-activated enzyme (Hatch, 1987). However, in contrast to PEPc, PPdK is inactivated by phosphorylation (Ashton *et al.*, 1984). The enzyme that regulates phosphorylation of PPdK (the PPdK regulatory protein) catalyzes both the de-phosphorylation of the inactive enzyme and the reverse

reaction. The PPdK regulatory protein is unusual, as ADP and not ATP is used as a phosphate donor (Burnell *et al.*, 1986). Although it is unclear how the PPdK regulatory protein is controled, high pyruvate levels appear to block inactivation (Burnell *et al.*, 1986). Due to a low maximum extractable activity, PPdK was previously implicated as having an important role in photosynthetic flux (Hatch, 1987). More recently however, transgenic analysis of *Flaveria* with suppressed levels of PPdK has showed that, even at high irradiances, PPdK is not dominant in controlling photosynthetic rates (Furbank *et al.*, 1997). With a control coefficient of 0.3, PPdK should only be considered a 'co-limiting' enzyme.

The regulatory role of enzymes in the PCR cycle of C_4 plants are not necessarily comparable to C_3 species, due to the compartmentation of the PCR cycle in C_4 plants and the physiology of the C_4 CO_2-concentrating mechanism. For example, the degree to which Rubisco limits photosynthetic rates in C_4 plants may be quite different to C_3 species, where the enzyme is confirmed as rate-limiting (Stitt & Schulze, 1994). Rubisco levels in C_4 plants are up to 50% lower than C_3 species, which is likely due to lower Rubisco requirements, owing to the effectiveness of the 'CO_2 pump' system in C_4 species (Hatch, 1987), and possibly due to a restriction of activity to the leaf bundle sheath (Dengler & Nelson, 1999). Nevertheless, antisense reduction of Rubisco activity in *Flaveria* has confirmed that, under a wide range of CO_2 concentrations and light levels, Rubisco activity exerts a high control over photosynthetic carbon flux (Furbank *et al.*, 1996). Even slight decreases in Rubisco have resulted in a substantial reduction in photosynthetic rates, indicating that, despite the high CO_2 levels attained in C_4 bundle sheath cells, there is sufficient Rubisco in *Flaveria* leaves to support maximum rates of photosynthesis (Furbank *et al.*, 1996). Reduced Rubisco levels were further linked to a decrease in activity of enzymes involved in the mesophyll C_4 cycle (Furbank *et al.*, 1996), suggesting the existence of a signaling system that coordinates both C_3 and C_4 cycles. The high control of C_4 photosynthesis by Rubisco, as well as a reduced capacity to alter Rubisco levels relative to C_3 species, was further shown to be a disadvantage at lower temperatures, and is perhaps symptomatic of the deficiency of C_4 species to inhabit cooler climates (Kubien *et al.*, 2003).

The elusive regulatory mechanisms involved in C_4 photosynthesis are slowly being unravelled, such that our understanding of this complex system is growing steadily. However, based on current knowledge, increased photosynthetic rates and crop yields

through direct transgenic manipulation of the C_4 photosynthetic pathway are unlikely at present. The complexity and abundance of metabolic regulatory mechanisms associated with Rubisco and PPdK, as well as other C_4-related enzymes, are indicative of the evolved optimization of the plant leaf photosynthetic machininery. The two enzymes which exert the most control over carbon flux, Rubisco and PPdK, are also the most expressed in the bundle sheath and mesophyll cells, respectively, thus the over-expression these genes with current technologies would not be a trivial endeavour (Furbank *et al.*, 1997).

2.2 Sugar metabolism: regulation and signaling mechanisms in the sucrolytic pathway

2.2.1 Introduction

In the majority of higher plant species, sucrose is the end-product of photosynthesis. Sucrose is the dominant long-distance transport form of carbon and energy and has also been shown to be a major participant of an intricate signaling network that regulates overall plant growth and development (Winter & Huber, 2000; Rolland *et al.*, 2002; Lunn & MacRae, 2003; Koch, 2004; Gibson, 2005). Considerable research has been focused on the mechanisms that control plant sucrose metabolism, and only now is the complexity of this system emerging. This chapter aims to highlight current knowledge of the regulatory points in this central metabolic pathway and to examine the signaling roles played by the metabolites involved, including sucrose and hexose.

Sucrose synthesis is essentially the same in both C_3 and C_4 plants and is restricted to the cytosol by compartmentation of key enzymes (Winter & Huber, 2000). In C_4 species, further compartmentation exists at the cellular level, with sucrose synthesis being located primarily in the leaf mesophyll cells (Lunn & Furbank, 1999). Typically, sucrose is initially synthesised in leaf tissue from triose-P exported out of the chloroplast in exchange for P_i during the day, while at night, starch mobilisation provides the substrate for sucrose biosynthesis, probably in the form of glucose which is derived from the amylolytic breakdown of starch (Schleucher *et al.*, 1998). During the day, several key metabolic control points exist in the sucrolytic pathway (Fig. 2.3), which are discussed in the following sections.

2.2.2 Fructose bisphosphatase

During the active photosynthetic period, triose-P dihydroxyacetone phosphate (DHAP) is converted to fructose-1,6-bisphosphate (F1,6P) by Ald and subsequently hydrolysed to form fructose 6-phosphate (F6P) by either the cytosolic FBPase isozyme (EC 3.1.3.11) or pyrophosphate-dependent phosphofructokinase (PFP; EC 2.7.1.90). While FBPase is considered to be an important light-dependent control point in the pathway (Stitt *et al.*, 1987; Quick, 1996), studies on PFP down-regulation in potato and tobacco suggest that PFP does not play an essential role in sucrose production (Hajirezaei *et al.*, 1994; Paul *et al.*, 1995).

As the principle irreversible step towards the eventual synthesis of sucrose, cytosolic FBPase activity is subject to a uniquely complex allosteric regulatory system (Stitt *et al.*, 1987; Quick, 1996). The activity of the enzyme is inhibited by decreases in the ratio of F1,6P to P_i and the localised levels of AMP (Stitt *et al.*, 1982). However, the inhibitory capacity of these two factors on FBPase is further regulated by the concentration of fructose 2, 6-bisphosphate (F2,6P). F2,6P has garnered much research interest over the past 25 years as a key signaling molecule (Sabularse & Anderson, 1981; Stitt, 1990, Nielsen *et al.*, 2004).

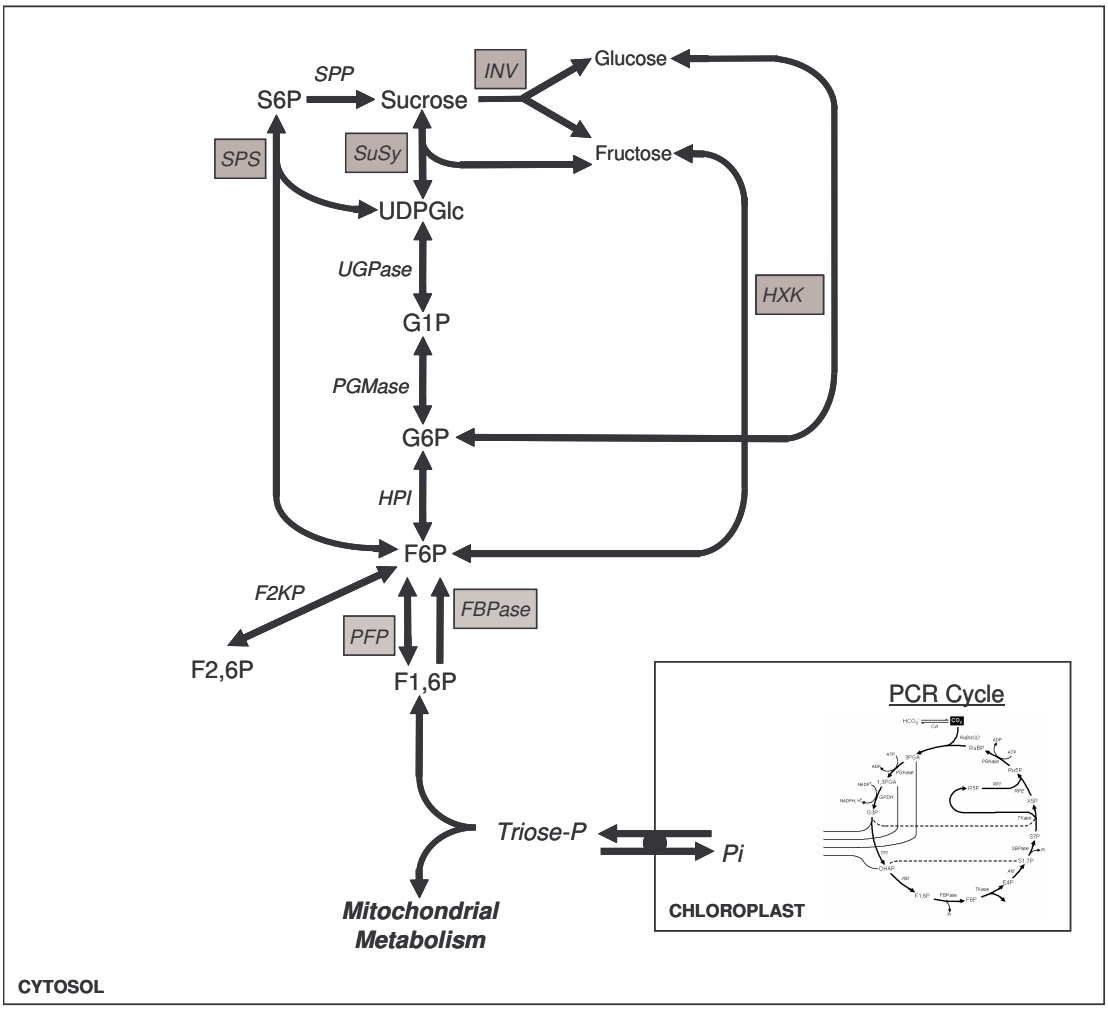

Fig. 2.3. Sucrose synthesis in the cytosol following export of triose-phosphate sugars from the chloroplast. Enzymes shown in italics are cytosolic fructose bisphosphatase (FBPase; EC 3.1.3.11), pyrophosphate-dependent phosphofructokinase (PFP; EC 2.7.1.90), the bifunctional fructose-2,6-bisphosphate (F2,6P) regulating protein F2KP (Nielsen *et al*. (2004)), hexose-phosphate isomerase (HPI; EC 5.3.1.9), phosphoglucomutase (PGMase; EC 5.4.2.2), uridine diphosphate-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) sucrose phosphate synthase (SPS; EC 2.4.1.14), sucrose phosphate phosphatase (SPP; EC 3.1.3.24), sucrose synthase (SuSy; EC 2.4.1.13), hexokinase (HXK; EC 2.7.1.1). Metabolite abbreviations indicate: triose-phosphates (triose-P), fructose-1,6-bisphosphate (F1,6P), fructose-2,6-bisphosphate (F2,6P), fructose-6-phosphate (F6P), glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), uridine diphosphate-glucose (UDPGlc) and sucrose 6-phosphate (S6P).

The regulation of F2,6P is catalyzed by a unique bifunctional enzyme which harbors both synthesis (fructose-6-phosphate,2-kinase (F6P,2K; EC 2.7.1.105)) and degradation (fructose 2,6-bisphosphatase (F26BPase; EC 3.1.3.46)) activities, and has subsequently been termed F2KP by Nielsen *et al*. (2004). The overall activity of F2KP is regulated by the levels of several primary metabolic intermediates, including triose-P, F6P, and P_i (Stitt, 1990). As photosynthesis increases, triose-P export from the chloroplast reduces F2,6P levels by inhibiting F6P,2K activity, while the decline of cytosolic P_i simultaneously stimulates F26BPase and inhibits F6P,2K (Stitt *et al*., 1987). However, at the end of the photoperiod, an increase in cytosolic hexose phosphates, resulting from a decrease in light-dependent sucrose-phosphate synthase (SPS; EC 2.4.1.14) activity, leads to an elevation in F2,6P levels. This results from the activation and inhibition of F26BPase by F6P and F6P,2K, respectively (Cseke & Bechana, 1983). In this way, the F2,6P system acts to depress sucrose production from triose-P in the dark and thus protects the metabolite levels of the glycolytic pathway and Calvin cycle from depletion, while also allowing sucrose synthesis and the recycling of P_i to be activated as photosynthate becomes available (Stitt *et al*., 1987).

More recent studies have examined the regulation of the two components of the bifunctional F2KP in more detail, and have shown that pyrophosphate (PP_i) and PEP strongly inhibit F6P,2K activity, while 6-phosphogluconate and F1,6P inhibit F26BPase activity (Villadsen & Nielsen, 2001; Markham & Kruger 2002). Furthermore, pyruvate has been shown to activate F6P,2K and inhibit F26BPase activities for F2KP in *Arabidopsis* (Villadsen & Nielsen, 2001). Although the overall impact of these metabolites on F2KP *in vivo* has yet to be observed, the multitude of regulatory factors involved does suggest a novel control system that operates under circumstances other than those of photosynthetic metabolism (Nielsen *et al*., 2004). Interestingly, transgenic studies on potato with reduced FPBase levels have lower levels of F2,6P (Zrenner *et al*., 1996), suggesting a counteracting effect to release the low levels of FBPase from inhibition. Thus, although photosynthetic rates were still depressed, plants retained normal growth rates. While the molecular basis of F26P regulation is understood, the phosphoregulation of FP2K, and a proper understanding of the interactions between FBPase and F2KP activity remain to be fully elucidated (Nielsen *et al*., 2004).

2.2.3 Pyrophosphate-dependent phosphofructokinase

The importance of PFP as an additional component of metabolic regulation at the F16P/F6P interconversion point remains a point of contention. Due to the low overall levels of PFP measured in maize leaves plus the decline in PFP activity throughout leaf development, it was initially suggested that the enzyme was not a major regulatory component of sucrose production during photosynthesis (Kruger *et al.*, 1986). PFP is highly sensitive to F2,6P, which can lead to increased activity in both the sucrolytic and glycolytic directions (Podestà & Plaxton, 2003). It is still not understood why PFP does not interfere with the regulation exerted over FBPase, or what further metabolic effectors may be involved (Nielsen *et al.*, 2004).

A likely physiological role suggested for PFP is as a fine regulator of glycolysis, gluconeogenesis or in PP_i formation or removal (Stitt, 1990). Certain CAM species do exhibit an increased activity of PFP during gluconeogenic carbon flux (Fahrendorf *et al.*, 1987). Additionally, in barley leaves, 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 P_i deficiency, anoxia, or where adenosine triphosphate (ATP) conservation is advantageous (Duff *et al.*, 1989; Mertens *et al.*, 1990; Perata & Alpi, 1993).

In sugarcane tissue, PFP has been suggested to have a more important function in plant carbohydrate metabolism (Heldt, 1997; Suzuki *et al.*, 2003), and furthermore to play a key role in the process whereby sugarcane adjusts its growth as a function of sucrose synthesis, export, import and utilisation (Groenewald & Botha, 2001; Suzuki *et al.*, 2003). Recently, transgenic sugarcane with reduced PFP levels was shown to have an increased culm sucrose content (Groenewald & Botha, 2007), perhaps suggesting a preference towards sucrose synthesis instead of glycolysis in these plants. Even a slight adjustment to the increase of sucrose flux would be favourable in sugarcane culm, and similar small changes have been detected in tobacco and potato (Nielsen & Stitt, 2001; Hajirezaei *et al.*, 1994).

2.2.4 Sucrose phosphate synthase

The first step in the committed pathway of sucrose synthesis begins with F6P and UDP-glucose, where SPS catalyses the synthesis of sucrose-6-phosphate (S6P), which is then irreversibly hydrolysed to P_i and sucrose by sucrose-phosphatase (SPP; EC 3.1.3.24). P_i is then recycled back to the chloroplast for the continued supply of triose-P to the cytosol (Lunn & ap Rees, 1990). As the 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), SPS activity is thought to contribute to the control of flux into sucrose (Fernie *et al.*, 2002). The expression of SPS is regulated by developmental, environmental and nutritional signals and, at least in some cases, at the translational level (Winter & Huber, 2000). Furthermore, it has been demonstrated that SPS activity is inversely proportional to starch synthesis (Silvius *et al.*, 1979; Huber & Isreal 1982), and thus SPS may also play an inadvertent role in regulating starch formation (Kerr *et al.*, 1984; Fernie *et al.*, 2002).

The expression of genes coding for SPS in maize and sugarcane are both light-dependent and developmentally regulated (Cheng *et al.*, 1996a; Sugiharto *et al.*, 1997), while the activity of the enzyme has been shown to be phosphoregulated by multiple serine protein kinases and phosphatases in a variety of species (Lunn & Furbank, 1997; Winter & Huber, 2000; Lunn & MacRae 2003). Phosphorylation has been implicated in the diurnal modulation of SPS activity in both soybean (*Glycine max* L.) and tomato (*Lycopersicon esculentum* Mill.), although the periods of fluctuation do differ between the species (Kerr *et al.*, 1985; Jones & Ort, 1997). Furthermore, there is evidence, based on an SPP binding site on SPS, for an association between SPS and SPP (a putative SPS-SPP complex) that might involve channeling of S6P (Echeverria *et al.*, 1997; Lunn *et al.*, 2000). A further complex between SPS and UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) has also been suggested (Toroser *et al.*, 1998; Winter & Huber, 2000). However, the composition, activity and regulation of such complexes remain to be determined (Lunn & MacRae, 2003).

Thus far, SPS protein kinases comprise two types: an SNF1-related protein kinase (SnRK1), which is so named due to a similarity to the sucrose non-fermenting 1 (SNF1) protein kinase from yeast, and a calmodulin-like protein kinase (CPDK) (Winter & Huber, 2000). A detailed study by Huang and Huber (2001) has shown that SPS from dicots is

primarily acted on by SnRK1, whereas both SnRK1 and CPDK could phosphorylate SPS from monocots. Transgenic studies have further revealed that a maize SPS gene expressed in tomato plants was active (Worrell *et al.*, 1991). However, in similar transgenic tobacco plants the enzyme was inactivated (Stitt & Sonnewald, 1995), suggesting that the endogenous tobacco SPS protein kinases were capable of phosphorylating maize SPS, but tomato protein kinases were not. This may be indicative of the importance of other amino acid residues around the phosphorylation site and at least some species specificity of the recognition sites of SPS for SPS protein kinases. In spinach (*Spinacia oleracea* L.), SPS protein kinases have been found to be inhibited strongly by G6P, while maize leaf SPS protein kinases are reported to be strictly Ca^{2+}-dependent, suggesting that changes in cytosolic Ca^{2+} concentration might be involved in a signaling transduction pathway (McMichael *et al.*, 1995). Phosphorylation of a unique serine site on SPS has also been reported for spinach leaves under osmotic stress (Huber & Huber, 1992).

More recent examinations of the complexity of expression of different SPS isoforms within a single species (Komatsu *et al.*, 1999; Langenkamper *et al.*, 2002; Chen *et al.*, 2005) revealed the need to re-examine certain previous interpretations of the enzyme, due to the assumption of 'uniformity' for SPS activity or expression levels being measured. It is now known that at least three families of SPS exist, with monocot/dicot divisions within each family, and it is predicted that at least one member of each family is expressed in an individual plant, although one isoform may predominate (Lunn & Macrae, 2003). For example, a reassessment of work done by Worrel *et al.* (1991) might show that maize SPS is less likely to be down-regulated by protein phosphorylation if it comes from a different family to the predominant form in the target species. Furthermore, several species have been shown to express at least two isoforms for SPP (Lunn *et al.*, 2000; Lunn, 2003), although the reasons for such multiplicity remain to be discovered. Future work on SPS and SPP will need to take the diversity of their isoforms into account.

2.2.5 Sucrose synthase

Several recent reviews have discussed the hypothesis that sucrose metabolism lies at the heart of a sensitive, self regulatory developmental system in plants, such that sucrose and its constituents (glucose and fructose) act not only as the substrates in

carbon metabolism, but also as signaling molecules for regulating several aspects of primary plant metabolism and growth (Loretti *et al.*, 2001; Rolland *et al.*, 2002; Koch, 2004; Gibson, 2005). If such is the case, then the control of this system will begin with the synthesis and degradation of sucrose. The cleavage of sucrose may proceed by one of two enzymatic pathways. The non-reversible invertase (EC 3.2.1.26) reaction produces glucose and fructose via sucrose hydrolysis. Alternatively, the cleavage of sucrose into fructose and uridine diphosphate-glucose (UDPGlc) may be catalyzed by sucrose synthase (SuSy; EC 2.4.1.13). The difference between these two reactions is significant, as cleavage by invertase comparably produces twice as many hexoses, while the products of SuSy initiate the fewest hexose-based signals (Wobus & Weber, 1999; Koch 2004). Such signals may be detrimental to tissues undergoing differentiation or maturation, and SuSy has been shown to predominate over invertase during such stages of organ development (King *et al.*, 1997; Wobus & Weber, 1999; Fernie & Willmitzer, 2002). The SuSy reaction is reversible, and there is also clear evidence from feeding experiments with labelled sugars that both SPS and SuSy pathways contribute to sucrose synthesis (Geigenberger & Stitt, 1991). It is suggested that the combined operation of these pathways with the invertase degradative pathway allows the cell to respond sensitively to both variations in sucrose supply and cellular demand for carbon for biosynthetic processes (Geigenberger *et al.*, 1997).

The crucial role of SuSy in carbon partitioning has been demonstrated in several species, including potato, tomato, carrot (*Daucus carota* L.), cotton (*Gossypium hirsutum* L.) and sugarcane (Zrenner *et al.*, 1995; D'Aoust *et al.*, 1999; Sturm & Tang, 1999; Botha & Black, 2000; Ruan *et al.*, 2003). SuSy is implicated in several important physiological process including starch synthesis (Nguyen-Quoc & Foyer, 2001), cell wall synthesis (Déjardin *et al.*, 1997; Chourey *et al.*, 1998), plant source-sink relations (Zrenner *et al.*, 1995), phloem loading and unloading (Hänggi & Fleming, 2001) and response to anoxia (Ricard *et al.*, 1998). Concerning the latter, SuSy has been shown to operate more effectively under conditions of low oxygen compared to invertase (Zeng *et al.*, 1999). Furthermore, recent evidence from transgenic potato has indicated that a normal developmental shift to sucrose cleavage by SuSy in growing tubers is beneficial to adenylate balance, starch biosynthesis and respiratory costs (Bologa *et al.*, 2003). Thus, the functional significance of SuSy as opposed to invertase is likely to be particularly important in the low-oxygen conditions of growing sinks, where it can help to conserve adenylate usage through production of UDPGlc instead of hexoses, compared

to invertase which utilizes ATP. This role for SuSy may be of particular interest with regards to sugar accumulation in sugarcane, as the internal physiology of mature internodal tissues is likely to be hypoxic (Watt, unpublished results). The regulatory importance of SuSy activity for sucrose synthesis in young sugarcane internodes has been discussed and modeled (Botha & Black, 2000; Schäfer *et al.*, 2004), however, the regulatory function of SuSy in older tissue has yet to be fully examined. Due to the correlation between peak activity of SuSy, as well as acid invertase during internode elongation, both enzymes are likely to be involved in the elongation growth process (Lingle, 1999).

An additional role for SuSy in phloem loading and unloading was initially based on consistent observations of localisation of SuSy expression to mature phloem cells in leaf tissues (Nolte & Koch, 1993). Although a report by Hänggi and Fleming (2001) concluded that there is a specific exclusion of SuSy transcript and protein accumulation from the sink phloem tissue of young maize leaves, more recent work has immunolocalised the enzyme to sieve-tube elements, as well as to companion cells (Wächter *et al.*, 2003). SuSy has now been observed in close proximity with the sieve-tube plasma membrane and a phloem specific ATPase that is believed to aid sucrose transport and compartmentalization (Wächter *et al.*, 2003). Besides being favorable under the anoxic conditions found in phloem tissues (van Dongen *et al.*, 2003), SuSy has been suggested to play a role in the direct supply of UDPGlc for the rapid wound induced biosynthesis of callose plugs (Wächter *et al.*, 2003).

Thus far, three isoforms of the SuSy gene have been identified in monocot species and two in dicot species (Fu *et al.*, 1995; Huang *et al.*, 1996). These genes have different spatial and temporal expression (Koch, 2004), are differentially regulated at the transcriptional and translational levels and are likely to perform different metabolic functions (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 (Winter & Huber, 2000). The maize SuSy isoform *Sh1*, for example, is maximally expressed under carbohydrate-limiting conditions (0.2% compared to 2% glucose), whereas the expression of *sus1* is induced by increasing glucose concentrations (Hellman *et al.*, 2000). *Sh1* also responds rapidly to low oxygen levels, with marked increases in both mRNA levels and enzyme activity especially under conditions of modest oxygen depletion (i.e. 3% oxygen) (Zeng *et al.*, 1998).

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 and cellular membranes (Winter *et al*., 1998; Hardin *et al*., 2004). Regulation of the membrane associated form has been linked to posttranslational modification by reversible phosphorylation (Winter *et al*., 1997), although the physiological significance of this is still unclear. Nevertheless several mechanisms that tightly control the activation and turnover of SuSy through phosphorylation by SnRKs and CPDKs have now become apparent. The first phosphorylation step (at the serine 15 residue) that is involved in activating the enzyme may be catalyzed by either kinase type (Huber *et al.*, 1996), however, this initial phosphorylation step is implicated in predisposing the enzyme to phosphorylation at a second site (serine 170). Phosphorylation of the second site can be catalyzed by CPDKs, but not SnRKs (Hardin *et al*., 2003). This second phosphorylation step, in turn, targets the enzyme for ubiquitin-mediated degradation via the proteosome (Hardin *et al*., 2003). In addition, the ubiquitin-mediated pathway of SuSy breakdown may be inhibited if the second phosphorylation site is blocked by the binding of an additional protein, known as ENOD40, which was initially discovered in soybean root nodules (Kouchi *et al*., 1999; Rohrig *et al*., 2002; Hardin *et al*., 2003). The protective role of ENOD40 proteins has been linked to the control of vascular function, phloem loading and assimilate import (Hardin *et al*., 2003). For example, ENOD40 mRNA expression is elevated at sites of high sink activity and at points of rapid unloading in the phloem (Rohrig *et al*., 2002; Hardin *et al*., 2003).

2.2.6 Invertases

As mentioned in section 2.2.5, the invertases catalyze the irreversible cleavage of sucrose into the two hexoses glucose and fructose, utilizing ATP and forming twice as many hexoses as SuSy in the process. The resulting shift in the sucrose:hexose ratio, and consequent signaling from sugar sensors, has been shown to alter the expression of diverse genes (Koch, 1996; Wobus & Weber, 1999; Sturm & Tang; 1999). Thus, invertases are involved in a wide variety of metabolic processes affecting plant development (Koch, 2004).

The invertases are a family of β-fructosidase enzymes that differ in pH optimum for activity (neutral or acidic) and solubility. The three major isoforms are cytosolic neutral invertase (NI), soluble vacuolar acid invertase (VAI) and insoluble cell wall invertase (CWI), which differ in both localization and function (Quick, 1996; Link *et al.*, 2004). Both VAI and CWI are of exceptional metabolic importance, as they are the only known enzymes able to cleave sucrose in extracellular compartments such as the vacuole (VAI) and apoplastic space (CWI) (Link *et al.*, 2004). However, the role of NI has previously been considered as a less important "maintenance" enzyme, involved in sucrose degradation when the activities of VAI and SuSy are low (Winter & Huber, 2000). Nevertheless, several recent studies in sugarcane have suggested that NI may play a prominent role in sucrose accumulation (Bosch *et al.*, 2004; Mao *et al.*, 2005).

2.2.6.1 Vacuolar acid invertase

The primary function of VAI can be characterized in terms of cell turgor regulation and the control of sugar balance in reproductive tissues and mature tubers (Greiner *et al.*, 1999; Sturm & Tang, 1999). VAI is also known to play a prominent role in both sucrose import and sugar signaling (Sturm & Tang, 1999), particularly during the initiation of sink growth and cell wall expansion, when there is a high need for sucrose hydrolysis (Richardo & ap Rees, 1970; Tymowska-Lalanne & Kreis, 1998). Several studies in sugarcane have linked VAI activity to the elongation and expansion rates of maturing internodes (Glasziou & Bull, 1965; Lingle, 1999).

The expression of VAI is dependent on a multitude of signals, including sugars (particularly hexoses), hormones and other environmental stimuli (Koch, 1996; Walker *et al.*, 1997; Koch 2004). A variety of studies have examined environmental signals, including the influence of gravity and indole acetic acid on bending stems (Long *et al.*, 2002), cold treatment on the sweetening of tubers (Greiner *et al.*, 1999), and drought and abscisic acid on hexose concentrations in leaves (Trouverie *et al.*, 2003). The repression of VAI expression during drought has also been linked to carbon resource management during reproduction (Andersen *et al.*, 2002). As sucrose cleavage in the early phase of maize kernel growth has been shown to be predominantly controlled by VAI before the up-regulation of other sucrose metabolism genes, the onset of water stress often causes younger kernels to abort, thus giving preferentiality to the survival and development of more mature kernels (Andersen *et al.*, 2002).

A variety of gene isoforms exist for VAI, which have been shown to have different developmental and tissue specific expression patterns. The precise function of each isoform remains to be fully elucidated. For example, the maize VAI isoform *Ivr1* is up-regulated by sugar depletion, whereas *Ivr2* exhibits increased expression levels when sugar supply is abundant (Koch, 1996). Furthermore, *Ivr2* is up-regulated in the leaves of drought stressed plants (Trouverie *et al*., 2003), but down-regulated in the ovaries and young kernels under similar conditions (Andersen *et al*., 2002). *Ivr2* is also highly expressed in various plant organs including root tips, prop roots, mature anther, silk, kernel base and adult leaves under water stress (Koch 1996), while *Ivr1* is mainly expressed in mature anther, and to a lesser degree in silk, kernel crown and root tips (Koch 1996; Xu *et al*., 1996; Kim *et al*., 2000a). In species other than maize, a considerable variation of VAI activity has been observed, particularly in photosynthesis of fully expanded leaves (Huber, 1989). Furthermore, Huber (1989) has demonstrated that only species with a low VAI activity accumulate sucrose in leaves, as an end product of photosynthesis (Huber, 1989). As sugarcane favours accumulation of photoassimilate in the form sucrose, a limited activity for VAI in sugarcane leaves is expected.

Several intriguing advances have been made in identifying novel regulators of VAI at the protein level, including the discovery of a pre-vacuolar regulatory system in *Arabidopsis* (Rojo *et al*., 2003). Prior to delivery in the vacuole, VAI may be compartmentalized for extended periods in a vacuole-associated endomembrane vesicle known as the precursor protease vesicle (PPV). The PPV is also home to an inactive form of a vacuolar processing enzyme (VPEy protease), which may be released into the vacuole together with VAI. VPEy protease auto-activates upon entering the vacuole and can then target VAI for degradation. Thus, the PPV compartment not only plays a role in regulating the time at which VAI activity commences, but also controls its vulnerability to subsequent turnover by VPEy protease (Rojo *et al*., 2003).

2.2.6.2 Neutral invertase

The soluble neutral invertases (NI) are involved in cytosolic sucrose hydrolysis. In comparison to studies on other invertases, NI has been largely neglected (Hawker, 1985). However, the enzyme has since been purified and characterized for a variety of

species (Chen & Black, 1992; Lee & Sturm, 1996; Vorster & Botha, 1998; Rose & Botha, 2000). The cycling of carbon between sucrose and hexoses, as a result of simultaneous synthesis and degradation of sucrose, has since been demonstrated in the cytosol of sink tissue in several species, including sugarcane (Geigenberger *et al.*, 1991; Whittaker & Botha, 1997; Nguyen-Quoc & Foyer, 2001). This 'futile cycling' of sucrose is believed to be primarily responsible for overall sucrose accumulation in sugarcane, which may be mediated by SuSy and/or NI (Batta & Singh, 1986). A more recent correlation between hexose levels and NI in younger tissue has suggested that the enzyme could contribute to the supply of hexoses in younger culm tissue, implicating the enzyme as an important component of growth metabolism (Rose & Botha, 2000; Mao *et al.*, 2005). cDNAs encoding an enzyme with neutral/alkaline invertase activity have been cloned in poison rye grass (*Lolium temulentum* Lam. Kuntze.) and carrot (Gallagher & Pollock, 1998; Sturm *et al.*, 1999). However, further cloning from other species will be required to elicit whether the neutral/alkaline invertases cover more than one family of enzymes.

Transcripts of NI have been found in all sugarcane tissues, albeit at relatively low levels (Bosch *et al.*, 2004). The highest levels of NI expression and activity were observed in maturing culm, where sucrose was low and hexoses were high, with decreasing activity as the culm matures (Rose & Botha, 2000). A similar trend in distribution was seen in carrot, where the highest steady state levels of NI favoured young, actively growing tissues (Sturm *et al.*, 1999). The relationship between NI and its substrates throughout the sugarcane growth season is still not clear (Batta & Singh, 1986; Rose & Botha, 2000). This has led to the suggestion that NI activity may only be linked to local environmental and cyclic factors, rather than development and maturation (Rose & Botha, 2000).

2.2.6.3 Cell wall invertase

The insoluble CWI is ionically bound to the extracellular cell wall and consequently plays a key role in phloem unloading and assimilate uptake, specifically in sink tissues where an apoplastic step is involved (Roitsch *et al.*, 2003; Koch, 2004). As CWI cleaves apoplastic sucrose, it ensures a steep concentration gradient of sucrose from source to sink (Escherich, 1980), and has consequently implicated as a pivotal component in establishing such metabolic sinks (Roitsch *et al.*, 1995). Furthermore, the enzyme has been implicated in maintaining a strong source-sink relationship (Roitsch *et al.*, 2003).

The role of CWI has been shown to be highly prominent in developing seeds and pollen, where a gap in plasmodesmatal connections between cells has been shown to exist, and apoplasmic sugar transfer is prominent (Wobus & Weber 1999). However, if at least some sucrose moves across the cell wall space, CWI can additionally influence sinks where the plasmodesmatal connections remain intact, such as in developing carrot root and potato tubers (Sturm & Tang, 1999, Wächter *et al.*, 2003). The importance of CWI in sugar partitioning has also been examined using a maize mutant (*miniature1*) which, due to an abolishment of endosperm specific CWI, showed a small seed phenotype (Cheng *et al.*, 1996*b*). Similarly, antisense inhibition of CWI in carrot resulted in the arrestment of tap-root formation (Tang *et al.*, 1999). In both cases, provision of apoplasmic hexoses was required for normal development.

A variety of isoforms of the CWI family have been isolated (Roitsch *et al.*, 2003). Similar to SuSy, CWI isoforms exhibit highly tissue-specific mRNA expression patterns. In maize, four isoforms that differ greatly in expression locales have been identified thus far (*Incw1*, *Incw2*, *Incw3* and *Incw4*) (Qin *et al.*, 2004). Expression of *Incw3* and *Incw4* is very low in grain, as it is detectable only by reverse transcription-polymerase chain reaction (RT-PCR) (Kim *et al.*, 2000b). In tomato, the expression of CWI isoform *Lin7* is observed solely in anther tapetum cells and pollen grains (Godt & Roitsch, 1997). Anther-specific isoenzymes of CWI are furthermore evident in both tobacco and potato (Maddison *et al.*, l 999; Goetz *et al.*, 2001), indicating the crucial function for extracellular invertases in providing carbohydrates to the male gametophyte. Recently, antisense reductions of CWI in pollen have demonstrated the important role of CWI in maintaining male fertility (Roitsch *et al.*, 2003).

Several regulatory mechanisms have been revealed for CWI, including tissue specific expression (Cheng *et al.*, 1999), differential transcript formation (Cheng *et al.*, 1999), exon skipping (Bournay *et al.*, 1996) and stimulation of activity by a variety of phytohormones (Roitsch *et al.*, 2003). Importantly, CWI expression and enzyme activity are also modulated by sugars. Roitsch *et al.* (1995) observed higher enzyme activity and increased levels of CWI mRNA in the presence of sucrose and glucose using photoautotrophic suspension cultures of *Chenopodium rubrum* (L.), whereas various isoforms of CWI have been up-regulated by glucose in tobacco and *Arabidopsis* (Krausgrill *et al.*, 1998; Tymowska-Lalanne and Kreis, 1998), and by sucrose in tomato (Godt & Roitsch, 1997). More recently, research in tomato suspension cultures has

observed up-regulation of a CWI isoform using non-metabolizable sucrose analogues, such as palatinose, turanose and flourosucrose (Sinha *et al.*, 2002). However, both turanose and palatinose are synthesised by plant pathogens, indicating that the invertase response may be linked to stress related stimuli, rather than a unique sugar-signaling mechanism. Nevertheless, as both metabolizable sugars and stress-related carbohydrate stimuli have been shown to regulate CWI expression, CWI is potentially an important marker gene for the analysis of converging signaling pathways (Roitsch *et al.*, 2003).

CWI activity is regulated through a protein-protein binding complex between CWI and invertase inhibitor proteins (INH) (Krausgrill *et al.*, 1998; Greiner *et al.*, 1999). Expression analysis of CWI and INH has indicated that, at certain stages of plant development, CWI activity is down-regulated by INH, the latter operating as a regulatory switch (Krausgrill *et al.*, 1998). INH was originally isolated and purified to homogeneity from tobacco leaves, while the genes encoding the INH have been cloned from *Arabidopsis* and tobacco (Greiner *et al.*, 1999). Recently, the isolation of INH-type isoforms in several plant species has provided evidence that independent INH-systems regulate both CWI and VAI activities (Greiner *et al.*, 2000; Link *et al.*, 2004). Although the exact physiological mechanism of INH is still unclear, one possibility is that INH may function to modulate CWI activity under adverse conditions, such as maintenance of a minimal, critical sucrose concentration during sugar starvation (Winter & Huber, 2000).

2.2.7 Hexokinase

In plant tissues, the ratio of sucrose to hexose concentrations is an important metabolic signal that affects almost every aspect of plant development, including programmed cell death (Balibrea Lara *et al.*, 2004; Koch, 2004). The invertases, and to a lesser degree SuSy, are involved in maintaining the sucrose:hexose ratio. However, sugar sensors, such as hexokinase (HXK; EC 2.7.1.1), play a principal regulatory role in sensing sugar status and initiating signaling cascades that influence overall plant metabolism. Although initially questioned (Halford *et al.*, 1999), the involvement of HXK in sugar sensing has now been widely accepted (Moore & Sheen, 1999, Loreti *et* al., 2001; Rolland *et al.*, 2002; Harrington & Bush, 2003).

Sugars are important regulators of gene expression. Sheen (1990) initially demonstrated in maize protoplasts that glucose, or sucrose, was able to repress the expression of photosynthetic genes in higher plants. Subsequent studies led to the hypothesis that the sugar signal was perceived by HXK (Jang & Sheen, 1994; Koch, 1996; Jang & Sheen, 1997). Although a variety of sugars and sugar analogs have been examined, only substrates that were phosphorylated by HXK resulted in the expected gene expression changes (Jang & Sheen, 1994). Phosphorylated hexose did not represent a signal for the plant, as demonstrated by the inability of glucose-6-phosphate, introduced into protoplasts via electrophoresis, to elicit the same effect as glucose (Jang & Sheen, 1994). Based on these results, it is thus likely the de-phosphorylated form of glucose that initiates the HXK signal. Furthermore, mannoheptulose, a competitive inhibitor of HXK, blocked the regulatory effects of all sugars (Koch, 1996). The role of HXK as a putative sensor of hexose signaling was further clarified using sense and antisense constructs of the *Arabidopsis* HXK isoforms *Hxk1* and *Hxk2* (Jang *et al*., 1997). Plants that overexpressed HXK genes exhibited glucose hypersensitive characteristics, whereas antisense plants were hyposensitive (Jang *et al*., 1997). Together, these data supported the hypothesis that HXK is the putative sensor for hexose signaling.

To isolate the hexose signaling feature of HXK, Moore *et al*. (2003) uncoupled the phosphorylation activity of the enzyme from its sensing/signaling activity by generating point mutations in the catalytic domains of the protein. Upon engineering a synthetic *Hxk1* isoform, which was not capable of hexose phosphorylation yet still exhibited glucose sensing activity, the protein construct was transferred to an *Arabidopsis Hxk1* knockout mutant (*glucose insensitive2-1*) [*gin2-1*]). Although typically exhibiting broad growth defects during both glucose-dependent reproductive and vegetative stages of development, *gin2-1* mutants carrying the synthetic *Hxk1* construct showed significantly restored growth and development, thus confirming a pivotal role for HXK in glucose signal transduction (Moore *et al*., 2003). These results also show that glucose signaling is not the result of the accumulation or depletion of downstream metabolic products, or of changes in the ATP:ADP ratio, as has been suggested (Jang & Sheen, 1997). Furthermore, as HXK-mediated glucose signaling is not depended on its catalytic activity, HXK appears to play two functionally distinct roles in plant metabolism (Harrington & Bush, 2003).

Many factors surrounding the HXK-glucose signaling function remain to be uncovered. As the binding site of glucose for glucose phosphorylation is not necessarily the same site involved in glucose signaling (Moore *et al.*, 2003), the precise location of the signaling mechanism needs to be isolated (Harrington & Bush, 2003). Furthermore, whether HXK senses free glucose in a linear, concentration-dependent manner, or whether it is a flux sensor that senses either upward or downward deviations from a fixed or dynamic set point, needs to be resolved (Koch, 1996). Although several other protein kinases and phosphatases are implicated as secondary messengers in yeast (Rolland *et al.*, 2002; Rolland *et al.*, 2006), their roles in higher plant systems are speculative, and other proteins could also be involved. In addition, very little is known about the intracellular or tissue specific localisation of the various HXK isoforms, or the relationship, if any, between HXK and other HXK-like enzymes, such as fructokinase or glucokinase, which are also implicated in sugar sensing (Pego & Smeekens, 2000; Loretti *et al.*, 2001; Hoepfner & Botha, 2003). Thus, although the function of HXK has been revealed, it seems that a complex cascade system, perhaps involving several other proteins and metabolites, must still be elucidated (Harrington & Bush, 2003).

2.2.8 Trehalose metabolism

An important role for the trehalose metabolic pathway in regulating a HXK-independent, sugar-signaling system has recently emerged (Pellny *et al.*, 2002; Eastmond *et al.*, 2003; Paul & Pellny, 2003; Paul, 2007) (Fig. 2.4). Previously, the trehalose pathway was not thought to be universal in plant species (Goddijn & Smeekens, 1998). A reason for this may lie in the fact that trehalose metabolites are found only in very low amounts in species other than resurrection plants, where their accumulation to high levels enables protection from desiccation (Wingler, 2002). Previously, the inability to detect trehalose even lead to the suggestion that most higher plants had lost the ability to produce it (Crowe *et al.*, 1992). However, the activity of trehalase, an enzyme responsible for the breakdown of trehalose to glucose, was found to be present in numerous plants (Müller *et al.*, 1995). Subsequent application of an inhibitor of trehalase successfully demonstrated detectable levels of trehalose in both tobacco and potato (Goddijn *et al.*, 1997).

Similar to sucrose, trehalose is typically produced in a two-step process (Fig. 2.4). Firstly, the synthesis of trehalose-6-phosphate (T6P) from UDP-glucose and G6P is

catalyzed by trehalose 6-phosphate synthase (TPS; EC 2.4.1.15). T6P is then dephosphorylated to trehalose by trehalose 6-phosphate phosphatase (TPP; EC 3.1.3.12) (Rolland *et al*., 2006). Functional genes encoding TPS and TPP have been detected in *Arabidopsis* (Blázquez *et al*., 1998; Vogel *et al*., 1998; Vogel *et al*., 2001), while T6P has been found in tobacco in the low µM range (Pellny *et al*., 2002).

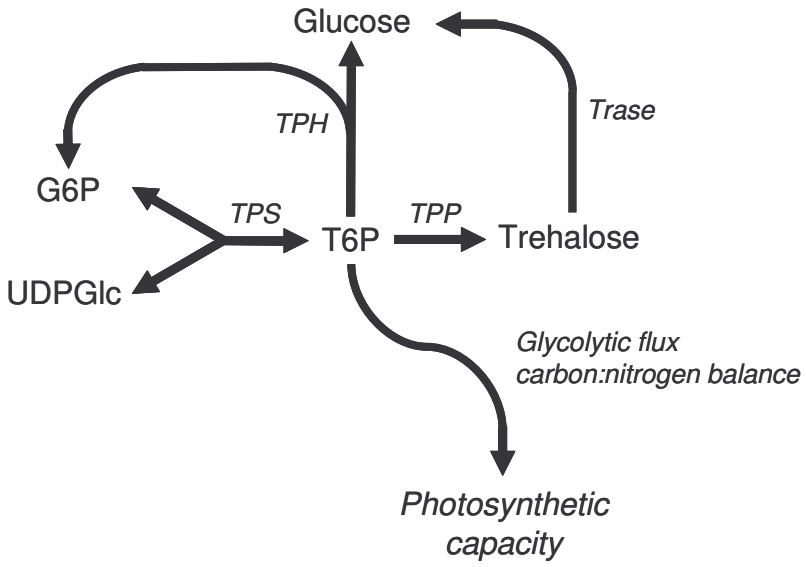

Fig. 2.4. Trehalose synthesis pathway and model for its relationship with regulating photosynthetic capacity (modified from Paul & Pellny, 2003). Enzymes indicated include trehalose phosphate synthase (TPS; EC 2.4.1.15), trehalose phosphate phosphatase (TPP, EC 3.1.3.12), trehalose phosphate hydrolase (TPH; EC 3.2.1.93), Trehalase (Trase; EC 3.2.1.28).

The regulatory role of the trehalose pathway in sugar signaling has now been examined in several plant systems. In *Arabidopsis*, external application of even small concentrations of trehalose has resulted in significant physiological changes, such as inhibition of root elongation (Wingler *et al*., 2000). Previous transgenic research in tobacco plants expressing the *E. coli otsA* and *otsB* genes, encoding TPS and TPP respectively, has produced effects consistent with an impact on sugar signaling (Paul *et al*., 2001). Further research has shown that transgenic plants expressing the *otsA* gene have smaller darker green leaves and greater photosynthetic capacity per unit leaf area than wild types, while plants expressing the *otsB* gene have larger and paler leaves with lower photosynthetic capacity (Pellny *et al*., 2002). Although producing no measurable

trehalose in transgenic leaves, the genetic modifications did perturb T6P levels, with increased photosynthetic capacity correlating with an increase in T6P content (Pellny *et al.*, 2002). Controlled alterations of T6P levels by expressing combinations of *E. coli* trehalose metabolism genes have since clearly demonstrated that T6P is indispensable for carbohydrate utilization for growth in *Arabidopsis* (Schluepmann *et al.*, 2003). The exact mechanisms whereby T6P influences carbon metabolism are still unclear, since unlike in yeast, T6P is not an inhibitor of HXK activity (Eastmond *et al.*, 2002). This indicates that T6P may be involved in a HXK-independent sugar-signaling pathway (Eastmond *et al.*, 2003).

A further interesting development has been the identification and isolation of a single gene system for the production of trehalose. In the basidiomycete fungi, *Grifola frondosa*, trehalose may also be synthesized directly from glucose and glucose-1-phosphate by a novel trehalose synthase (Saito *et al.*, 1998; Zhang & Zheng, 1999). Transgenic transfer of the gene into tobacco and sugarcane resulted in plants that accrued high levels of trehalose and exhibited increased drought and salt tolerance (Zhang *et al.*, 2005; Zhang *et al.*, 2006). Transgenic sugarcane had no obvious morphological changes and no growth inhibition in the field, whilst exhibiting an increased tolerance of drought and improved yield under drought conditions (Zhang *et al.*, 2006). Although the exact physiological effects of trehalose in this system are not known, these results demonstrate that sugarcane may tolerate high amounts of trehalose. This raises interesting questions regarding the sensitivity of the trehalose signaling system between different plant species.

Although the mechanisms of trehalose and T6P regulation in higher plants requires further investigation, it appears the T6P may perturb glycolytic carbon flow and the carbon: nitrogen balance implicated in the regulation of leaf photosynthesis (Paul & Pellny 2003; Paul, 2007). Further research has also shown that the TPS gene is required during embryogenesis, where it appears to enable *Arabidopsis* embryos to respond to sucrose (Eastmond *et al.*, 2002). These findings implicate the trehalose pathway in an even broader sugar-sensing role.

2.3 The relationship between sink and source

2.3.1 Introduction

A firm grasp of the control mechanisms of photosynthesis (see section 2.1) and sucrose-related plant growth (see section 2.2) may clarify certain facets of plant metabolism; however, without knowledge of the interrelationship between the leaf and growing regions of the plant, a proper understanding of how plant development is regulated cannot be fully realised. Carbon assimilation is a function of the balance between the supply by leaf photosynthesis and the demand from growth processes. As such, to properly conceptualise the manner in which plants assimilate and partition carbon, an understanding of the paradigm of 'sink' and 'source' tissues is required.

The hypothesis that photoassimilate accumulation plays a role in regulating photosynthesis rates is not new. Such a connection was initially highlighted by Boussingault (1868), who suggested the existence of a putative link between leaf (sources) and storage tissues (sinks), instead of a one-way relationship. Close co-ordination of source photosynthetic activity with carbon demand of sink organs has now been clearly demonstrated in several species, where a decrease in assimilation rate is observed when sink demand for carbohydrate is limited (Gucci *et al.,* 1994; Iglesias *et al*., 2002; De Groot *et al*., 2003; Quilot *et al*., 2004; Franck *et al*., 2006). As plants are sessile, autotrophic organisms, the need to integrate metabolism and growth with external environmental signals is crucial. To do so efficiently, plants must rigorously coordinate both source activity and sink demand, or else risk a fatal 'economic crisis' from over-investing in either one or the other. This involves both fine (substrate and allosteric) and coarse (gene expression) regulation, as well as specific sugar-signaling mechanisms (Rolland *et al*., 2006).

The elucidation of a regulatory relationship between the demand for carbon from growing areas of the plant and the supply from leaves has become a novel and exciting field of molecular and physiological research (Wardlaw, 1990; Pego *et al*., 2000; Paul & Foyer, 2001; Rolland *et al*., 2002; Paul *et al*., 2001; Paul & Pellny, 2003). Evidence increasingly supports a sink-dependent relationship (Paul & Foyer, 2001), whereby the demand for carbon from sink tissues, or sink-strength, influences the net photosynthetic activity and carbon status of source organs (Paul *et al*., 2001). However, the dominant

mechanisms through which the sink regulates the source appear not to be simple linear pathways, but rather a set of complex networks with many points of reciprocal feedback control, which together determine the limits within which photosynthesis can be productive and underpin the source-sink interaction (Paul & Foyer, 2001; Minchin & Lacointe, 2004; Rolland et al., 2006).

The precise metabolic mechanisms that give rise to feedback control are still not fully understood (Paul & Pellny, 2003). However, recognition of the role of sugar transporters and associated regulatory metabolic enzymes in the loading and unloading of sucrose in the phloem has led to the proposition of several models that serve to illustrate the carbon transport pathways between source and sink tissue. Using the source-sink paradigm, this chapter will discuss existing understanding of the physiological and metabolic means by which plants regulate carbon partitioning and accumulation. Furthermore, it will highlight current mechanistic modeling efforts to predict the observed complexity of the source-sink relationship, with particular reference to sugarcane models**.**

2.3.2 Phloem transport

Long-distance transport of carbohydrates between sources and sinks occurs in specific cells of the vascular system called the phloem sieve elements. During development, most organelles (including vacuoles and nuclei) of sieve elements are degraded, resulting in an intimate connection via numerous branched plasmodesmata with neighboring phloem companion cells, which in turn supply energy and proteins to the sieve elements (Williams *et al.*, 2000, Stadler *et al.*, 2005). Phloem transport functions via bulk flow, where accumulation of sugars inside the sieve element-companion cell complex (SE-CCC) results in the osmotic uptake of water which then drives sap along the sieve tube. The unloading of sugars at the sink results in a loss of water, and thus a gradient of pressure is maintained (Williams *et al.*, 2000). Despite the occurrence and importance of other phloem solutes, such as amino acids, raffinose and stachyose, hexitols, inorganic ions and the most recently identified component, fructans (Wang & Nobel, 1998), sucrose is the osmotically dominant compound in the sieve tube sap of most species, including sugarcane (Hartt *et al.*, 1963; Huber *et al.*, 1993; Komor, 2000). Thus, in these species, sucrose is not only the main transport metabolite, but also the

primary contributor to the osmotic driving force for phloem translocation (Hellman *et al.*, 2000).

2.3.2.1 Phloem loading

Recently, there have been several advances in understanding of the processes involved in phloem loading at the source (Lalonde *et al.*, 2003; van Bel, 2003; Minchin & Lacointe, 2004). Depending on species, phloem loading is now believed to involve an apoplastic step, a symplastic step, or both, between the site of sucrose synthesis and the sieve tubes involved in transport (Komor, 2000). Apoplastic loading involves flow of sucrose from the leaf mesophyll into the apoplastic space in the vicinity of the vascular tissue, from where it is actively taken up across the cell membrane by sugar transporter proteins into the SE-CCC, or directly into the sieve element (Hellman *et al.*, 2000; Minchin & Lacointe, 2004). However, with symplastic loading, sucrose flows from cell-to-cell to the SE-CCC entirely through plasmodesmata, probably via diffusion, and thus does not cross the membrane (van Bel & Gamalei, 1992). However, it should be noted that plasmodesmata are not simply intracellular 'holes' which facilitate passive transport, but rather dynamic, complex structures through which the transport of macromolecules is highly regulated (Lucas *et al.*, 1993). An association of cytoskeletal elements, such as actin- and myosin-like proteins, with plasmodesmatal structures has been demonstrated (White *et al.*, 1994; Radford & White, 1998). These cytoskeletal elements may play a role in the targeting and transport of macromolecules through the plasmodesmata (Radford and White, 1998).

As most studies of the leaf 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). Similar to other active transport processes, the rate of active sucrose export would 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 has been shown by manipulating sucrose content with varying light intensities (Fader & Koller, 1983). A similar $^{14}CO_2$-labelling study was undertaken using leaves of a variety of species, with particular emphasis on the difference between C_3 and C_4 plants (Grodzinski *et al.*, 1998). C_4 plants were clearly shown to have higher leaf solute concentrations and export rates (Grodzinski *et al.*, 1998).

In C_4 plants, such as sugarcane, where a two cell-type (exterior mesophyll and interior bundle sheath) configuration exists, sucrose is still produced primarily in the mesophyll (Lunn & Furbank, 1997). Thus, in C_4 plants, sucrose must additionally pass through the bundle sheath cells to be loaded into the phloem through either a plasmodesmatal or apoplastic step, or both (Fig. 2.5) (Lunn & Furbank, 1999; Walsh *et al.*, 2005). However, in sugarcane, the conducting cells of the phloem are not connected to other cells of the leaf by plasmodesmata (Robinson-Beers & Evert, 1991), suggesting that phloem loading occurs from the apoplast (Rae *et al.*, 2005a).

2.3.2.2 Phloem unloading

Phloem unloading in most species is believed to favour symplastic movement, through plasmodesmata linking the cells in the sink region (Minchin & Lacointe, 2002). In immature potato tubers, phloem unloading is predominately apoplastic, although the onset of tuber development and starch accumulation is accompanied by a switch to symplastic transfer of solutes to storage parenchyma cells (Viola *et al.*, 2001). The destination of the symplastic flow is not the terminal sieve elements, but rather within the receiver cells, where the sink osmotic pressure is kept low by metabolic utilization of the carbohydrates or conversion into less osmotically active polymorphic forms, such as starch (Minchin & Lacointe, 2002). Growing evidence indicates that the region of highest flow resistance is not within the transport phloem linking sources and sinks, but rather within the symplastic pathway of the receiver cells (Gould *et al.*, 2004; Walsh *et al.*, 2005).

Importantly, symplastic unloading rarely operates independently, as the pathway of unloading in storage tissues, in which soluble sugars are accumulated, often includes an apoplastic step at the periphery of the phloem or in subsequent cell layers (Lalonde *et al.*, 2003). A preference for apoplastic unloading has been demonstrated in tomato and developing fruits by the movement of tracer dyes (Patrick, 1997). Thus, in sugarcane, where sugars accumulate in tissues that are relatively mature, the apoplastic pathway may be pre-eminent (Rae *et al.*, 2005a; Rae *et al.*, 2005b). The sugarcane culm contains high levels of apoplastic sucrose (Hawker & Hatch, 1965; Welbaum & Meinzer, 1990), which suggests that sucrose is unloaded from the phloem complex directly into the apoplast. Sucrose is then cleaved in the apoplast by CWI and taken up by surrounding parenchyma storage tissues, where it may be re-synthesized (Patrick,

1997). At culm maturity, the concentration of sucrose would generate very high turgor pressures, if retained in the parenchyma cells. Measurements of turgor, wall extensibility and membrane conductivity in sugarcane parenchyma tissue have suggested that low turgor is maintained by partitioning of solutes between the symplastic and apoplastic compartments (Moore & Cosgrove, 1991).

An added complexity occurs within the sugarcane culm due to the structure of the vascular bundles, which are surrounded by a sheath of lignified fibre cells that serves to isolate the xylem water from the apoplast of culm storage tissues (Fig. 2.5) (Jacobsen *et al.*, 1992; Welbaum & Meinzer, 1990). As the culm matures, the sheath cell layers progressively lignify to form a barrier to apoplastic movement of solutes during the period of sucrose accumulation (Jacobsen *et al.*, 1992). Thus, sucrose probably cannot reach the parenchyma cells through a purely apoplastic path (Rae *et al.*, 2005a). The symplastic passage of sucrose through the fiber sheath has been examined by Walsh *et al.* (2005) and is suggested to be a likely point of rate limitation in sucrose transport from phloem to storage parenchyma. Upon export from the vascular bundle, it is possible that sucrose may then move symplastically throughout the parenchyma, as has been partially demonstrated by tracer dyes (Rae *et al.*, 2005b). However, in order to maintain a gradient for sucrose flow, sucrose may be excluded from the symplastic continuum by export to the apoplast or into the vacuole (Rae *et al.*, 2005a). Backflow of apoplastic sucrose into the vascular system would then be restricted by the hydrophobic nature of the lignified and suberized cell walls surrounding the vascular bundle, thus forming an isolated apoplastic compartment (Jacobsen *et al.*, 1992). Consequently, a comprehensive model for sucrose unloading in sugarcane should incorporate both symplastic and apoplastic transfer.

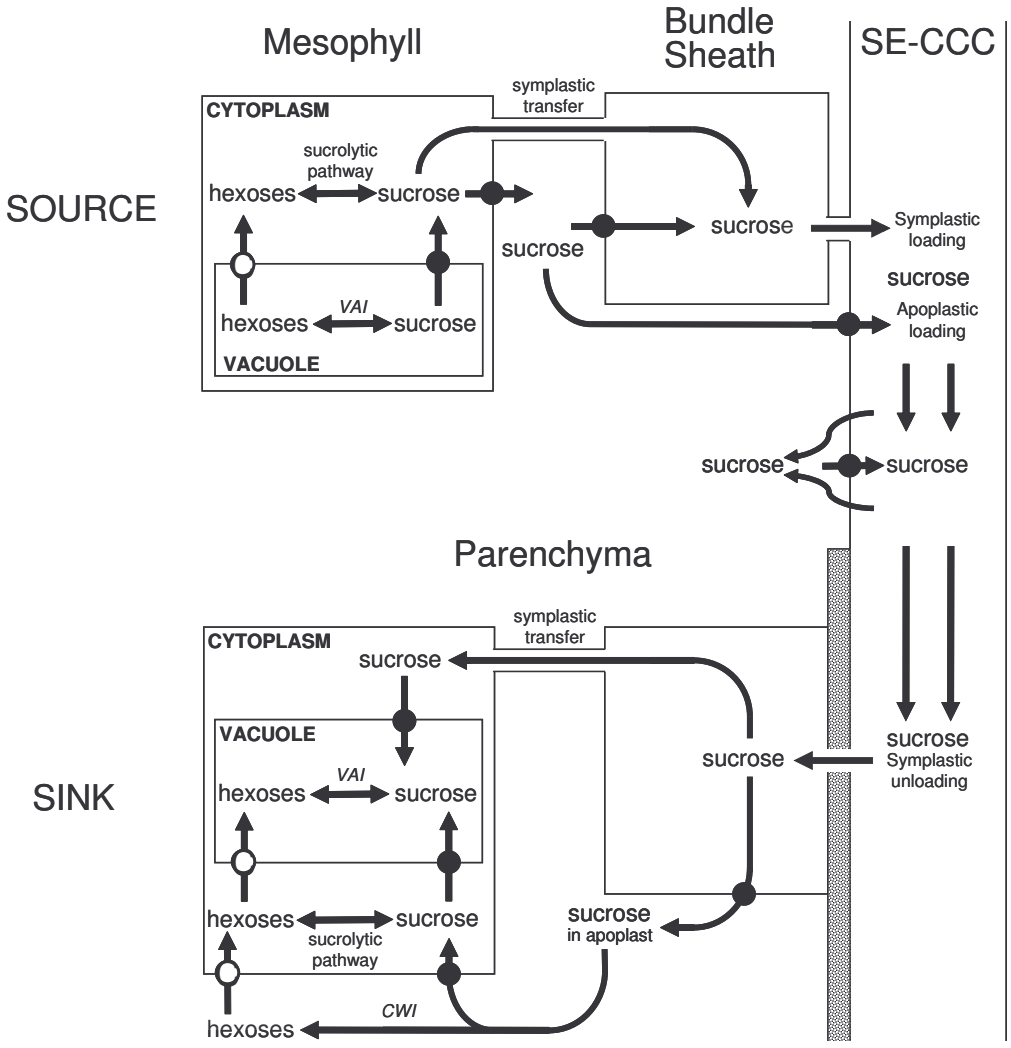

Fig. 2.5. Possible pathways for sucrose flux from source to sink in sugarcane. Sucrose is produced in the source mesophyll and either loaded symplastically via the bundle sheath or exported to the apoplasm and imported into the sieve element companion cell complex (SE-CCC). Sucrose leakage and re-uptake may occur along the SE-CCC. Sucrose may then be unloaded in the culm sink symplastically through the fibre sheath, whereupon it flows from cell-to-cell through the plasmodesmata. Alternatively, sucrose may be exported from the symplastic continuum and then actively removed from the apoplast by surrounding parenchyma tissue via sugar transporter proteins (●), or hydrolyzed to hexoses, taken up via hexose transporters (○), and then resynthesized within the cell. Interconversion of sucrose and hexoses ('futile cycling') may co-exist within the vacuole and/or cytoplasm, and even indirectly extend into the apoplastic space due to sugar transporter activity (modified from Hellman *et al.*, 2000 and Rae *et al.*, 2005b).

2.3.3 Sugar transporters

Sugar transporter proteins play a pivotal role in carbon supply pathways via regulating the membrane transport of carbon assimilates, as well as their distribution throughout the plant. As discussed in chapter 2.3.2, sucrose loading may occur symplastically, although sucrose is often released from source mesophyll cells and actively loaded from the apoplastic space into the SE-CCC (Hellman *et al.*, 2000; Williams *et al.*, 2000). Plasma membrane sucrose-H^+ symporters are fundamental to this process. Sucrose unloading can also occur via a symplastic or apoplastic route, which is often species-specific and can depend on the developmental stage of organs or tissues (Williams *et al.*, 2000). Within the apoplastic pathway, sink parenchyma may either import sucrose directly from the apoplast via sucrose-H^+ symporters, or alternatively as hexoses via monosaccharide-H^+ co-transporters after sucrose hydrolysis by CWI.

Sugar transporters are categorised into two family types, *viz.* monosaccharide (MST) and disaccharide (DST) transporters, with several forms existing within each family. In addition, depending on plant species, each transporter may be expressed at different developmental stages and under varying environmental conditions in different tissues (Williams *et al.*, 2000). Although DSTs and MSTs share little homology at the amino acid level, expression studies in yeast and *Xenopus* oocytes on some of the family members have suggested that both families function as proton symporters (Sauer *et al.*, 1990; Sauer & Stolz, 1994; Boorer *et al.*, 1996; Zhou *et al.*, 1997). Generally the DSTs are highly specific for sucrose, although some have been shown to transport maltose (Lemoine, 2000). MSTs, on the other hand, display broader substrate specificity, transporting a range of hexoses and pentoses (Büttner & Sauer, 2000; Hellman *et al.*, 2000).

2.3.3.1 Disaccharide transporters

The primary role of DSTs is reported to be the apoplastic loading and long-distance transport of sucrose from the source. DST proteins are found in the companion cells of the SE-CCC complex (Stadler & Sauer, 1996) and the sieve element itself (Kühn *et al.*, 1997). Notably, DSTs found in the sieve elements have been shown to result from gene transcription in the companion cell, however, the precise signals and mechanisms that regulate their final destination remain to be uncovered (Williams *et al.*, 2000; Stadler *et*

al., 2005). In *Solanaceae* species, DST mRNA is localized in the sieve elements, suggesting that transcription occurs in the companion cells and might be followed by transportation of the mRNA through the plasmodesmata and translation within the sieve element (Kühn *et al*., 1997).

The first complete cDNAs for DST genes were isolated from spinach (*SoSUT1*) and potato (*StSUT1*) using yeast complementation (Reismeier *et al*., 1992; 1993). Both genes showed high genetic similarity (Reismeier *et al*. 1993). The importance of *SUT1*-type DSTs in sucrose translocation was further demonstrated in potato and tobacco using antisense technology (Reismeier *et al*., 1993; Bürkle *et al*., 1998). Transgenic potato plants with lowered *SUT1* expression have reduced tuber yields and accumulate sugars in mature leaves (Reismeier *et al*., 1993), while transgenic tobacco plants have a dwarf phenotype and show drastically impaired ability to export sugars from leaves (Bürkle *et al*., 1998). Several *SUT1*-type genes have since been isolated and their tissue-specific expression patterns analysed from a variety of plants (Kühn *et al*., 1999; Williams *et al*., 2000). As DSTs continue to be expressed in phloem tissue, an additional role for phloem-localised *SUT1* homologues might be in the retrieval of sucrose lost to the apoplast from leaking sieve elements along the translocation pathway (Lemoine *et al*., 1996; Lalonde *et al*., 2003). Notably, a sucrose-retrieval DST has been identified in tissues surrounding the mature culm vascular bundles in sugarcane (*ShSUT1*) (Rae *et al*., 2005b). Recent characterization studies have indicated that *ShSUT1* may play an important role in the accumulation of sucrose, even though it exhibits a relatively low affinity for the substrate (Reinders *et al*., 2006).

Additional DST homologues have been identified, but are not as well documented as *SUT1*-type transporters. A total of nine DST homologues are known in the *Arabidopsis* genome, however, most have not been characterised in detail (Eckhart, 2003). The role of *SUT2* (sometimes referred to as *SUC3*) homologues is still not clearly defined, as they do not appear to have a high affinity for sucrose (Eckhart, 2003). It is debatable whether their roles are linked to sucrose transport or a putative sucrose sensing function (Eckhart, 2003).

There are several mechanisms that have been proposed for the regulation of DST transcription and activity, including a phosphorylation-dephosphorylation system in sugar beet (*Beta vulgaris* L.), perhaps via SnRK1- or CPDK-type kinases (Roblin *et al*., 1998).

In rice, a local lipid-based modification of DST activity has been postulated (Delrot *et al.*, 2000). Different regulatory systems may also affect DST transcript levels, depending on the transporter homologue and expression location (Williams *et al.*, 2000). An analysis of two DSTs genes in carrot (*DcSUT1* and *DcSUT2*) showed expression, albeit at different levels, in both source leaf tissue and non-photosynthetic sink tissues (Shakya & Sturm, 1998). Although *DcSUT1* was predominately expressed in leaf, the expression of *DCSUT1* and *DcSUT2* was linked to photosynthesis, and transport activity appeared to be regulated in a diurnal fashion (Kühn *et al.*, 1997; Shakya & Sturm, 1998). However, in root tissues *DcSUT1* and *DcSUT2* showed no diurnal fluctuation, with *DsSUT2* exhibiting much higher steady-state transcript levels (Shakya & Sturm, 1998). As *DcSUT2* expression was found in both sink phloem and parenchyma tissue, it is still not known whether this transporter is involved in phloem unloading or import of sucrose into parenchyma cells (Shakya & Sturm, 1998). A similar study of *CitSUT1* and *CitSUT2* expression patterns in citrus suggested that *CitSUT2* is more strongly linked to sink activity, whereas *CitSUC1* is believed to be primarily involved in phloem loading and may be regulated by sugars, perhaps via the HXK-mediated signaling pathway (Li *et al.*, 2003).

2.3.3.2 Monosaccharide transporters

Most reports have located MST to sink tissues (Sauer & Stadler, 1993; Williams *et al.*, 2000). Studies have suggested that monosaccharide transport is a moderately flexible system for carbohydrate allocation, as the MST family is regulated by numerous mechanisms, including phytohormones, abiotic stress and wounding (Truernit *et al.*, 1996; Ehneβ & Roitsch 1997). Furthermore, MST activities have been identified from several species for a variety of substrates, including D-glucose, 3-O-methyl-glucose (3Omeg), 2-deoxy-D-gluose, D-mannose and D-fructose (for review see Hellman *et al.*, 2000). Cloning of the first higher plant MSTs was accomplished by heterologous hybridisation with hexose transporter genes from the green alga, *Chlorella kessleri* (Sauer & Tanner, 1989; Sauer *et al.*, 1990). Numerous genes and gene subfamilies from several species have since been isolated (Lalonde *et al.*, 1999; Williams *et al.*, 2000; Johnson *et al.*, 2006)

The regulation of MST expression suggests that these membrane proteins function in hexose uptake in sink tissues, specifically an apoplastic mechanism for phloem or post-

phloem unloading (Sauer & Stadler, 1993). This mechanism is particularly pertinent in symplastically isolated sinks, such as developing pollen and seeds, which do not have plasmodesmatal connections to the surrounding tissue (Weber *et al.*, 1997; Schneidereit *et al.*, 2003). In *Vicia faba* (L.) seed embryos, the MST *VfSTP1* is expressed specifically in the mitotically active parenchyma, indicating that this transporter serves to supply substrate for metabolism (Weber *et al.*, 1997). Once sucrose is released from the phloem it may then be hydrolyzed by extracellular CWI and subsequently taken up into sink cells by the corresponding MST (Lalonde *et al.*, 1999). An expected requirement for this mechanism in plants might be the mutual regulation of expression for CWI and MST genes. This has been demonstrated in *Chenopodium* cell suspension cultures (Ehneß & Riotsch, 1997) and more recently in soybean seedlings (Dimou *et al.*, 2005), indicating the presence of a regulatory network to control the co-ordination of different protein activities in the process of phloem unloading (Roitsch & Gonzalez, 2004). Sugarcane is also believed to unload sucrose into the apoplast, where it is cleaved by CWI to produce hexoses, which are then taken up by MSTs in culm parenchyma cells (Rae *et al.*, 2005a). This contention supports previous evidence provided by sugarcane cell suspension studies, which express high levels of CWI and take up reducing sugars but not sucrose (Komor *et al.*, 1981).

MSTs are also associated with defense-responses following infection by micro-organisms. The expression of *Arabidopsis* transporter *AtSTP4* has been linked to the transportation of monosaccharides into sink tissues, especially under stress conditions (Truernit *et al.*, 1996). However, induction of *AtSTP4* is also observed during plant and fungal biotrophic interaction (Fotopoulos *et al.*, 2003). The signaling pathway and putative role of this transporter is still unclear, but it is suggested that an increase in carbohydrates into infected tissues may assist the activation of defense systems, or serve to recover sugars from the apoplast and thus reduce the loss of carbohydrates to the pathogen (Williams *et al.*, 2000). Interestingly, MSTs have even been detected in the plasma membrane vesicles of sugar beet leaves (Tubbe & Buckhout, 1992). The function of these transporters may be to prevent the accumulation of hexose in the source apoplast, however this hypothesis has not yet been fully tested.

2.3.4 Source-sink modeling

To fully understand the mechanisms and regulation involved in complex physiological processes, such as the accumulation and partitioning of carbon in plants, information on every facet of the system, including the complex interactions amongst components, is required. As this knowledge is often not readily available, the ability to characterise and predict the actions of such systems is often best accomplished through the construction of simplified simulation models. The application of simulation models in agriculture is widespread across a variety of industries, all of which input a number of measured environmental and crop-related factors, with the aim of predicting future growth and yield values (Jones & Richie, 1990; Chapman *et al.*, 1993; McCown *et al.*, 1996; Dhakhwa *et al.*, 1997; Singels & Bezuidenhout, 2002).

The partitioning of carbon in crops is an important yield factor. Although comprising a large body of experimental data, previous work on carbon partitioning has not readily encompassed a detailed mechanistic understanding of the source-sink relationship (Wardlaw, 1990). Instead, a variety of empirical carbon allocation models have been developed, which incorporate partitioning algorithms derived from observational data (Marcelis *et al.*, 1998; Lacointe, 2000; Le Roux *et al.*, 2001). The algorithmic approach is effective in simulating carbon partitioning within the range of conditions appropriate to calibration data, but is much less successful for extrapolating into other conditions, and gives no insight as to the processes involved (Bancal & Soltani, 2002; Minchin & Lacointe, 2004). To advance the calibration data sets to encompass, for example, different locations, environments and crop management practices, it has been suggested that the mechanistic understanding of partitioning is an essential foundation for more complex models (Thornley, 1998; Bancal & Soltani, 2002; Minchin & Lacointe, 2004).

Previously, a mechanistic model was formulated by Thornley (1972) for quantifying root: shoot ratios, and comprised a simple transport-resistance (TR) archetype to describe carbon translocation through the phloem between a single source and sink. The TR model used parameters based on the now widely accepted Münch theory, which described the formation of a solution flow by an osmotically generated pressure gradient (Minchin *et al.*, 1993). Münch theory states that the solute flow rate between source and sink is proportional to the hydrostatic pressure between source and sink (ΔP), and

inversely proportional to the pathway length (*L*) (Lacointe, 2000). Using Van Hoff's osmotic equation to describe the relationship between solute concentration *C* and the osmotically generated hydrostatic pressure *P*, the solute flow rate can be expressed as J_s = constant $\times \frac{\Delta C}{R}$, where the resistance *R* is proportional to *L* (Minchin & Lacointe, 2004). Thus, distance effects, such as sink being supplied by the nearest available source, are qualitatively explained using the resistance term (Lacointe, 2000). According to a critique by Thornley (1998), the TR model should ideally be the starting point of all plant growth models.

However, the TR model proposed by Thornley (Thornley, 1972) is not without shortcomings. For example, to maintain a constant pressure gradient the model requires the phloem to be non-leaking, with osmotic flow only occurring at the source and sink regions; an assumption which is unrealistic given current knowledge of phloem physiology (van Bel, 2003). Furthermore, several parameters are difficult to estimate (Minchin *et al.*, 1993). Nevertheless, the basic TR model has since been improved in several respects, including phloem loading parameters, possible changes in sap viscosity with solute concentration, and the ability to model two or more sinks simultaneously (Minchin *et al.*, 1993; Bancal & Soltani, 2002). Regarding the latter enhancement, unloading of assimilate at sink regions was incorporated using Michaelis-Menten kinetics specified by the maximum unloading rate (V_m) and a Michaelis constant (K_m) (Minchin *et al.*, 1993). This approach was used to explain and quantify sink priority, or sink strength, for both equivalent and non-equivalent sinks, based on changes in sink function (v_m), changes in *R*, or source function (*C* at source) (Minchin *et al.*, 1993). This modified model has since been tested and validated in supplementary experiments (Minchin & Thorpe, 1996; Minchin *et al.*, 2002), and has also clarified the results of several published studies involving cold-girdling between source and sinks, which previously explained the observed changes in partitioning according to a presumed, but as yet unidentified, biochemical signal *(*Grusak & Lucas, 1985; Pickard *et al.*, 1993). Minchin *et al.* (1993) predicted that adjustments in partitioning were, in fact, due to changes in the common pathway flow resistance, as the lowered temperatures caused partial blockage of the phloem pathway. Interestingly, these new outcomes demonstrated that solute flow into sinks is not just a property of the sink strength, but rather of the source, pathway and sink properties combined (Minchin & Lacointe, 2004).

Metabolic control theory (MCT) is additionally a highly effective mechanistic modeling tool for unraveling complex systems (Kacser & Burns, 1973; Fell, 1997). Research has demonstrated that plants are highly adept at 'buffering' changes in the levels of various metabolites wrought via transgenic manipulation, including attempts to increase sucrose accumulation in sugarcane (Ma *et al*., 2000; Groenewald & Botha, 2007). This is most likely a result of the difficulty in detecting correlations between enzyme activity and flux in plants (Stitt & Sonnewald, 1995). In yeast, MCT studies of glycolysis have suggested that rate-limiting enzymatic steps do not exist, but rather that the control of flux is shared among all enzymes in the metabolic system (Schaaf *et al*., 1989). In MCT, the extent to which any catalytic step (e.g. an enzyme-catalyzed reaction) controls a steady state variable (flux or concentration) is quantified by a 'control coefficient' (Kholodenko *et al*., 1995). Control analysis of the interactions between control coefficients in a metabolic system allows for the identification of the significant affectors of that system. MCT provides a powerful, theoretical method for not only identifying, but also testing the points of control in metabolically complex systems, for example, establishing correlations between enzyme activity and carbon flux in plants. Using thermodynamic and kinetic data of the appropriate enzymes, Rohwer & Botha (2001) utilized this approach to detect the steps in glycolysis that most affect futile cycling of sucrose in the sugarcane culm. Results indicated that HXK and NI had the highest positive control efficients, implying increased futile cycling upon an increase in activity of those enzymes. Although limited to a single internode and only incorporating cytosolic reactions, this analytical technique provided a novel way to examine the dynamics of a complex system involving numerous fluxes and metabolic intermediates (Rohwer & Botha, 2001). Currently, this model is being extended to include the cell vacuole and possibly interactions with the phloem involving sucrose transport and unloading. It may thus be a useful tool for combining with the TR model to examine source-sink relations down to the localized activity of sink and/or source enzymes.

2.3.4.1 Sugarcane models

Sugarcane growth simulation models have been used by sugar industries for several decades. Presently, there are two main models used throughout the world: the South African model, CANEGRO (Inman-Bamber, 1995), and the Australian model APSIM-Sugarcane (Keating *et al*., 1995). The origins of CANEGRO date back to the early seventies with the development of the equations for photosynthesis and respiration

(McCree, 1970, Inman-Bamber & Thompson, 1989). CANEGRO has since greatly evolved to include, among others, components for carbon simulation, crop development, energy and water simulation (Inman-Bamber & Kiker, 1997). Conversely, the APSIM-Sugarcane model is an advancement of two previous models (AUSCANE and EPIC), which not only addresses soil erosion, crop productivity and economic factors, but also models soil water and nitrogen (Probert *et al.*, 1998). Both models represent the collected efforts of a large number of agronomists, physiologists and programmers.

In the past, such sugarcane simulation models have not included a mechanistic approach for predicting sucrose yield and purity, but have, instead, relied on empirical calibration factors (O'Leary, 2000). This approach was considered a more practical alternative, primarily due to the lack of knowledge regarding carbon partitioning in sugarcane (Muchow *et al.*, 1996). Furthermore, sugar accumulation was not the main factor contributing to the development of these models. APSIM-Sugarcane is based on an agronomically soil-centric modeling system (Keating & Huth, 1995), while CANEGRO was originally designed to help determine the optimal harvesting age due to the widespread predation of sugarcane by the stalk borer *Eldana saccharina* in the South African sugar industry (Inman-Bamber, 1995). As such, both models have shown limitations in ability to accurately predict sucrose yield, due to both an apparent lack in complexity and mechanistic explanation (Keating *et al.*, 1999; O'Leary, 2000; Singels & Bezuidenhout, 2002).

A more mechanistic source-sink approach was originally pursued by Inman-Bamber (unpublished data) for the CANEGRO model, but without success. However, based on the limitations of contemporary sugarcane models, it has since been suggested that the inclusion of a source-sink component may improve the accuracy of sucrose predictions (O'Leary, 2000). Recently, a model for dry matter partitioning, based on environment-linked rate changes in partitioning, rather than just states, such as crop age or time of year, has been suggested (Singels & Bezuidenhout, 2002). This model attempts to bring source-sink dynamics into the CANEGRO model by basing growth algorithms on a variety of source-sink concepts and definitions. The exact meaning of sink strength is still contentious (Ho, 1988; Chamont, 1993), however Singels & Bezuidenhout (2002) have included several aspects of the sink to develop partitioning variables for this model. Sugarcane stalks are defined in terms of sink activity (SSR), sink capacity (FCAP) and sucrose sink strength (ΔSUC). FCAP represents the potential structural growth when

source strength is not limited, while SRR represents the ability of the crop to fill the FCAP. The product of SSR and FCAP result in the overall structural sink strength of the stalk (ΔFNS), such that ΔSUC can be defined by the equation ΔSUC = ΔSK – ΔFNS, where ΔSK represent the daily change in stalk mass. Interestingly, in this model, source strength is defined as the ability of crop canopy to export carbon to the stalk, and thus ΔSK can be taken as the accumulated source strength over the crop life span and is thus a reflection of source history (Singels & Bezuidenhout, 2002).

Validation tests for this source-sink model showed large improvements in both stalk and sucrose mass predictions. Inclusion of partitioning variables into the CANEGRO model has since been suggested to improve yield forecasting, and management support for harvest and irrigation Furthermore, in the model, changes in daily environmental conditions cause a fluctuation in FCAP, allowing the model to simulate subtle sink-related responses to external factors, such as water stress and temperature shifts on a large scale (Singels & Bezuidenhout, 2002).

That model represents a step forward towards accurate modelling of the complexities of the source-sink relationship in sugarcane. Instead of relying simply on plant states, the model has introduced the concept of rate changes in carbon accrual and partitioning. Currently however, the model does not include variables accounting for competition for carbon between roots, stalk and meristematic tissue, or possible feedback from those sinks. Such improvements may be of particular help in predicting yield changes resulting from *Eldana* infestation or crop performance on different soils. As such, FCAP fluctuations may be linked not only to environmental shifts, but also internal changes in metabolism.

Future advancement in modeling the source-sink relationship may include a more detailed understanding of sink demand at the enzymatic level (Rohwer & Botha, 2001). However, a mechanistic model of any system needs to be done with a thorough awareness of the processes involved. This requires prudent use of applied knowledge regarding what to include and what to leave out. For example, if too many of the included parameters are estimated by fitting to observations, a model can be made to perform in any way (Minchin & Lacointe, 2004). As such, a minimalist description of a complex system is often the recommended approach to identify the dominant regulatory processes (Young, 1999; Minchin & Lacointe, 2004). It will be fascinating to see how

future models will include both physiological measurements and the ever-increasing wealth of molecular data to interpret and predict plant growth and development.

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Chapter 3:
Sink strength regulates photosynthesis in sugarcane

3.1 Summary

- The relationship in sugarcane between photosynthetic source tissue and sink material was examined through manipulation of the sink:source ratio of field-grown *Saccharum* spp. hybrid *cv.* N19 (N19).
- To enhance sink-strength, all leaves, except for the third fully-expanded leaf, were enclosed in 90% shade cloth for varying periods of time. Variations in sucrose, glucose and fructose concentrations were measured and the effects of shading on the leaf gas exchange and fluorescence characteristics recorded. Changes in carbon partitioning due to shading were examined based on the uptake and translocation of fixed $^{14}CO_2$.
- Following a decline in sucrose concentrations in young internodal tissue and shaded leaves, significant increases in the CO_2 saturated photosynthetic rate (J_{max}), carboxylation efficiency (*CE*) and electron transport rate were observed in unshaded leaves after 8 d of shading treatment.
- It was concluded that up-regulation of source leaf photosynthetic capacity is correlated with a decrease in assimilate availability to acropetal culm sink tissue. Furthermore, a significant relationship was revealed between source hexose concentration and photosynthetic activity.

Keywords: C_4, partitioning, photosynthesis, sink, source, sucrose, sugarcane

3.2 Introduction

The accumulation of phenomenal levels of sucrose by sugarcane has been the focus of intense study (Moore, 1995; Lakshmanan *et al.*, 2005; Moore, 2005). Sugarcane is a C_4 species that accumulates high sucrose concentrations in the mature internodes with less accumulation in younger internodes. The differences in sucrose accumulation between young and mature culm tissues are the consequence of varying rates of cycling of sucrose between vacuole, cytosol and apoplasm (Sacher *et al.*, 1963; Batta & Singh, 1986). Much research has focussed on culm-specific processes (Whittaker & Botha,

1997; Casu *et al.*, 2003; Walsh *et al.*, 2005), but the integration of source (photosynthetic) and storage (culm) processes in plants is still not fully understood (Koch *et al.*, 2000; Pego *et al.*, 2000).

For many plant species, the activities of source photosynthetic production and sink growth appear to be closely co-ordinated, such that a balance is maintained between source supply and sink demand (Wardlaw, 1990; Ho, 1992; Foyer *et al.*, 1995). Evidence increasingly supports a sink-dependent relationship (Paul & Foyer, 2001), whereby sink-strength influences the net photosynthetic activity and carbon status of source organs (Paul *et al.*, 2001). Apart from possible feedback through product accumulation, there is increasing evidence that the activity of photosynthesis-related enzymes and expression of associated gene transcripts is modified by sink demand (Sheen, 1990; Sheen, 1994; Black *et al.*, 1995; Koch, 1996; Pego *et al.*, 2000; Paul & Foyer, 2001; Rolland *et al.*, 2002).

Although there have been limited studies on sugarcane focusing on the relationship between source and sink tissue (Marcelis, 1996; Pammenter & Allison, 2002), in various other plant species the dominant influence of sink activity on source photosynthesis and carbon partitioning has been demonstrated. In *Solanum tuberosum*, a high sink demand in the form of rapidly growing tubers caused increased rates of photosynthesis (Dwelle *et al.*, 1981) and enhanced translocation of photosynthate (Moorby, 1978). Removal of the tubers led to a marked decrease in net photosynthesis due to the imbalance between source and sink activity (Nosberger & Humphries, 1965). Irrespective of the presence or absence of water stress conditions, plants with artificially lowered sink-strength (tuber excised) accumulated carbohydrate in the leaves and displayed a considerably reduced maximum photosynthetic rate (A_{max}), electron transport rate (ETR) and quantum yield (F_v/F_m) (Basu *et al.*, 1999). Cold girdling of the leaves of *Citrus unshiu* to reduce carbon export and defruiting have also reduced rates of photosynthesis (A), and this reduction coincided with an accumulation of carbohydrate in the source leaf (Iglesias *et al.*, 2002). Sugar accumulation in leaves also represses the expression of photosynthetic genes (Sheen, 1990). In transgenic *Nicotiana tabacum* leaves, the expression of a yeast invertase in the cell wall resulted in increased carbohydrate content, especially soluble sugars, which gradually inhibited photosynthetic levels as sugars accumulated (Von Schaewen *et al.*, 1990; Stitt *et al.*, 1991). Similarly, mature

leaves of *Spinacia oleracea* supplied with glucose through the transpiration stream lost Rubisco activity over a 7 d period (Krapp *et al.*, 1991).

In sugarcane, the sucrose accumulating processes within the maturing stem are likely to be strong sinks for photoassimilate (Marcelis, 1996). Sucrose accumulation in the sugarcane culm has recently been shown to receive high priority in the allocation of assimilates (Pammenter & Allison, 2002). Coincidently, large differences in photosynthetic rates have, in the past, been reported for individual sugarcane leaves related to the age of the plant, with young plants typically assimilating at significantly higher rates than older plants (Hartt & Burr, 1967; Bull & Tovey, 1974). Gross photosynthesis has been found to be lower in eight-month-old sugarcane plants compared to four-month-old plants, regardless of the light intensity (Allison *et al.*, 1997). Another study reported that three-month-old sugarcane exhibited photosynthetic rates of 45 μmol m^{-2} s^{-1} under intense illumination, while young leaves on ten-month-old plants only photosynthesised at 25 μmol m^{-2} s^{-1} (Amaya *et al.*, 1995).

In plants, sugar status modulates and coordinates growth and development (Smeekens, 2000) and, although the regulatory role of sugar on photosynthesis and metabolism is well known, progress has only recently been made in determining the molecular mechanisms of sugar sensing and signaling (Rolland *et al.*, 2002; Gibson, 2005). Components of sugar sensing systems that have been identified include glucose, sucrose and trehalose sensing systems. For example, hexokinase (HXK) functions as a glucose sensor that modulates gene expression and sucrose non-fermenting 1 (Snf1)-related protein kinases (SnRKs), which are known to have diverse functions in carbon metabolism and sugar signaling (Rolland *et al.*, 2002). Since the details of sink regulation of photosynthetic source relations in C_3 plants are only now emerging, the picture is even less clear in the more complex C_4 species, such as sugarcane. In part, regulation of C_4 photosynthesis is achieved through compartmentation of the process between mesophyll and bundle sheath cells and control of metabolite transfers through a set of cell-specific organelle metabolite translocators (e.g. dicarboxylic acid transporters) together with symplastic connections (Edwards *et al.*, 2001). Various specialisations have been demonstrated for C_4 leaf carbon metabolism, including bundle sheath cell-specific storage of starch for a range of species (Downton & Tregunna, 1968; Laetsch, 1971; Lunn & Furbank 1997) and preferential localisation of genes involved in sucrose biosynthesis in the mesophyll cells (Lunn & Furbank, 1999). Studies continue to

uncover new aspects of the control mechanisms involved in C_4 photosynthesis (Kubien *et al.*, 2003; von Caemmerer *et al.*, 2005), however little is known about the unique regulatory interactions that determine assimilatory flux in C_4 plants, such as sugarcane. However, many of the controls elucidated for C_3 systems also operate in C_4 plants (Sheen, 2001); for example carbamylation of Rubisco by Rubisco activase has been shown to be essential for photosynthesis in the C_4 dicot, *Flaveria bidentis* (von Caemmerer *et al.*, 2005).

The existence of a sugar-dependent relationship between source and sink tissues in sugarcane could represent a potentially fundamental limiting factor for sucrose accumulation in the stalk and consequently play a major role in overall sucrose accumulation and crop yield. In the current study, the relationship between photosynthetic source tissue and sink material was examined through manipulation of sink demand and total sink-strength in field-grown sugarcane. To artificially increase sink-strength by manipulating the sink:source ratio, all leaves, except for the third fully expanded leaf, were enclosed in 90% shade cloth. In this way leaves that served as source were converted to sinks, producing an overall increase in plant sink-size. The effects on gas exchange characteristics and PSII efficiency were investigated and changes in photosynthesis were explained on the basis of leaf sugar levels and variations in sugar partitioning based on the uptake of a $^{14}CO_2$ label.

3.3 Materials and methods

3.3.1 Plant material

Nine- to twelve-month-old field-grown *Saccharum* spp. (L.) hybrid *cv.* N19 (N19) cultivated at Mount Edgecombe, KwaZulu-Natal, South Africa, on a 5 x 15 m plot was used in this study, which was conducted during summer (December 2004). The plot was located on a north-east facing slope with a gradient of *ca.* 10^{o}.

3.3.2 Manipulation of sink capacity

To increase plant sink:source ratios, all leaves except the third fully-expanded leaf (leaf 6) (Fig. 3.1) were covered in a black sleeve constructed from 90% shade cloth. Leaf 6 was chosen as the most suitable intermediate between mature and maturing stalk tissue. Shade cloth was selected so as not to totally impede gas flow to the plant or to elicit changes in photomorphogenesis. Treated plants were selected based on similar height and stalk width, and were separated by at least two unshaded plants to negate potential shading effects of the shade cloth on neighboring plants. Treatments were applied between 1 and 14 d prior to the measurements and sampling, effectively rendering leaf 6 the sole light receiving source for photosynthetic carbon assimilation for this variable period prior to analysis. Light conditions were measured regularly throughout the experiment using a LI-6400 portable photosystem unit (LI-COR Biosciences Inc., Nebraska, USA) to ensure that leaf 6 from control and partially shaded plants received similar levels of light exposure. These plants that had been shaded for variable periods of time were compared to “control” plants that were not shaded.

3.3.3 Sugar determination

Following shading treatments for 1, 3, 6 and 14 d, treated and unshaded plants (n=7) were concurrently harvested at 12h00. In this way, all plants were exposed to the same environmental factors immediately prior to harvest. To decrease the risk of potential sucrose hydrolysis, time taken between harvest and processing was kept to a minimum. Stalks were kept intact and internodes 4, 6, 8, 10 and 12 were excised sequentially from top to bottom. The rind was removed and the underlying tissue, spanning the core to the periphery of the entire internode, was cut into small pieces (*ca.* 2 x 5 mm). Leaf material representing the meristematic leaf roll (designated leaf 0), first fully-expanded leaf (designated leaf 3) and third fully expanded leaf (designated leaf 6) was sliced thinly. Tissues were then milled in an A11 Basic Analysis Mill (IKA®, Staufen, Germany) and frozen in liquid nitrogen (–196° C). The samples were stored in 50 ml centrifuge tubes at –80° C. Prior to analysis, leaf and culm tissues were incubated overnight in 20 volumes of sugar extraction buffer (30 mM HEPES [pH 7. 8], 6 mM $MgCl_2$ and ethanol 70% [v/v]) at 70° C. Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and

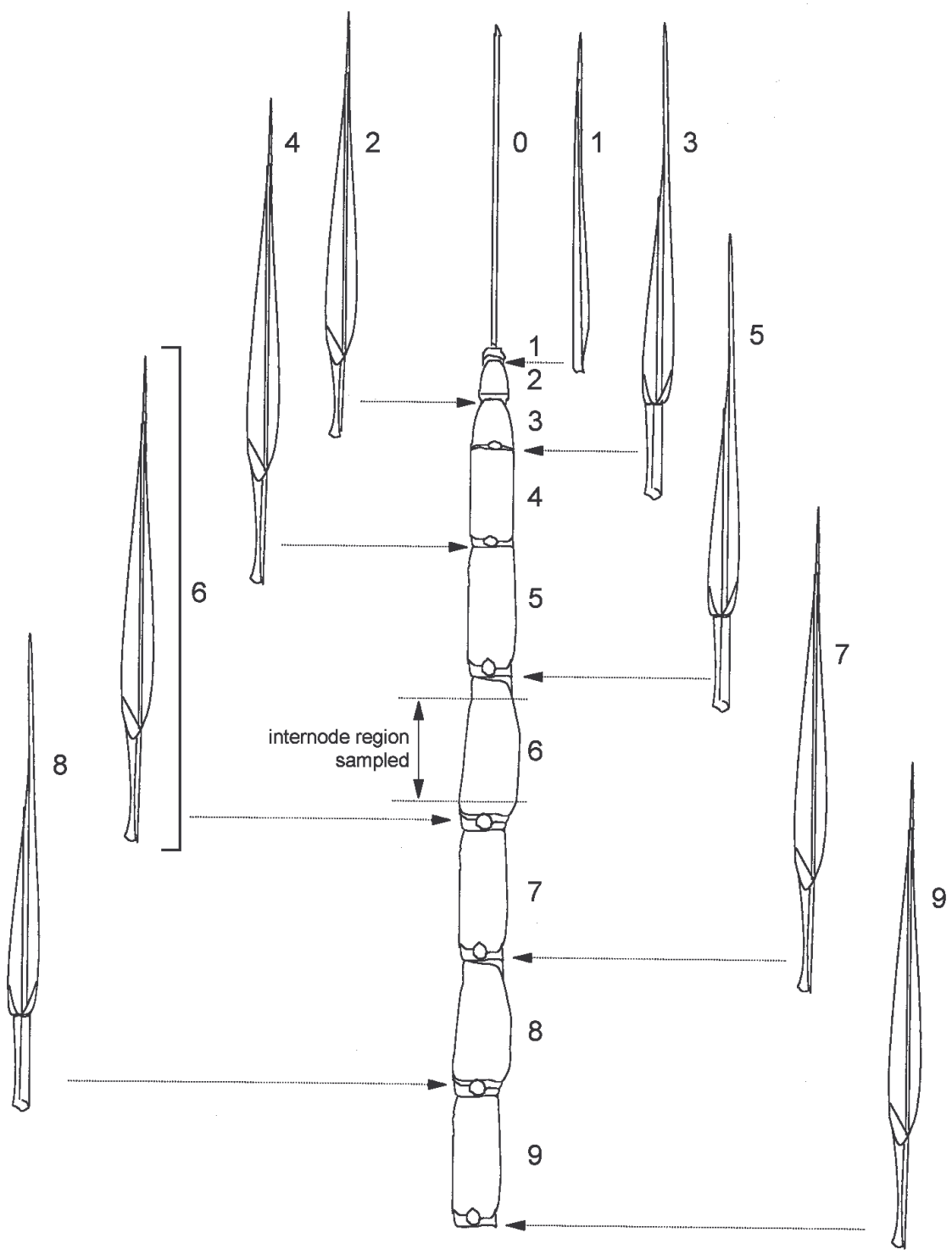

Fig. 3.1. The upper section of a sugarcane stalk showing leaves 1 to 9 and internodes 1 to 9. Leaves are consecutively numbered and attached to the bottom of their corresponding internode. The third fully-expanded leaf (leaf 6) is indicated in brackets. Adapted from van Dillewijn (1952).

glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic coupling assay modified from Jones *et al.* (1977). The phosphorylation of glucose by hexokinase/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Roche Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5.3.1.9) (Roche) was quantified by following the reduction of $NADP^+$ to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader (Biotek Instrument, Inc., Winooki, VT, USA) using KC4 software (Biotek Instrument, Inc), respectively.

3.3.4 Gas exchange and fluorescence determinations

A LI-6400 portable photosystem unit was used to measure photosynthetic assimilation (A), transpiration rate (E), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and leaf temperature of leaf 6 between 9h00 and 12h00. Comparative measurements were performed on the day of harvest for plants that were unshaded or had previously been partially shaded for 1 to 14 d (n=4). Partially shaded plants were further measured over a period of 2, 4 and 8 d (n=4). The latter experiment was repeated at least once to confirm results. The response of A to C_i (A:C_i) was measured by varying the external CO_2 concentration from 0 to 1 000 µmol mol^{-1} under a constant photosynthetically active radiation (PAR) of 2 000 µmol m^{-2} s^{-1}. An equation $A = a\left(1 - e^{(-bC_i)}\right) - c$ was fitted to the A:C_i data using least squares. The portion of the curve where the slope approaches zero due to limitation in the supply of substrate (ribulose-1,5-bisphosphate), which is equivalent to the CO_2- and light-saturated photosynthetic rate(J_{max}) (Lawlor, 1987), was calculated from this equation (a, J_{max}; b, curvature parameter; c, dark respiration (R_d)). Linear regression was performed on the data between a C_i of 0 and 200 µmol mol^{-1} to determine the efficiency of carboxylation (CE; Lawlor, 1987). The assimilation rate in the absence of stomatal limitations (A_a) was as calculated as A interpolated from the response curve at C_i = 380 µmol mol^{-1}.

Chlorophyll fluorescence was determined concurrently with A:C_i gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0.8 s, 6 000 µmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm') at varying external CO_2 concentrations (0 - 1 000 µmol mol^{-1}). The electron transport rate (ETR), defined as the actual flux of photons

driving photosystem II (PSII) was calculated from $ETR = \left(\frac{Fm' - Fs}{Fm'} \right) fI\alpha_{leaf}$, where *Fs* is "steady-state" fluorescence (at 2 000 µmol m^{-2} s^{-1}), *Fm'* is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by PSII, typically assumed to be 0.4 for C_4 plant species (Edwards & Baker, 1993), *I* is incident photon flux density and α_{leaf} is leaf absorptance (0.85, LI-COR manual). The component fluorescence variables were derived as described by Maxwell & Johnson (2000).

3.3.5 $^{14}CO_2$ labelling

The influence of shading treatments on carbon allocation was measured by supplying leaf 6 of unshaded and partially shaded plants (4 d and 10 d) (n=3) with $^{14}CO_2$ using a protocol modified from Hartt *et al.* (1963). A portion of leaf (5 x 20 cm) weighing approximately 5 g was sealed in an air-inflated polythene bag containing 50 µl $NaH^{14}CO_3$ (specific activity, 55 mCi $mmol^{-1}$, ICN Radiochemicals, Irvine, CA, USA) to which 1 ml 10% (v/v) lactic acid was added to release $^{14}CO_2$. The sealed bags were then gently palpated to ensure equilibration of released $^{14}CO_2$ and even distribution of uptake over the leaf surface. After 1 h, bags were removed and a leaf disc (*ca.* 10 mg) of the labelled region of leaf 6 was excised and stored in liquid nitrogen. The plants were harvested 24 h after $^{14}CO_2$ supply and tissue samples milled in an A11 Basic Analysis Mill (IKA®) and incubated overnight in twenty volumes of sugar extraction buffer (30 mM HEPES [pH 7. 8], 6 mM $MgCl_2$ and ethanol 70% [v/v]) at 70°C. The radioactivity in the 70% (v/v) alcohol-soluble component was measured with a Tri-Carb Liquid Scintillation Analyzer (Packard, Massachusetts, USA) using Ultima Gold™ XR (Packard, Milford, MA, USA).

Labelled sugars in the 70% alcohol-soluble component were spotted onto 10 x 20 cm silica gel plates (Merck, Darmstadt, Germany) using a semi-automatic Thin Layer Chromatography (TLC) sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland) and fractionated using a mobile phase consisting of 50% ethyl acetate (v/v), 25% acetic acid (v/v), and 25% filter-sterilized water for 3 h. Silica plates were dried at 70°C for 10 min, sealed in polyethylene film and exposed to high-resolution phosphor screens (Packard). After 24 h exposure, the images on the phosphor screens were captured and analysed by means of a Cyclone Storage Phosphor Screen imaging system (Packard) using Optiquant Ver. 03.10 (Packard).

3.3.6 Statistical analysis

Results were subjected to analysis of variants (ANOVA) or Student's *t* tests to determine the significance of difference between responses to treatments. When ANOVA was performed, Tukey's honest significant difference (HSD) *post-hoc* tests were conducted to determine the differences between the individual treatments (SPSS Ver. 11.5, SPSS Inc., Chicago, IL, USA). SPSS was also used to calculate the Pearson's correlation coefficients for correlation analyses.

3.4 Results

3.4.1 Effect of source: sink variations on sugar levels

Glucose concentrations in the unshaded leaf (leaf 6) declined over the duration of the shading treatment (Fig. 3.2), whereas fructose concentrations remained constant until day 6, declining subsequently. Apart from a temporary increase in sucrose after 6 d, there were no changes in sucrose concentration in leaf 6. Sucrose concentrations in shaded leaf 3 decreased over the initial 24 h period, and then remained constant at 21 μmol g^{-1} (Fig. 3.2). No significant changes were observed in glucose or fructose levels of leaf 3 over time.

Internodes above and below leaf 6 responded differently to the shading treatment, although internode 6 had consistently higher concentrations of hexoses and sucrose than internode 4 (Fig. 3.2). A comparable decline in sucrose levels over time was seen in both internodes 4 and 6; however, this trend was stronger in internode 4. There were no significant changes in hexose concentrations in internodes 4 and 6.

Sucrose levels in mature internodes (internode 8, 10 and 12) were consistently highest in internode 12, followed by internodes 10 and 8 (Fig. 3.2). Sucrose concentrations decreased at day 3 across all mature internodes, followed by a sharp increase at day 6. This trend was more substantial in internodes closer to leaf 6. Hexose levels in internodes 8 and 10 also declined significantly during the course of the time treatments while hexose levels in internode 12 remained constant throughout. Levels of hexose in

mature internodes were consistently highest in internode 8, followed by internodes 10 and 12 respectively.

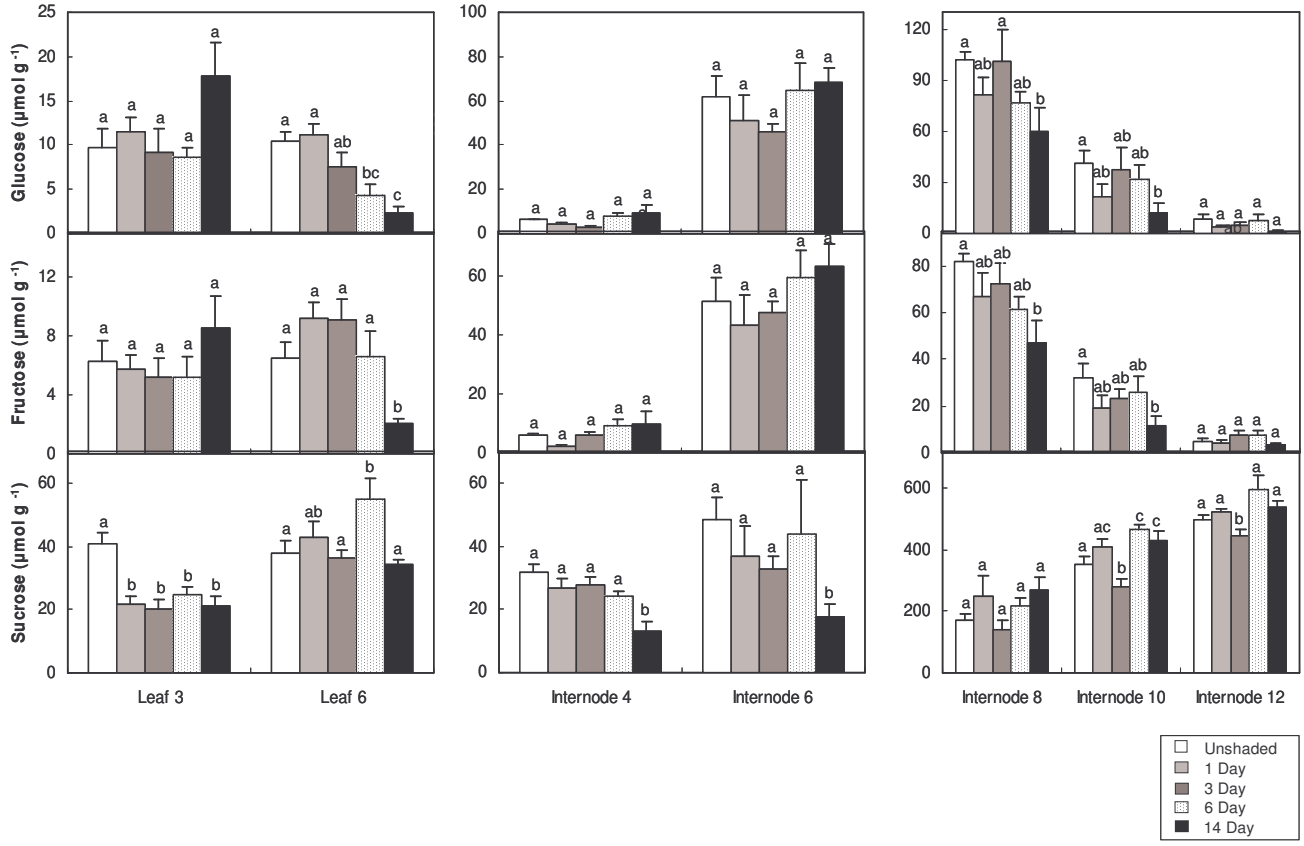

Fig. 3.2. Glucose, fructose and sucrose (µmol g^{-1} FW) measurements for field-grown N19 plants that were unshaded (0 d) and partially shaded (leaf 6 not shaded) for 1, 3, 6 and 14 d prior to sampling (n=7). All plants were harvested and processed concurrently. Sugar levels are shown for leaf 6 and leaf 3; internode 4 and internode 6; internode 8, internode 10 and internode 12. Letters above the SE bars indicate whether the treatment had a significant influence within each tissue type ($P<0.05$) as determined by ANOVA followed by Tukey's honestly significant difference (HSD) tests.

3.4.2 *Partial shading effect on ^{14}C partitioning*

The ^{14}C detected in a sample of leaf 6 immediately after the 1 h feeding period was statistically indistinguishable between the sample groups (unshaded, 4d or 10 day partially shaded) although variations might have been anticipated due to variation in total

leaf size and weight (Table 3.1). After the 24 h chase period, the amount of ^{14}C in leaf 6 was significantly less in the shaded than in the unshaded treatments. The allocation of ^{14}C labelled assimilate to the leaf roll and leaf 3 (shaded) increased with increased duration of shading. Likewise, the amount of ^{14}C allocated to internodes 4 also increased with increased duration of shading. In contrast, the amount of label in internode 8 was reduced by shading, while internode 10 received a small amount of label, which was not influenced by the shading treatment. The amount of ^{14}C recovered from internode 12 was negligible (data not shown).

Shading (4 d and 10 d) prior to $^{14}CO_2$ exposure reduced the amount of sucrose retained by leaf 6. However, labelled hexose levels in leaf 6 only showed a significant reduction in plants previously shaded for 10 d (Fig. 3.3). Shading caused a significant increase in ^{14}C allocation to sucrose in the leaf roll, leaf 3 and in internodes 4 and 6. In contrast, there was a reduction in allocation to sucrose in internode 8 after 4 d of shading. Changes in ^{14}C-hexose concentrations were generally smaller than the changes in ^{14}C-sucrose. However, shading increased ^{14}C allocation to hexoses in leaf 3 and internode 6, but reduced allocation to ^{14}C-hexose in internode 8.

Table 3.1. Incorporation and distribution of a ^{14}C label in field-grown sugarcane plants that were either unshaded or previously partially (leaf 6 not shaded) shaded for either 4 or 10 d prior to exposure to $^{14}CO_2$. The plants were supplied with 100 µCi $^{14}CO_2$ to leaf 6 followed by a 24 h chase period. The means ± standard errors (kBq g^{-1} FW, n=3) are followed by letters indicating for each tissue type whether the treatments had a significant influence ($P<0.05$) as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests.

Tissue	Unshaded	4 days	10 days
Leaf 6 * (kBq g^{-1} FW)	789.3 ± 146 a	668.6 ± 114 a	569 ± 38 a
Internode 4	1.5 ± 0.7 a	2.2 ± 0.5 a	2.7 ± 0.3 b
Internode 6	1.7 ± 0.9 a	2.6 ± 0.8 a	2.4 ± 0.5 a
Internode 8	2.7 ± 0.1 a	0.8 ± 0.1 b	1.1 ± 0.4 b
Internode 10	0.8 ± 0.4 a	0.9 ± 0.1 a	0.8 ± 0.3 a
Leaf Roll	1.5 ± 0.4 a	3 ± 0.3 b	5 ± 1.3 b
Leaf 3	0.3 ± 0.1 a	3.2 ± 2.3 a	6.1 ± 1.0 b
Leaf 6	55.5 ± 6.8 a	32.3 ± 12.4 a	32.9 ± 1.8 b

Leaf 6* samples taken directly after labelling.

FW, fresh weight.

3.4.3 Source leaf photosynthesis and sugar correlations

Photosynthetic gas exchange characteristics and leaf chlorophyll fluorescence activities were determined on leaf 6 of unshaded plants and plants partially shaded for 1, 3, 6 and 14 d (Fig. 3.4). For partially shaded plants, a striking increase in both photosynthetic assimilation (A) and electron transport rate (ETR) across all C_i values was observed over the duration of the shading. Interestingly, plants shaded for 6 d exhibited a 37% higher J_{max} compared to day 3 (Fig. 3.4). This was associated with a significant increase in leaf sucrose levels between day 3 and 6 (Fig. 3.2). The gas exchange variables and leaf ETR variables derived from A:C_i and ETR:C_i curves increased over the duration of the shading treatment (Table 3.2). After 8 d, significant increases in the substrate-limited photosynthetic rate ($J_{max,}$ 42%) and carboxylation efficiency (CE, 28%) were observed in comparison to unshaded plants, while the assimilation rate (A) and assimilation rate in the absence of stomatal limitation (A_a) were 48% and 51% higher, respectively, than in unshaded plants. The ETR of leaf 6 at ambient CO_2 was also elevated by 29% after 8 d.

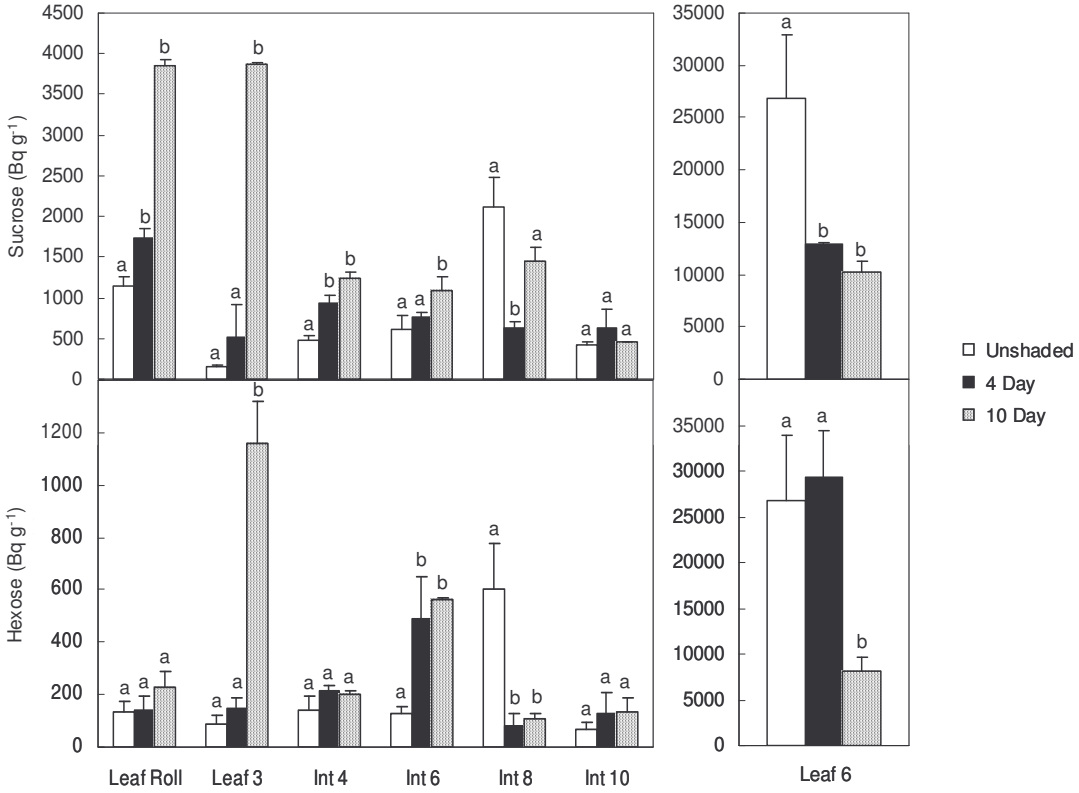

Fig. 3.3. Allocation of ^{14}C label (Bq g^{-1}) to hexose and sucrose pools of various tissues for field-grown N19 sugarcane plants either unshaded or previously partially (leaf 6 not shaded) shaded for 4 or 10 d prior to exposure to $^{14}CO_2$ (n=3). Bars represent labelled hexose or sucrose 24 h after label incorporation (mean ± SE). See Table 3.1 for $^{14}CO_2$ labelling details. Letters above SE bars indicate whether the treatment had a significant ($P<0.05$) influence within each tissue type as determined by ANOVA followed by post-hoc Tukey's honest significant difference (HSD) tests.

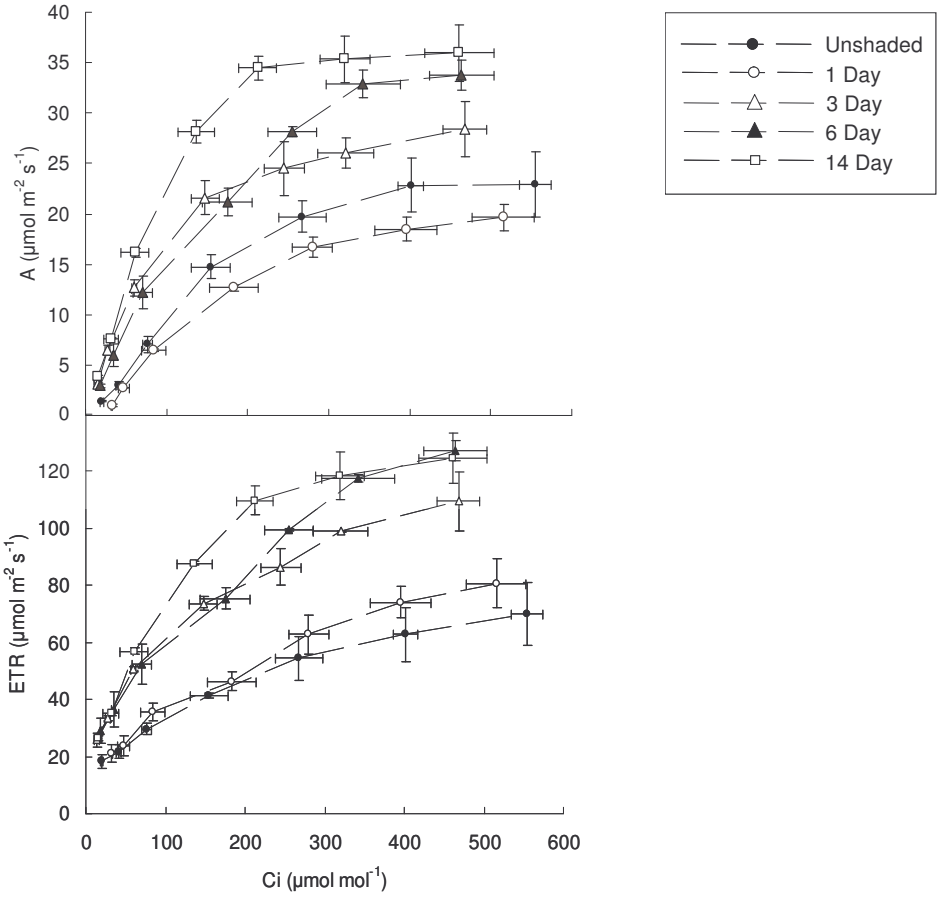

Fig. 3.4. Changes in photosynthetic CO_2 assimilation (µmol m^2 s^{-1}) and photosynthetic electron transport rate (ETR) for unshaded leaf 6 versus intercellular CO_2 concentration (C_i, µmol mol^{-1}) for different times (1 to 14 d) from initiation of shading for both unshaded and partially shaded (all leaves shaded except for leaf 6) twelve-month-old field-grown N19 sugarcane (n=4). Measurements were made at an average ambient RH of 44.6% ± 3.6 and an irradiance of 2000 µmol m^{-2} s^{-1}.

Table 3.2. Variables from A:C_i curves based on gas exchange analysis and leaf fluorescence of leaf 6 from unshaded and partially (leaf 6 not shaded) shaded (2, 4 and 8 d) twelve-month-old field-grown N19 sugarcane: substrate supply limited assimilation (J_{max}), dark respiration (R_d), carboxylation efficiency (CE), photosynthetic rate in the presence (A_i) and absence of stomatal limitation (A_a), stomatal conductance (G_s), intercellular CO_2 concentration at ambient CO_2 (C_i at C_a = 380) and electron transport rate (ETR) at C_a = 380. Measurements were performed over a period of 8 d at an ambient RH of 35.9% ± 0.8 (mean ± SE) and an irradiance of 2000 µmol m^{-2} s^{-1}. The shade treatment values are the mean ± SE (n=4) and are followed by letters indicating for each tissue type whether the treatments had a significant influence ($P<0.05$), as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests. For each measurement, unshaded plants were measured as controls, but there were no significant differences between the photosynthetic parameters for the unshaded plants over time and thus the overall mean ± SE (n=12) is presented for this group.

Photosynthetic parameter	Unshaded	2d	4d	8d
J_{max} (µmol m^{-2} s^{-1})	22.7 ± 2.0 a	23.4 ± 1.1 a	29.7 ± 4.5 a	32.2 ± 0.7 b
R_d (µmol m^{-2} s^{-1})	3.7 ± 0.8 a	2.2 ± 0.3 a	2.6 ± 0.3 a	2.3 ± 0.4 a
CE (mmol m^{-2} s^{-1})	129 ± 36.6 a	130 ± 39 a	153 ± 34 a	164.5 ± 5.4 b
A_a (µmol m^{-2} s^{-1})	18 ± 2.5 a	19.3 ± 0.7 a	21.2 ± 0.4 b	27.1 ± 0.6 b
A_i (µmol m^{-2} s^{-1})	13.8 ± 2.6 a	14.2 ± 1.1 a	16.3 ± 3.0 a	20.4 ± 0.5 b
G_s (mmol m^{-2} s^{-1})	131 ± 26 a	137 ± 10 a	119 ± 27 a	145 ± 7 a
C_i at C_a = 380 (µmol mol^{-1})	162 ± 11.8 a	168 ± 9 a	167 ± 19 a	187 ± 17 a
ETR at C_a = 380 (µmol mol^{-1})	55 ± 6.2 a	62 ± 3.0 a	67 ± 8.1 a	71 ± 2.0 b

A comparison between changes in sugar levels and photosynthetic activity variables of unshaded leaf 6 over 14 d of shading treatment further revealed a strong negative correlation between hexose concentrations in source leaf tissue, and J_{max} and CE (Fig. 3.5). This relationship was not evident for these variables with sucrose. Further analysis revealed significant relationships between sucrose and hexose concentrations, and leaf 6 photosynthesis levels in the internodal tissue sampled (Table 3.3; Fig. 3.6). All sampled internodes produced a positive correlation between glucose and fructose levels. Immature sink tissue (internodes 4 and 6) was characterised by decreased sucrose levels which were correlated with an increase in J_{max} (and CE for internode 4) over the 14 d period. The decreased hexose concentrations in internode 8 were negatively correlated with both sucrose and photosynthetic variables J_{max} and CE, while

the increased levels of sucrose in mature internodes (10 and 12) were positively correlated with changes in *CE*.

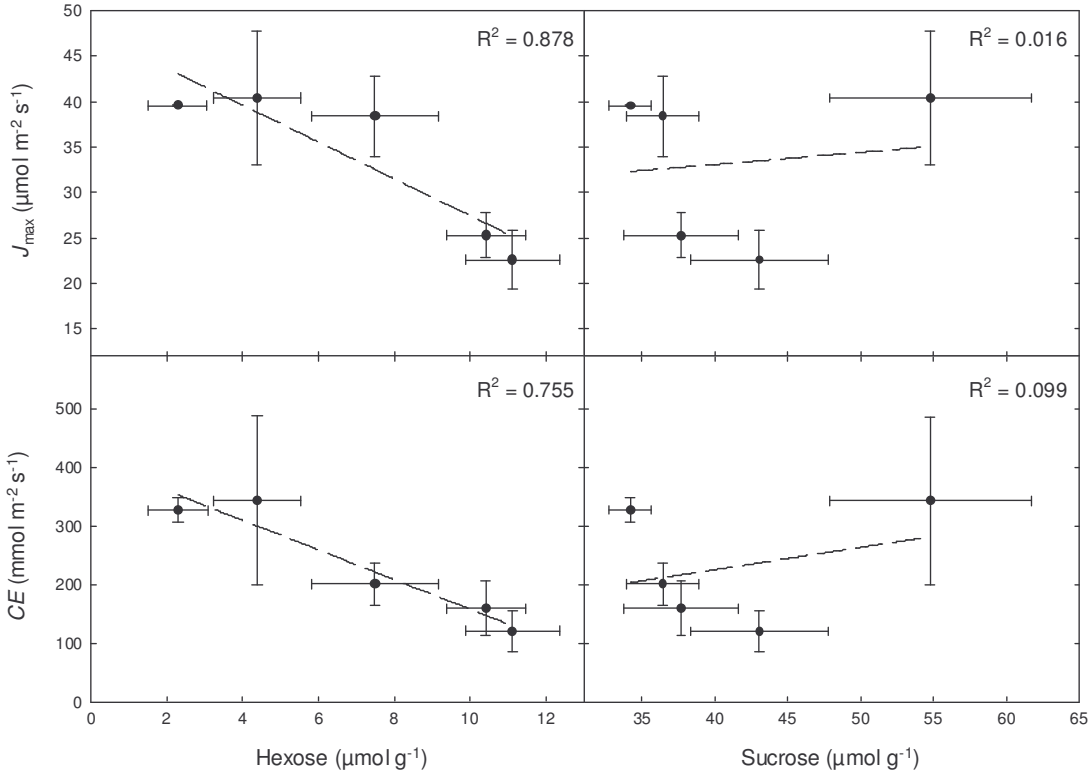

Fig. 3.5. Correlations between sugar concentration (hexose and sucrose) and photosynthetic gas exchange variables (substrate supply limited assimilation, J_{max}; carboxylation efficiency, *CE*) for leaf 6 of field-grown sugarcane (hybrid cv. N19) either unshaded (0 d) or partially shaded 1, 3, 6, 14 d.

Table 3.3. Bivariate Pearson's correlation coefficients between sugar concentrations (sucrose, glucose, fructose) of leaf or culm tissue and photosynthetic variables J_{max} and *CE* (see Table 3.2 for variable declarations) of leaf 6 for field-grown N19 sugarcane either unshaded or partially shaded for between 1 and 14 d. Significance levels (P) are reported for the Pearson's correlation coefficients (in brackets).

	Leaf 6	Leaf 3	Int 4	Int 6	Int 8	Int 10	Int 12
Glucose:Fructose	0.66 (0.00)	0.79 (0.00)	0.85 (0.00)	0.96 (0.00)	0.74 (0.00)	0.76 (0.00)	0.82 (0.00)
Sucrose:Hexose			-0.34 (0.04)		-0.61 (0.00)	-0.36 (0.03)	
Hexose:J_{max}	-0.67 (0.00)		0.39 (0.02)		-0.36 (0.04)		
Hexose:*CE*	-0.74 (0.00)		0.37 (0.03)		-0.35 (0.04)		
Sucrose:J_{max}			-0.61 (0.00)	-0.34 (0.04)			
Sucrose:*CE*			-0.59 (0.00)			0.40 (0.02)	0.38 (0.03)

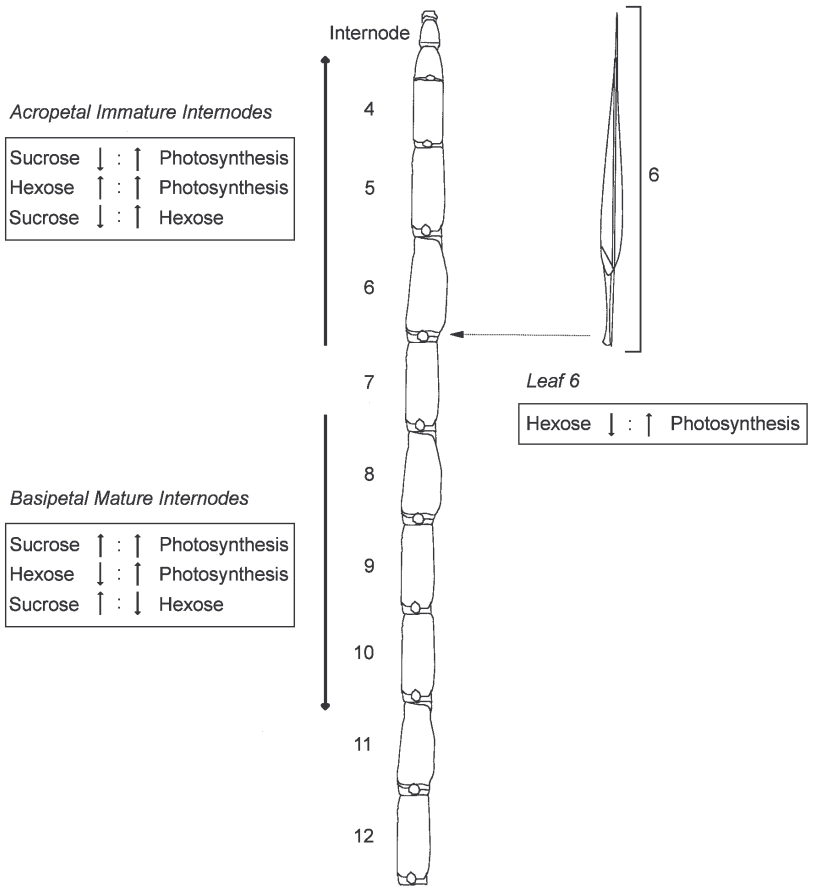

Fig. 3.6. Diagram illustrating the relationship between changes in sugar concentrations and leaf photosynthetic activity. Arrows represent significant (P<0.05) linear correlations between paired variables, according to Pearson's correlation coefficient analysis.

3.5 Discussion

Although sucrose concentrations in internodes followed the well-established pattern of greater sucrose concentration in older internodes (Whittaker & Botha, 1997), the manipulation of sink-strength through partial shading produced significant changes in plant sugar levels and assimilate partitioning. In general, young internodal tissue was characterized by low levels of hexose and shading treatments produced a decrease in sucrose over time. This trend was more evident in the youngest internode sampled (internode 4) than in internode 6, which may be due to the location of internode 6 relative to leaf 6, and/or the lower rate of sucrose accumulation observed in younger internodal tissue (Whittaker & Botha, 1997). Overall, shading produced an initial (day 3) decrease in sucrose concentrations in internodes 10 and 12. Shading also resulted in a reduced sucrose concentration in shaded leaf 3 after 24 h that was sustained for the duration of the treatments. The relatively short period of time taken to reduce sucrose levels in both shaded leaf and internodal tissue indicated that, for sugarcane, partial shading was a practical means to evaluate the effects of changes in sink demand on the remaining unshaded source material.

Leaf 6 of unshaded plants distributed more ^{14}C to internode 8 than internodes 6, 4, 10 and 12 (in order of diminishing distribution). Thus shading of the entire plant, except leaf 6, would seem likely to reduce the sugar levels in all other internodes more significantly than internode 8. Label analyses of partially shaded plants further revealed a significant shift in ^{14}C partitioning to immature culm and shaded leaf tissue. Distribution of ^{14}C allocated to sucrose followed a similar pattern to total $^{14}CO_2$ distribution, while trends for labelled sucrose and hexoses were generally comparable (Fig. 3.3), indicating sucrose and hexose as the two major pools for labelled assimilate.

The observed shifts in assimilate partitioning for partially shaded plants emphasise the roles of phloem loading at the source (van Bel, 1993; 2003) and unloading at the sink (Patrick, 1997; Walsh *et al.*, 2005) as crucial links in the sink:source relationship (Kühn *et al.*, 1999). Shading treatments produced a significant drop in the total sucrose pools of shaded leaf and immature culm tissue, while ^{14}C analysis confirmed a shift in assimilate partitioning to these tissues, which are typically not supplied by leaf 6 (MacDonald, 2000). Plants partially shaded for 4 d showed ^{14}C allocation patterns for treated plants prior to any significant change in photosynthesis in leaf 6, while 10 d

shaded plants showed distribution after adaptation of leaf 6 to changes in sink demand. The increased allocation of leaf 6-derived ^{14}C to the leaf roll and leaf 3 of shaded plants further indicated prioritisation of young leaf tissues as sinks for carbon from leaf 6. This change in typical leaf 6 partitioning patterns not only indicated a sink-strength-related response to the decreasing levels of sucrose measured in young shaded leaf tissue, but also a change in physiological state from source to sink. Such an event is not uncommon in infected leaves following pathogen attack, where an increase of import to the infected sites occurs (Farrar, 1992; Wright *et al.*, 1995; Ayres *et al.*, 1996), but has not yet been reported in shading experiments. While no overall change in sucrose was observed in leaf 6 after shading for 14 d, a decreased level of ^{14}C-labelled sucrose after 24 h was evident in plants shaded for both 4 d and 10 d. As partial shading did not produce any significant variation in stomatal conductance in leaf 6, reduced labelled sucrose might be indicative of increased sucrose turnover and a higher assimilate transport rate in the phloem of treated plants. This study has thus illustrated the ability of the phloem transport of sugarcane to respond to changes in environment and alter assimilate translocation patterns between various sink and source tissues. These results substantiate the role of sucrose as a signaling molecule in assimilate partitioning (Chiou & Bush, 1998), however, the signaling mechanisms which link phloematic, apoplastic and intracellular sucrose concentrations remain to be fully elucidated (Gibson, 2005).

The changes observed in sugar levels over time in maturing internodes (8, 10 and 12; Fig. 3.2) are indicative of the many complex factors influencing the overall physiological environment of the plant in shading treatments. Although partial shading produced an acropetal shift in assimilate partitioning from leaf 6 to younger internodes and young leaf tissue, the effect of this on the overall sugar content of mature internodes would be further confounded by the acclimation of leaf 6 to increased sink demand over time and the overall drop in available assimilate for the entire plant. Shading treatments would additionally influence plant water relations. Assuming that water loss from shaded leaves was reduced, this would increase water potential (Ψ_P) and possibly reduce the flow of nutrients to culm and leaf tissue. This could influence shaded leaf and root metabolic activities which could in turn reduce the overall demand for CH_2O and consequently affect carbon accumulation in mature internodes which typically supply root tissue. As a number of factors may thus affect mature internodal tissue under the present shading treatment, more detailed study is required before accurately interpreting

the observed changes and correlations between sugar levels and photosynthesis observed.

Significant increases in photosynthetic rate, carboxylation efficiency and PSII efficiency were measured in leaf 6 over the duration of the shading treatment. A significant linear relationship was further elucidated between maximum photosynthetic assimilation rates (J_{max}) of leaf 6 and decreasing levels of sucrose in immature culm tissue (internodes 4 and 6) over the partial shading time treatments. This supports evidence that decreased sucrose at the sink is a likely physiological signal to the source for increased assimilate requirements (van Bel, 2003). A similar effect has previously been observed in pot-grown sugarcane plants, where partial defoliation resulted in only small decreases in culm dry mass (Pammenter & Allison, 2002). However, the dramatic photosynthetic increase in leaf 6 observed here may have been compounded by the sustained presence and required maintenance of other leaves. Furthermore, the depletion or excess of sugars has previously been shown to respectively activate or repress the expression of genes for photosynthetic components and ultimately influence photosynthesis itself (Stitt, 1991; Krapp *et al*., 1993; Van Oosten & Besford, 1994; 1995; Basu *et al*., 1999). The plasticity of leaf assimilation capacity over time observed in sugarcane may thus be linked to regulation of C_4 leaf metabolism at the molecular level, such as regulatory phosphorylation of PEPc activity (Vidal & Chollet, 1997) and/or adjustments in several other C_4 photosynthetic control mechanisms (Furbank & Taylor, 1995). It is important to note that this study has 'simulated' an increase in plant sink strength via an increased demand for carbon from leaf 6. Thus, although it is feasible that the overall sink activity of internodal tissue my have, in fact, declined due to the lack of source supply, this research has provided evidence for the physiological ability of the source to adapt to increased sink requirements. The *Saccharum* complex is potentially capable of storing more than 25% sucrose on a fresh weight basis (Bull & Glasziou, 1963; Moore *et al*., 1997). As this estimate is still almost double current commercial yields (Grof & Campbell, 2001), further understanding of source regulation may assist in the eventual utilisation of a greater portion of the potential sink strength of sugarcane.

Interestingly, no relationship was observed between sucrose levels in either unshaded or shaded leaves, and photosynthesis in this study. These results are comparable to studies on maize leaves, where changing sucrose concentrations were shown to have no significant short-term feedback inhibitory effects on the synthesis of sucrose itself in

the leaf (Lunn & Furbank, 1997). Instead, a strong negative correlation was found between hexose and photosynthetic gas exchange variables J_{max} and *CE* in unshaded leaf 6, which implicated hexoses, rather than sucrose, as possible signal factors involved in photosynthetic feedback regulation. In the past, hexoses have been shown to be inhibitors of photosynthesis (Goldschmidt & Huber, 1992). For example, the external supply of glucose (50 mM) to excised *Spinacea oleracea* leaves over 4 d lead to inhibition of the light harvesting complex (LHC) II-encoding chlorophyll a/b binding protein (*cab*) genes and a 60% decrease in Rubisco content (Kilb *et al.*, 1995). Thus in sugarcane, a decreased leaf glucose pool could constitute a signal of increased demand from sinks. More recently, hexoses have been shown to play an important role in regulating photosynthesis and leaf development (Ehness *et al.*, 1997; Paul & Pellny, 2003). Hexokinase has been implicated as a putative receptor (Jang et al., 1997), however, the mechanisms involved in hexokinase sensing remain contentious. It has also been demonstrated that glucose itself, and not an analogous phosphorylated metabolite, may be the primary signal that interacts with some putative receptor involved in transduction of the carbohydrate signal (Ehness et al., 1997). Progress has been made (Rolland *et al.*, 2002), but further efforts will be required to fill in the gaps in this complex network, especially for C_4 species. Compared to C_3 species, relatively little is known about the control of sugar biosynthesis in the leaves of C_4 plants, however, sugar induced changes in gene expression are likely to be as important in C_4 as in C_3 in balancing sink:source interactions (Lunn & Furbank, 1999).

Although it is likely that the hexose concentrations in the leaf tissue are under strict metabolic control, it will ultimately be difficult to elucidate the actual mechanisms of hexose responses, as sugars can act by affecting osmotic potentials as well as by functioning as signal molecules (Gibson, 2005). This may be further complicated by the interactions between carbon and nitrogen levels in leaf developmental processes (Paul & Pellny, 2003). For instance, the application of moderate (111 mM) concentrations of glucose stimulates the senescence of *Arabidopsis*, but only under limited nitrogen conditions (Wingler *et al.*, 2004). Furthermore, since most plants can synthesise sucrose when fed with hexoses, it is difficult to attribute the effects of hexoses to their direct sensing, as sucrose sensing could possibly occur. However, our correlations indicate that, although sucrose must play a key role in regulating sink assimilate partitioning, hexoses may be a more proximal component of the signaling mechanism between photosynthetic source activity and sink requirements in sugarcane. The

regulatory effects of hexose sensors such as HXK are well documented in C_3 plants (Rolland *et al.*, 2002) and may provide a useful starting point for examining the control of photosynthesis in C_4 sugarcane.

3.6 Concluding remarks

Sugarcane, like other plants, exhibits a robust soluble carbohydrate-dependent relationship between source and sink tissue based on assimilate demand and partitioning needs. This study has provided good evidence for a sink-dependent relationship between source and sink tissues as well as an important role for sugars in this relationship. Further study will be required to substantiate the molecular basis of these correlations, specifically the observed effects of hexose on photosynthesis, as the molecular pathways involved in regulating such a relationship are not yet fully understood. Future research may include comparison of different sugarcane cultivars, however, the general patterns observed here with the model N19 hybrid are likely to pertain for other cultivars. The existence of sink regulation of source activity in sugarcane should inform biotechnological efforts to modify culm metabolism to improve sugar accumulation. The fact that sink demand limits source activity indicates that the signal feedback system reporting sink sufficiency and regulating source activity may be important loci for investigation/modification in sugarcane. We are currently attempting to elucidate which genes and enzymes in sugarcane leaves are responsive to changes in the sink:source ratio, with particular emphasis on links between photosynthesis and sugar sensing/signaling.

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Chapter 4:
Changes in leaf gene expression during a source-sink perturbation in sugarcane

4.1 Abstract

In crops other than sugarcane there is good evidence that the size and activity of carbon sinks influence source activity via sugar-related regulation of the enzymes of photosynthesis, an effect that is partly mediated through coarse regulation of gene expression. In the current study, leaf shading treatments were used to perturb the source-sink balance in nine-month-old *Saccharum* spp. hybrid *cv.* N19 (N19) by restricting source activity to a single mature leaf. Changes in leaf photosynthetic gas exchange variables and leaf and culm sugar concentrations were subsequently measured over a 14 d period. In addition, the changes in leaf gene response to the source-sink perturbation were measured by reverse Northern hybridisation analysis of an array of 128 ESTs related to photosynthetic and carbohydrate metabolism. Sucrose concentrations in immature culm tissue declined significantly over the duration of the shading treatment, while a 57% and 88% increase in *A* and ETR, respectively, were observed in the source leaf. Several genes (27) in the leaf displayed a greater than two-fold change in expression level, including the up-regulation of several genes associated with C_4 photosynthesis, mitochondrial metabolism and sugar transport. Changes in gene expression levels of several genes, including Rubisco (EC 4.1.1.39) and hexokinase (HXK; EC 2.7.1.1), correlated with changes in photosynthesis and tissue sugar concentrations that occurred subsequent to the source-sink perturbation. These results indicate that sink demand may limit source activity through a kinase-mediated sugar-signaling mechanism that correlates to a decrease in source hexose concentrations, which, in turn, correlate with increased expression of genes involved in photosynthesis and metabolite transport. The signal feedback system reporting sink sufficiency and regulating source activity may be a potentially valuable for target for future genetic manipulation to increase sugarcane sucrose yield.

Keywords: hexose, gene, leaf, photosynthesis, sucrose, sugarcane

4.2 Background and aims

Sugarcane (*Saccharum* L. spp. hybrids) is the most important source of sucrose worldwide and accounts for more than 70% of global sucrose production (Lunn and Furbank, 1999). It has been suggested that the accumulation of high concentrations of sucrose in sugarcane is regulated principally at the level of the sink, where the balance between simultaneous synthesis and degradation of sucrose, often referred to as futile-cycling, is believed to be responsible for overall sucrose accumulation (Sacher *et al.*, 1963; Batta and Singh, 1986; Whittaker and Botha, 1997). The high sucrose level (up to 650 mM) in storage tissues of some *Saccharum* spp. hybrids (Welbaum and Meinzer, 1990) makes it an important model genus in the study of the interactions between source (leaf) and sink (culm) tissues. However, sugarcane presents a major challenge for such studies, as sucrose is stored in the culm parenchyma tissue and not in specialized storage organs. As such, identifying and interpreting events regulating sucrose partitioning is hampered by the fact that the storage organs are also the primary growth sink.

In addition to improving biomass yield, increasing the concentration of sucrose in the culm is a key objective of most sugarcane breeding programmes. However, recent improvements to sugarcane varieties have been achieved almost entirely through increased cane yield rather than increased culm sucrose content (Jackson, 2005). Attempts to increase sucrose accumulation depend on a thorough understanding of sucrose metabolism, transport and source-sink interactions that govern sucrose accumulation. Despite extensive research, the dynamics and interactions amongst these processes are not well characterised and are only beginning to be explored (Carson and Botha, 2002; Watt *et al*., 2005; Casu *et al*., 2007).

Strategies to increase sucrose concentrations in sugarcane have focused on the manipulation of single enzymes involved in culm sucrose metabolism, primarily those catalyzing sucrolytic reactions (Lakshmanan *et al*., 2005). These genes include those encoding the various isoforms of invertases (EC 2.7.1.90) (Ma *et al*., 2000; Botha *et al*., 2001) and pyrophosphate-dependent phosphofructokinase (PFP; EC 2.7.1.90) (Groenewald and Botha, 2007). The mixed success of these attempts may be due to the ability of plants to physiologically compensate for small changes in their genetic environment (Halpin *et al*., 2001; Luguang and Birch, 2007). A kinetic model of sucrose

metabolism in sugarcane culm tissue, developed by Rohwer and Botha (2001), predicts only a limited control on sucrose metabolism for individual genes widely regarded as having a crucial regulatory role. Generally, control over a metabolic flux is shared by several enzymes of a pathway and large increases in flux cannot be expected from the manipulation of single enzymes, but rather several sites on the pathway (Fell and Thomas, 1995). Without a better understanding of the underlying mechanisms that govern feedback regulation, both within metabolic pathways and between source and sink activity, it may prove difficult to identify potential targets for the effective manipulation of stalk sucrose content.

A key step in understanding the control of sucrose accumulation in sugarcane will be to unravel the complex metabolic and signaling networks that mediate the source-sink relationship. The physiological nature of this relationship has previously been examined in C_3 (Fellows *et al.*, 1979; Wright *et al.*, 1995; Basu *et al.*, 1999; Borrás and Otegui, 2001; Minchin *et al.*, 2002; Franck *et al.*, 2006) and C_4 species (McCormick *et al.*, 2006 [Chapter 3]) and good evidence now exists to support a sink-dependent relationship, whereby carbon levels in storage organs influence the net photosynthetic activity and carbon assimilation of source leaf tissues (Paul and Foyer, 2001; Paul and Pellny, 2003). There is also increasing evidence that the activity of photosynthesis-related enzymes and expression of associated gene transcripts in the leaf, among others, are modified by the local status of the primary transport sugar, sucrose, and/or its constituent hexoses (Pego *et al.*, 2000; Rolland *et al.*, 2002; Franck *et al.*, 2006). However, the mechanisms whereby sugars act to regulate source gene expression are just beginning to be discovered (Rolland *et al.*, 2002; Gibson, 2005), and for C_4 plants, including sugarcane, these remain relatively unexamined (Lunn and Furbank, 1999). It is essential that research into source-sink relationships in C_4 plants is directed towards the identification of regulatory elements unique to C_4 plants and examines metabolism across hierarchical scales, from the molecular (transcript, enzyme and metabolites) to the crop level (Edmeades *et al.*, 2004).

Study of sugarcane physiology has revealed that the demand for carbon from source tissues is related to the sucrose concentration and age and condition of sugarcane culms (Hartt and Burr, 1967; Marcelis, 1996). Gross photosynthesis is higher in eight-month-old sugarcane plants compared to four-month-old plants, regardless of light intensity (Allison *et al.*, 1997). Furthermore, three-month-old sugarcane leaves have

photosynthetic rates of 45 μmol m^{-2} s^{-1} under intense illumination, while young leaves of ten-month-old plants have a maximum rate of 25 μmol m^{-2} s^{-1} (Amaya *et al.*, 1995). Investigations using partial defoliation and shading techniques, revealed the existence of a bias in carbon allocation during growth towards culm sucrose accumulation, at the expense of structural growth (Pammenter and Allison, 2002). Gutiérrez-Miceli *et al.* (2004) have additionally demonstrated that partial defoliation of sugarcane plants produces no significant change in culm sucrose concentration compared to control plants, indicating that the remaining intact leaves were capable of maintaining a nominal supply of carbon based on the demand from sink tissues. In a recent study in which source activity was limited to a single leaf by a shading treatment, a significant increase in photosynthetic rates was observed in the sole source leaf, which was, in turn, negatively correlated with sucrose concentrations in the immature culm tissue (McCormick *et al.*, 2006 [Chapter 3]). Although providing good evidence for sink-regulation of photosynthesis in sugarcane, these studies did not address the molecular mechanisms that mediate communication between the source and sink in sugarcane.

Good progress has been made recently in the use of expressed sequence tags (ESTs) as a tool to examine gene expression in sugarcane. EST analysis has been used to examine gene expression behaviour during culm development, with associated increases in sucrose accumulation (Carson and Botha 2002; Grivet and Arruda, 2002; Casu *et al.*, 2004; Watt *et al.*, 2005). However, while such approaches provide valuable information, they may be ineffective as a sole means to identify factors regulating sucrose storage in the sugarcane culm (Watt *et al.*, 2005). Focus on gene regulation during culm maturation does not provide insights into feed-back mechanisms that may operate between the culm and leaf. Hence, experimental systems that permit the study of the expression of genes associated with carbohydrate metabolism in parallel to that of overall plant physiological responses may provide a means to detect mechanisms that mediate the relationship between source and sink tissues (Edmeades *et al.*, 2004).

In the current study on mature sugarcane plants, shading treatments were used to restrict source activity to a single leaf, thereby perturbing the source-sink balance. Gas exchange variables and tissue sugar concentrations were measured in parallel to reverse Northern macroarray analysis, which was used to determine relative changes in mRNA expression levels in the sole source leaf over 14 day period following the source-sink perturbation. A set of ESTs representing 128 genes of photosynthesis and

carbohydrate metabolism was used in the gene expression analyses, which were subsequently correlated to changes in photosynthesis and tissue sugar concentrations. This study represents an attempt to determine the changes in the expression of carbohydrate metabolism-related genes that are associated with a source-sink perturbation.

4.3 Methods

4.3.1 Plant material

Twelve-month-old field-grown *Saccharum* spp. hybrid *cv.* N19 (N19), cultivated at Mount Edgecombe, KwaZulu-Natal, South Africa on a 5 x 15 m plot, was used in this study, which was conducted during December 2004. The plot was located on a north-east facing slope with a slope of *ca.* 10°. Tissue from the third fully expanded leaf (leaf 6) and culm tissue (internodes 4–6) were sampled as described previously (McCormick *et al*., 2006 [Chapter 3]). The harvested material was immediately frozen in liquid nitrogen (–196°C). The frozen tissue was then reduced to powder using an A11 Basic Analysis Mill (IKA®) and stored in 50ml polypropylene centrifuge tubes (Corning®) at –80°C until used for further experimentation.

4.3.2 Plant treatment

To modify plant source-sink balance, all leaves except leaf 6 of seven plants per treatment were enclosed in a black sleeve made of 90% shade cloth. Shade cloth was used so as not to totally impede gas flow to the plant or to cause photomorphogenic effects. Treatments were carried out for 1, 3, 6 and 14 d, effectively rendering leaf 6 the sole light receiving source for photosynthetic carbon assimilation over these periods. Treated plants were selected based on similar height and stalk width, and were separated by at least two unshaded plants to negate potential shading effects of the shade cloth on neighbouring plants. Control plants were completely unshaded. Light conditions were checked daily throughout the experiment using a LI-6400 portable photosystem unit (LI-COR Biosciences Inc., Lincoln, NE, USA) to ensure that leaf 6 from control and treated plants received similar levels of light exposure. The start of the

shading treatments was staggered so that leaves and culms from all treatments were harvested on the same day at 12h00.

4.3.3 Sugar determination

Approximately 100 mg powdered tissue was incubated overnight at 70°C in 10 volumes of sugar extraction buffer consisting of 30 mM HEPES (pH 7. 8), 6 mM $MgCl_2$ and ethanol 70% (v/v). Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic-coupled assay modified from Jones *et al.* (1977). The phosphorylation of glucose by hexokinase/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Roche, Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5.3.1.9) (Roche) was quantified by following the reduction of $NADP^+$ to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader using KC4 software (Biotek Instrument, Inc., Vermont, USA).

4.3.4 Gas exchange and fluorescence determinations

A LI-6400 portable photosystem unit (LI-COR Biosciences Inc., Lincoln, NB, USA) was used to measure photosynthetic assimilation (*A*), transpiration rate (*E*), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and leaf temperature of the source leaf. Gas exchange measurements were made on 2 cm^2 portions of leaf tissue. Light was provided by a red/blue LED light source (LI-COR Biosciences Inc.) at photon irradiance of 2 000 µmol m^{-2} s^{-1}. All leaf measurements were performed at a constant leaf temperature of 28°C.

Measurements were performed between 9h00 and 12h00 on the day of harvest for plants that were unshaded or had previously been partially shaded for 1 to 14 d (n=4). The response of *A* to C_i (*A*:C_i) was measured by varying the external CO_2 concentration from 0 to 1 000 µmol mol^{-1} under a constant photosynthetically active radiation (PAR) of 2 000 µmol m^{-2} s^{-1}. An equation $A = a\left(1 - e^{(-bC_i)}\right) - c$ was fitted to the *A*:C_i data using least squares. The portion of the curve where the slope approached zero due to limitation in the supply of substrate (ribulose-1,5-bisphosphate), which is equivalent to the CO_2- and light-saturated photosynthetic rate (J_{max}) (Lawlor, 1987), was calculated

from this equation (a, J_{max}; b, curvature parameter; c, dark respiration (R_d)). Linear regression was performed on the data between a C_i of 0 and 200 µmol mol^{-1} to determine the efficiency of carboxylation (*CE*) (Lawlor, 1987). The assimilation rate in the absence of stomatal limitations (A_a) was as calculated as *A*, interpolated from the response curve at C_i = 380 µmol mol^{-1}.

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0.8 s, 6000 µmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm') at varying external CO_2 concentrations (0 – 1 000 µmol mol^{-1}). The electron transport rate (ETR), defined as the actual flux of photons driving photosystem II (PSII) was calculated from $ETR = \left(\frac{Fm' - Fs}{Fm'} \right) fI\alpha_{leaf}$, where *Fs* is "steady-state" fluorescence (at 2 000 µmol m^{-2} s^{-1}), *Fm'* is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by PSII, typically assumed to be 0.4 for C_4 plant species (Edwards and Baker, 1993), *I* is incident photon flux density and α_{leaf} is leaf absorptance (0.85, LI-COR manual). The component fluorescence variables were derived as described by Maxwell and Johnson (2000).

4.3.5 Array target preparation

Target cDNA populations were prepared using mRNA isolated from leaf 6 of unshaded plants (control) and partially shaded plants where leaf 6 was the sole source leaf for 1, 3, 6 and 14 d. Total RNA was extracted using a modified extraction protocol from Bugos *et al.* (1995). RNA concentration and quality were calculated from ultra-violet (UV) spectrophotometric absorbance measurements using standard photometric equations (Beckman DU-7500 spectrophotometer, USA) and confirmed via agarose gel electrophoresis (Ingelbrecht *et al.*, 1998). RNA samples from each time treatment (n=6) were then pooled. To permit accurate and quantifiable comparisons between the array signal intensities produced from each sample, RNA samples (100 µg) were then spiked with two internal mRNA standards, *A. thaliana* Rubisco activase (RCA; GenBank accession no. X14212) and *A. thaliana* Rubisco large subunit (rbcL; GenBank accession no. U91966), each at 0.5 ng as per the SpotReport®-3 Array Validation System (Stratagene, La Jolla, CA, USA). Poly A$^+$ RNA (mRNA) was isolated from each RNA sample using a Dynabeads Oligo $(dT)_{25}$ mRNA Purification kit (Dynal, Oslo, Norway).

Single stranded (ss) cDNA fragments, radiolabelled with a α^{33}P dCTP (Amersham BioSciences, UK), were then generated from each mRNA population (1 µg) using a LabelStar™ Array Kit (QIAGEN, Hilden, Germany). Unincorporated dNTPs were removed with a LabelStar™ Array Cleanup Module (QIAGEN).

4.3.6 Array probe preparation and printing

A suite of 128 carbohydrate metabolism and photosynthesis-related expressed sequence tags (ESTs) were collected to probe cDNA populations synthesised from total leaf mRNA [**Supplementary Information**]. EST homologies were confirmed by gel analysis and random partial sequence analysis. Sequences were cross-referenced to the accession numbers of the putative identities contained within the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). Arrays were printed onto a positively charged nylon Hybond-N+ membrane® (Amersham, Germany) by means of a 96-pin manual gridding device (V&P Scientific Inc., San Diego), which facilitated the transfer of aliquots (2 µl) of each sample at a final concentration of 10 ng. For replication, aliquots of each probe were delivered to two adjacent addresses on the array at two randomly selected locations, permitting a total of four query events per probe. External standards were integrated into each array (10 ng), including three heterologous bacterial genes encoding a δ-endotoxin crystal protein (*cry1A(b)*), phosphothricin acetyltransferase (*bar*) and β-glucoronidase (*gus*), and human Cot-1 DNA®, with the latter standard being from the SpotReport®-3 Array Validation System. Furthermore, two internal standards (RCA and rbcL, SpotReport®-3 Array Validation System) were included at final concentrations of 1, 10, 50 or 100 ng**.** 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.

4.3.7 Array querying and analysis

Array membranes were incubated for approximately 18 hours in 20ml Church and Gilbert buffer (0.5 M sodium phosphate (pH 7.2); 7% (w/v) SDS; 0.94 mM EDTA) (Church and Gilbert, 1984) containing 10 µg ml^{-1} denatured fragmented salmon or herring sperm DNA (Sigma-Aldrich Inc., MO, USA). Incubation was performed at 65^{o} C in 300 ml volume hybridisation bottles within a Hybaid Micro-4 rotary hybridisation oven

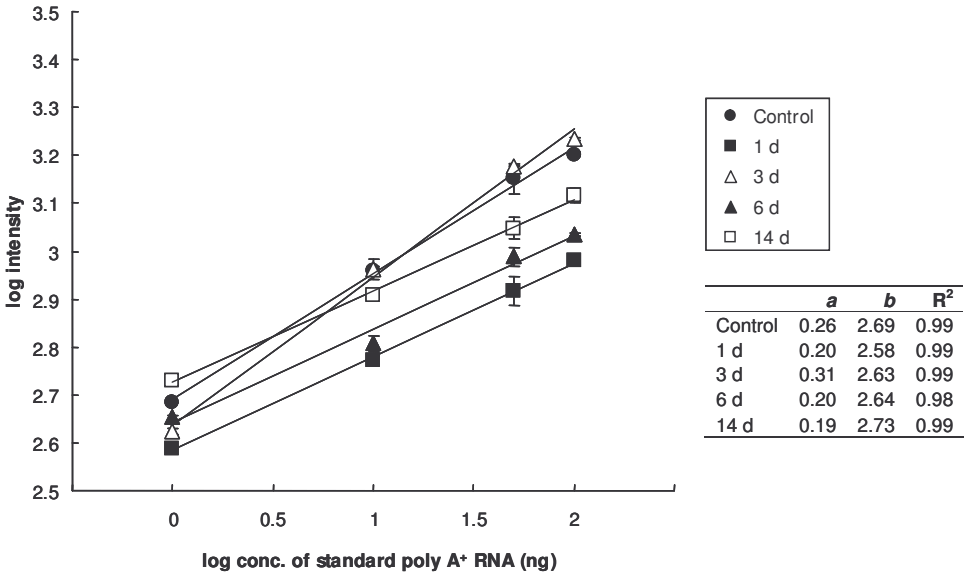
(Hybaid Ltd., UK). After prehybridisation, the original solution was discarded and an aliquot of fresh, pre-warmed (65°C) hybridisation buffer containing the cDNA target population was added in the absence of denatured salmon or herring sperm DNA. Following overnight hybridisation at 65°C, the membranes were washed twice in aliquots of 1X SSC (155 mM tri-sodium citrate; 150 mM NaCl), 0.1% (w/v) SDS solution for 10–20 min until unbound labelled target cDNA were removed.

Arrays were exposed to high-resolution Cylcone phosphor screens (Packard Instruments Company, Connecticut, USA) and captured by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard Instruments Company). Array images were analysed using QuantArray® MicroArray Analysis Software (version 3.0, Packard Bioscience). This software was used to quantify the spot hybridisation intensity and corresponding background intensity for each of the probes contained on the array membranes in response to each querying event. Images were visually inspected to identify spots with poor morphology or high local background. These spots were flagged and omitted from further analysis. Array data sets, representing spot and background intensity values, were then imported into Excel spreadsheets for evaluation and comparative analysis.

Overall background consistency was validated by confirming that the coefficient of variation (CV) for the mean background intensity between all query events was less than 10% for each array data set. Based on the average ratio of background to spot intensity of external standard query events, a lower intensity-specific threshold was established (Yang *et al*., 2002), below which probe query events were excluded from further analysis. Following individual background subtraction from query events, replicate query events were compared and excluded from inter-array comparison if their CV exceeded 5%. Standard curves were then generated for each treatment group from signal intensity data derived from the internal standard query events (Fig. 4.1). To normalise between data from differing arrays, the equation $C = e^{\left(\frac{\ln I - b}{a}\right)}$ was used, where I is average query event intensity and C is expression level relative to the internal control (a and b are curvature parameters). FiRe software was used to detect differential expression between treatments (FiRe Ver. 2.2, Fribourg, Switzerland) (Garcion *et al*., 2006). To reduce the possibility of generating false-positive results, only query events with a greater than two-fold change in expression between treatments were considered for further correlation analysis.

4.3.8 Statistical analysis

Results were subjected to analysis of variants (ANOVA) or Student's *t* tests to determine the significance of difference between responses to treatments. When ANOVA was performed, Tukey's honest significant difference (HSD) *post-hoc* tests were conducted to determine the differences between the individual treatments (SPSS Ver. 11.5, SPSS Inc., Illinois, USA). SPSS was also used to calculate the Pearson's correlation coefficients for correlation analyses.



	a	*b*	R^2
Control	0.26	2.69	0.99
1 d	0.20	2.58	0.99
3 d	0.31	2.63	0.99
6 d	0.20	2.64	0.98
14 d	0.19	2.73	0.99

Fig. 4.1. Standard curves generated from the average log intensity values of two mRNA standards that were spiked into the total cDNA population during the array query events (*A. thaliana* Rubisco activase (RCA) and Rubisco large subunit (rbcL)). Each array contained DNA probe standards at concentrations of 1, 10, 50 and 100 ng. The SE bars represent the average of four intensity values. Curvature parameters *a* and *b* were used for normalisation between array data sets.

4.4 Key results

4.4.1 Effect of source: sink variations on sugars and photosynthesis

Sugars levels, photosynthetic gas exchange characteristics and leaf chlorophyll fluorescence activities were determined on the sole source leaf of the partially shaded

plants and the corresponding leaf of control plants at days 1, 3, 6 and 14. Hexose concentrations in leaf 6 of partially shaded plants declined over the duration of the shading treatment (Fig. 4.2). Apart from an increase in sucrose detected at 6 d, there were no significant changes in sucrose concentration in leaf 6. In immature internodal tissue, a decline in sucrose over time was observed, while there were no significant changes in hexose concentrations.

A significant increase in maximum photosynthetic assimilation rates (J_{max}), carboxylation efficiency (*CE*) and electron transport rate (ETR) measured at ambient CO_2 (380 µmol m^2 s^{-1}) were observed over the duration of the source-sink perturbation (Table 4.1). Of note is that plants shaded for 6 d exhibited a 37% higher J_{max} compared to day 3, which was associated with a significant increase in leaf sucrose levels over the same period (Fig. 4.2).

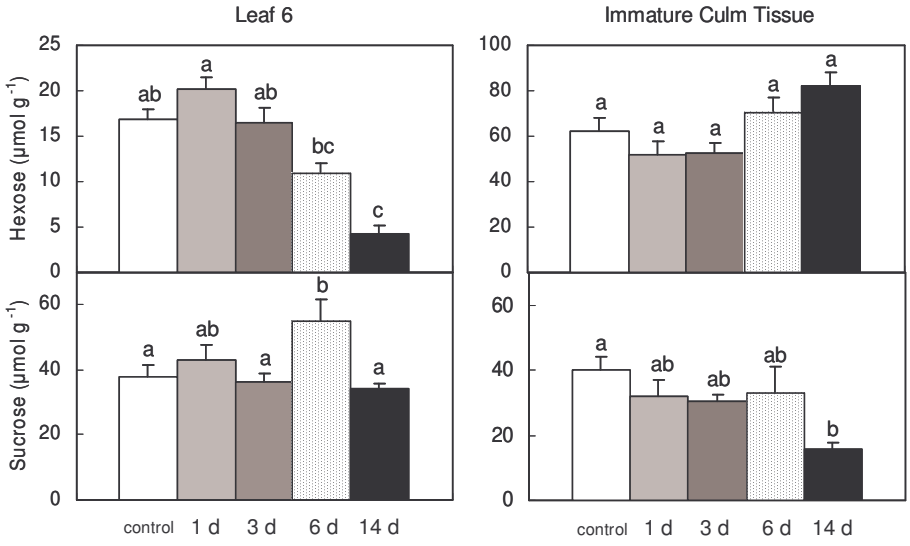


Fig. 4.2. Comparison of sugar levels in the leaves and immature culm of plants subjected to a source-sink perturbation. Hexose and sucrose (µmol g^{-1} FW) measurements for field-grown plants that were completely unshaded (control) and those in which all but leaf 6 (sole source leaf) were shaded for 1, 3, 6 and 14 d prior to sampling (n=7). All plants were harvested and processed concurrently. Sugar levels are shown for leaf 6 and immature culm tissue (internodes 4–6). Letters above the SE bars indicate whether the treatment had a significant influence within each tissue type ($P<0.05$) as determined by ANOVA followed by Tukey's honestly significant difference (HSD) tests.

Table 4.1. Variables from A:C_i curves based on photosynthetic gas exchange and chlorophyll fluorescence variables following a source-sink perturbation. Measurements were taken on leaf 6 of plants in which all the other leaves had been shaded for 1, 3, 6 and 14 d. The control represents measurements taken on leaf 6 of the plants not subjected to shading treatments. All measurements were taken on the same day. Abbreviations are as follows: substrate supply limited assimilation (J_{max}), dark respiration (R_d), carboxylation efficiency (CE), photosynthetic rate in the presence (A_i) and absence of stomatal limitation (A_a), stomatal conductance (G_s), intercellular CO_2 concentration at ambient CO_2 (C_i at C_a = 380) and electron transport rate (ETR) at C_a = 380. Measurements were performed at an ambient RH of 44.6% ± 3.6 (mean ± SE) and an irradiance of 2000 µmol m^{-2} s^{-1}. Values represent means ± SE (n=4) and are followed by letters indicating whether treatment time had a significant influence (P<0.05), as determined by Student's *t* tests.

	Control	1d	3d	6d	14d
J_{max} (µmol m^2 s^{-1})	25.2 ± 2.6 a	22.3 ± 4.5 a	28.7 ±3.2 a	38.4 ± 3.4 b	39.6 ± 0.1 b
R_d (µmol m^2 s^{-1})	3.8 ±.0.9 a	2.3 ± 0.3 a	2.5 ±.0.8 a	3.7 ± 0.4 a	3.1 ± 1 a
CE (mmol m^2 s^{-1})	159 ± 47 a	120 ± 36 a	231 ±.34 a	343 ± 72 b	326 ± 21.1 b
A_a (µmol m^2 s^{-1})	19.8 ±2.2 a	17.5 ± 1.3 a	26 ±2.5 a	32.9 ± 3.2 b	36 ± 1 b
A_i (µmol m^2 s^{-1})	13.2 ±1.2 a	11.7 ± 1.9 a	20.3 ± 0.6 b	20.9 ± 0.8 b	21.3 ± 1.7 b
G_s (mmol m^2 s^{-1})	154 ± 10 a	132 ± 12 a	177 ± 32 a	194 ± 19 a	147 ± 11 a
C_i at C_a=380 (µmol mol^{-1})	125.8 ± 43.2 a	183 ± 30.1 a	131.7 ± 27.9 a	153.4 ± 37.8 a	119.8 ± 25.8 a
ETR at Ca=380 (µmol mol^{-1})	41.4 ± 7.6 a	46.4 ± 3.3 a	73.7 ± 2.6 b	87.7 ± 9.6 b	78 ± 6.2 b

4.4.2 Hybridisation analysis of leaf transcript abundance

Changes in transcript abundance of selected genes in the source leaf were monitored following the source-sink perturbation induced by the partial shading treatment. Macroarrays were prepared bearing 128 cDNA probes (ESTs), derived primarily from graminaceous species, with known involvement in carbohydrate and photosynthetic metabolism (Table 4.2, [**Supplementary Information**]). The genes represented on the array were specifically selected to target metabolic activities most likely to be involved in the source-sink relationship. The expression of this set of genes was monitored in leaf 6 of the control plants and in the plants in which all other leaves were shaded at day 1, 3, 6 and 14.

Table 4.2. Functional classification of ESTs used in gene expression analysis. The number of genes cited reflect both different genes and variants of single genes.

General classification of gene product function	Number of genes under analysis
Cell Wall Biosynthesis	9
Sugar Sensing and Signaling	12
Carbon (Starch) Metabolism	5
Sucrose Metabolism	23
Glycolysis	20
Triose-phosphate metabolism	13
Photosynthesis	16
Mitochondrial metabolism	13
Sugar Transport	17

Hybridisation between total cDNA populations from leaf 6 and the gene probes on the array was highly consistent between replicate query events. Comparison of the average intensities generated from replicate query events produced R^2 values > 0.99 for each treatment, indicating consistent target-probe hybridisation (Fig. 4.3). Following the implementation of a low threshold cutoff, background subtraction and CV analysis, a total of 116 valid gene query events (89.9 %) were produced. The majority of query events did not exhibit any substantial difference in hybridisation signal intensity between array data sets. However, 27 ESTs showed a 2 to 9 fold change in intensity over time (Table 4.3). Within this group, the majority (22) displayed increased hybridisation intensity, indicating an overall up-regulation of expression in leaf tissue over time.

Within the gene categories represented on the macroarray, a number of trends were evident. During the source-sink perturbation there was a general trend towards the up-regulation of genes encoding products involved in photosynthesis, mitochondrial metabolism and sugar transport (Fig. 4.4). Expression of genes within other EST categories showed little change over time.

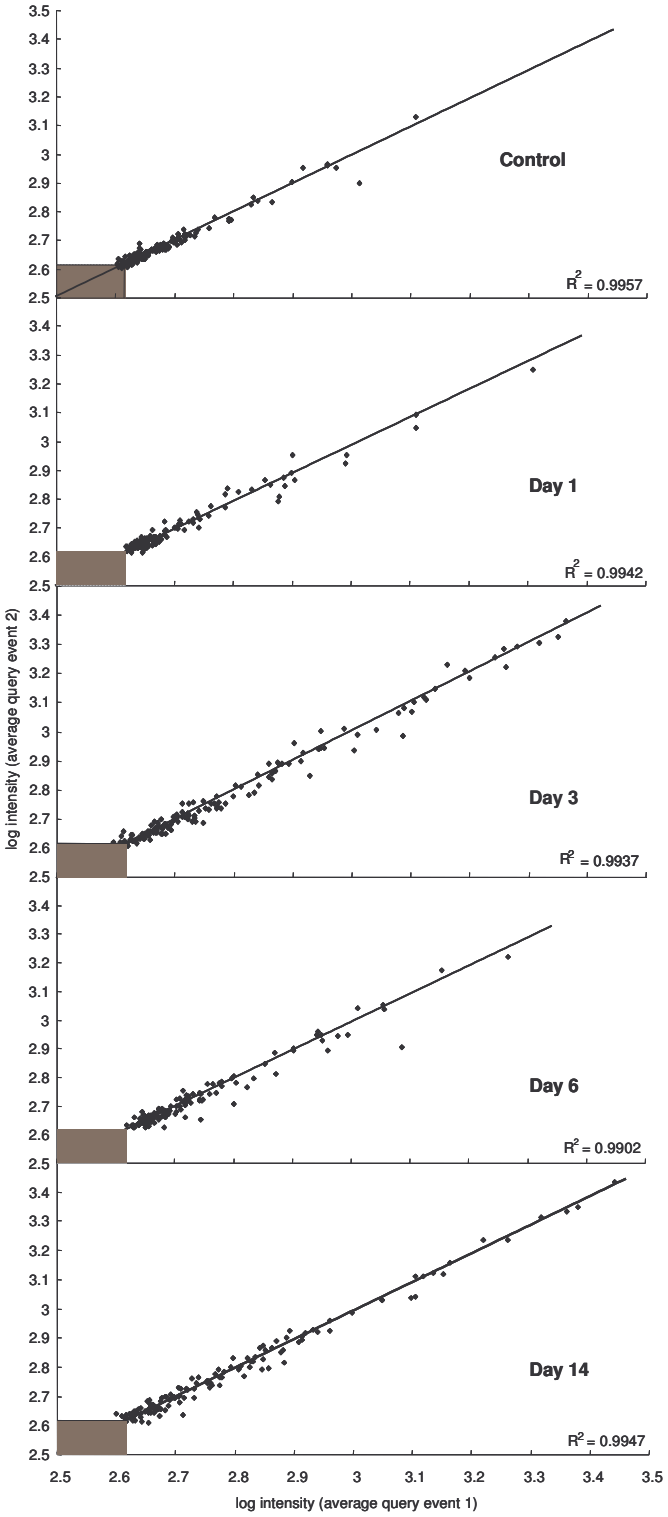


Fig. 4.3. Comparison of probe-target hybridisation intensities of replicate macroarray query events. Log data represent signal intensities of leaf 6 total cDNA populations hybridized to 128 probes on the macroarray. The data represent the average probe hybridisation of two adjacent probes compared to a second set placed probe pair at another location on the array. Intensity values below the lower threshold value (shown in grey) were excluded from data analysis.

Many of the ESTs that depicted an increase in expression over time were homologous to genes associated with the C_4 photosynthetic pathway (Table 4.3). Overall, this EST group showed a 5-fold increase in mRNA abundance (Fig. 4.4), while, within this group, NADP-dependent malic enzyme (NADPME; EC 1.1.1.40) and pyruvate orthophosphate dikinase (PPdK; EC 2.7.9.1) showed the greatest increase in expression during the source-sink perturbation. Two ESTs related to mitochondrial metabolism, *viz*. malate dehydrogenase (MDH; EC 1.1.1.82) and citrate synthase (CS; EC 2.3.3.1), exhibited increased expression, as did triose metabolism-related ESTs for fructose bisphosphate aldolase (ALD; EC 4.1.2.13) and glyceraldehyde phosphate dehydrogenase (GPDH; EC 1.2.1.12). Increased expression levels were also observed for six ESTs homologous to putative transporter proteins, including three putative monosaccharide transporters, an ADP/ATP plastidic transporter and two triose phosphate transporters.

Included in the small group of five ESTs that were down-regulated were those representing fructokinase (FK; EC 2.7.1.4) and hexokinase (HXK; EC 2.7.1.1) (Table 4.3). In addition, ESTs homologous to a mitogen-activated protein kinase (MAPK; EC 2.7.1.37), UTP-glucose dehydrogenase (UTP-GD; EC 1.1.1.22) and alcohol dehydrogenase (AD; EC 1.1.1.1) showed reduced expression levels during the perturbation.

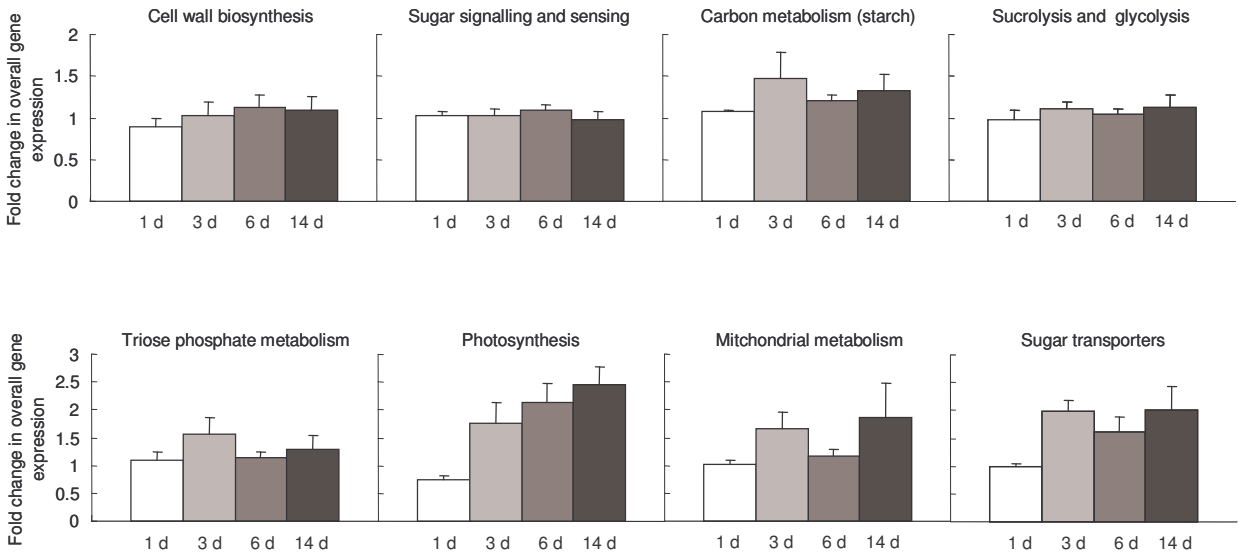


Fig. 4.4. Average fold changes in gene expression per functional category over time (□ 1d; ■ 3d; ■ 6d; ■ 14d) in the sole source leaf of partially shaded sugarcane plants compared to an unshaded control. See Table 4.2 for EST quantities per category and Supplementary Information for EST details.

In some instances, not all ESTs assigned the same identity displayed consistent changes in expression. For example, only one of the six sucrose synthase (SuSy; EC 2.1.4.13) ESTs (AU173014) showed a greater than 2-fold increase in expression, while only one of the three MAPK ESTs showed reduced expression. This could be due to significant sequence divergence between the ESTs isolated from different species, or alternatively, these ESTs may represent distinct isogenes or gene family members particular to specific tissues.

Table 4.3. Fold changes in gene expression between leaf 6 mRNA of an unshaded plant (control) and leaf 6 (sole source leaf) at days 1, 3, 6 and 14 following a source-sink perturbation induced by partial shading. Putative ID indicates the homology match of the particular EST probe referenced to accession number records of the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). See Supplementary Information further details.

Putative ID	Accession No.	Fold change			
Up regulated		**Day 1**	**Day 3**	**Day 6**	**Day 14**
ATP/ADP transporter	CD423751	0.6	2.4	1.9	9.1
Fructose bisphosphate aldolase	AW745533	1.0	1.9	1.8	2.9
Glyceraldehyde-P dehydrogenase	BG947834	1.7	4.1	2.0	3.4
	PQ0178	0.9	2.1	1.5	1.9
Malate dehydrogenase	AU093830	1.2	1.4	1.5	2.5
NADP-dependent malic enzyme	CN136258	0.9	2.3	1.3	3.6
	CN146318	0.7	3.0	2.1	8.1
Phenolenolpyruvate carboxylase	AU088696	0.8	1.3	0.8	2.2
	BG158755	0.9	2.7	2.4	4.3
psbA chloroplast protein	CD212978	0.9	1.7	1.5	3.2
Pyruvate orthophosphate dikinase	CF071996	0.6	2.6	2.5	8.5
Rubisco (large subunit)	AW678375	0.3	1.1	1.2	3.0
Rubisco (small subunit)	CN150664	0.9	2.7	1.5	2.4
Rubisco activase	BM318446	1.1	1.2	1.4	2.2
Rubisco transition peptide	CN142383	1.1	4.4	3.4	6.9
Citrate synthase	BE363510	1.6	3.9	1.6	2.3
Sucrose synthase	AU173014	1.6	1.8	1.2	2.6
Sugar transporter (monosaccharide)	CD231617	1.1	3.5	4.9	6.7
	AU163471	0.9	1.9	1.1	3.0
	AU094600	0.9	1.3	1.3	2.0
Triose phosphate transporter	CN149774	1.0	3.6	2.4	4.3
	CN149403	1.1	3.0	3.1	3.0
Down regulated					
Alcohol dehydrogenase	AU091741	1.0	1.1	1.4	2.3
Fructokinase	CN140006	1.1	1.0	1.2	2.0
Hexokinase	AU057562	1.0	1.1	1.4	2.2
Mitogen-activated protein kinase	CN132740	1.1	0.9	1.2	2.0
UDP-glucose dehydrogenase	AA525658	1.1	1.2	1.4	2.0

4.4.3 Correlation analysis

To uncover possible regulatory events induced in leaf 6 by the source-sink perturbation, the observed changes in photosynthesis, sugar concentration and gene expression over time were subjected to Pearson's correlation analyses. With regard to photosynthesis and sugar concentrations, a strong negative correlation between hexose concentrations in source leaf tissue, and J_{max} and *CE* was revealed (Table 4.4). This relationship was not evident for sucrose. Furthermore, significant correlations between sucrose and hexose concentrations in immature culm tissue and leaf 6 photosynthesis levels were observed. Immature sink tissues were characterised by decreased sucrose levels which were correlated with an increase in J_{max} over the 14 d period.

Of the 27 genes that exhibited marked changes in expression over time in leaf 6, 20 showed strong correlations to leaf 6 photosynthetic variables (J_{max} and *CE*) and leaf 6 hexose concentrations (Table 4.4). Within this group no correspondence was evident with leaf sucrose concentrations, however, nine genes showed significant correlation with the decreasing sucrose levels observed in immature culm tissue although a secondary correlation through leaf hexose may be indicated. Notably, these included ESTs homologous to the C_4 photosynthetic enzymes phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31), NADPME and PPdK, and two putative transporter proteins.

Table 4.4. Bivariate Pearson's correlation coefficients between leaf 6 photosynthetic variables J_{max} and *CE* (see Table 4.1 for variable declarations), leaf 6 and immature culm sugar concentrations (hexose and sucrose) and gene expression data of leaf 6 from either unshaded plants or partially shaded plants (sole source leaf 6) between 1 and 14 d. Significance levels (P) are reported for the Pearson's correlation coefficients (in brackets). Absent values indicate genes that were up or down regulated but not significantly correlated.

		Leaf 6			
		J_{max}	*CE*		
CE		0.979 (0.00)			
Leaf 6 - hexose		- 0.694 (0.00)	- 0.655 (0.00)		
Leaf 6 - sucrose					
Immature culm - hexose		0.336 (0.04)			
Immature culm - sucrose		- 0.372 (0.03)			
Up regulated	**Accession No.**	**Leaf 6**			**Immature Culm**
		J_{max}	*CE*	**Hexose**	**Sucrose**
ATP/ADP transporter	CD423751	0.708 (0.00)	0.621 (0.00)	0.631 (0.00)	- 0.487 (0.03)
Fructose bisphosphate aldolase	AW745533	0.827 (0.00)	0.799 (0.00)	- 0.675 (0.00)	
Glyceraldehyde-P dehydrogenase	BG947834				
	PQ0178				
Malate dehydrogenase	AU093830	0.772 (0.00)	0.699 (0.00)	- 0.632 (0.01)	- 0.478 (0.03)
NADP-dependent malic enzyme	CN136258	0.618 (0.01)	0.587 (0.01)	- 0.565 (0.01)	- 0.515 (0.02)
	CN146318	0.730 (0.00)	0.662 (0.00)	- 0.651 (0.00)	- 0.493 (0.03)
Phenolenolpyruvate carboxylase	AU088696	0.514 (0.02)	0.581 (0.01)	- 0.542 (0.01)	- 0.485 (0.03)
	BG158755	0.826 (0.00)	0.815 (0.00)	- 0.630 (0.00)	
psbA chloroplast protein	CD212978	0.775 (0.00)	0.719 (0.00)	- 0.649 (0.00)	- 0.465 (0.04)
Pyruvate orthophosphate dikinase	CF071996	0.775 (0.00)	0.699 (0.00)	- 0.647 (0.00)	- 0.452 (0.05)
Rubisco (large subunit)	AW678375	0.785 (0.00)	0.706 (0.01)	- 0.664 (0.00)	
Rubisco (small subunit)	CN150664	0.826 (0.00)	0.815 (0.01)	- 0.489 (0.00)	
Rubisco activase	BM318446	0.830 (0.00)	0.744 (0.01)	- 0.648 (0.00)	
Rubisco transition peptide	CN142383	0.789 (0.00)	0.783 (0.00)	- 0.603 (0.01)	
Citrate synthase	BE363510				
Sucrose synthase	AU173014				
Sugar transporter (monosaccharide)	CD231617	0.946 (0.00)	0.934 (0.00)	- 0.668 (0.00)	
	AU163471	0.591 (0.01)	0.555 (0.01)	- 0.563 (0.01)	- 0.532 (0.02)
	AU094600	0.819 (0.00)	0.774 (0.00)	- 0.685 (0.00)	
Triose phosphate transporter	CN149774	0.683 (0.00)	0.716 (0.00)	- 0.501 (0.00)	
	CN149403	0.683 (0.00)	0.716 (0.00)	- 0.501 (0.00)	
Down regulated					
Alcohol dehydrogenase	AU091741				
Fructokinase	CN140006				
Hexokinase	AU057562	- 0.730 (0.00)	- 0.605 (0.01)	0.576 (0.01)	
Mitogen-activated protein kinase	CN132740				
UDP-glucose dehydrogenase	AA525658	- 0.561 (0.01)	- 0.449 (0.04)	0.511 (0.02)	0.5 (0.03)

4.5 Discussion

Disturbance of the source-sink balance by partial shading of all source leaves, bar one, produced significant changes in the sugar levels of the leaf and subtending internodes (Fig. 4.2), as well as in photosynthetic activity of the single unshaded leaf (Table 4.1), in which significant increases in photosynthetic rate, carboxylation efficiency and PSII efficiency were observed. Conversely, shading treatments resulted in decreased sucrose levels in the young immature internodal tissue. A significant negative linear relationship was observed between maximum photosynthetic assimilation rates (J_{max}) of the source leaf and sucrose concentrations in these immature internodes (Fig. 4.2; Table 4.4). These observations support reports that reduced carbon supply to sink tissue is a physiological signal to the source of increased assimilate demand (van Bel, 2003). Partial defoliation has been shown to produce a similar effect in sugarcane, which results in preferential partitioning of available carbon to sucrose culm storage (Pammenter and Allison, 2002). Partial defoliation also has no effect on overall plant sucrose yields (Gutiérrez-Miceli *et al.*, 2004), suggesting that the assimilation capacity of sugarcane leaves is robust and flexible and can readily adjust carbon supply relative to sink demand. In contrast to defoliation studies, the marked increases in photosynthesis resulting from the source-sink perturbation achieved in this study may have been exacerbated by the continued presence of other leaves. Previous work has shown a significant increase in partitioning of a ^{14}C label to shaded leaves, indicating that these leaves were converted to additional sinks during shading treatments (McCormick *et al.*, 2006 [Chapter 3]).

A strong negative correlation was observed between hexose concentrations and J_{max} and *CE* in unshaded source leaves. In contrast, no relationship was observed between source leaf sucrose levels and photosynthesis (Table 4.4). This suggests that hexoses, rather than sucrose, may participate in a feed-back system for photosynthetic regulation. This contention is supported by observations from maize (*Zea mays* L) in which sucrose concentrations were shown to have no significant short-term feedback inhibitory effects on the synthesis of sucrose itself in leaf tissue (Lunn and Furbank, 1999). Furthermore, hexoses have been shown to inhibit photosynthesis in numerous C_3 species and consequently are believed to play a significant role in regulating carbon accumulation and leaf development (Goldschmidt and Huber, 1992; Kilb *et al.*, 1995; Ehness *et al.*, 1997; Paul and Pellny, 2003). A decreased leaf hexose pool may serve as a signal for

increased sink demand, and also reduce negative feedback regulation of photosynthesis (Foyer, 1987), an effect which has been observed previously in sugarcane (McCormick *et al*., 2006 [Chapter 3]). Due to the compartmentation of enzymes between mesophyll and bundle sheath cells in C_4 species (Edward *et al*., 2001), the regulation of sucrose accumulation and signaling mechanism may be more complex than for C_3 species. Nevertheless, sugar-mediated regulation of gene expression may be as important in C_4 as in C_3 species for maintaining the balance between the source and sink activity (Lunn and Furbank, 1999).

In the current study, physiological and metabolic effects of a source-sink perturbation have been examined in parallel to changes in the expression of genes associated with photosynthesis and carbohydrate metabolism. To permit comparison of replicate array query events within each array, the hybridisation signal intensity values for each EST probe were normalised amongst replicates. To compare array data generated by replicate array hybridisations a method for the normalisation of hybridisation signal intensity data was used. An internal standard was introduced into the leaf RNA samples prior to cDNA synthesis and labelling and then used to normalise hybridisation signal intensity data amongst replicate query events. This is an improved means of normalisation compared to standard, comparative normalisation techniques, such as generation of relative expression values (Cui and Churchill, 2003) in which all measured values are divided by the sum of the values and then compared between arrays. This latter method is not ideal as it is based on the assumption that the amount of mRNAs per sample is constant (Velculescu *et al*., 1999). A further drawback is that large changes in relative gene expression may impact on the expression of unchanged genes, leading to the generation of false-positive results (Yang *et al*., 2002). In contrast to such analytical approaches, the method used in this study provides a more stringent approach to the detection of specific changes in gene expression.

During photosynthesis in NADPME-type C_4 species, such as sugarcane, malate is translocated to bundle sheath cells where NADPME catalyses its decarboxylation (Lunn and Hatch, 1995; Edwards *et al*., 2001). The three key enzymes of C_4 photosynthesis, *viz*. PPdK, PEPC and NADPME, are strongly regulated by light (Hatch, 1992; Furbank and Taylor, 1995). However, only PEPC has previously been shown to respond to changing sugars levels (Chollet *et al*., 1996; Sima and Desjardins, 2001). This study has revealed an increase in gene expression of all three of these enzymes, as well as an

increase in expression of Rubisco (both RbcL and RbcS) and Rubisco-related proteins (Table 4.3). Increased abundance of these transcripts correlated to an increase in photosynthetic activity and decreasing leaf hexose concentrations (Table 4.4). These observations indicate that hexoses may play a key role in regulating the expression of these enzymes. Sheen (1990) demonstrated that supplying maize protoplasts with glucose or sucrose lead to the repression of genes encoding products involved in photosynthesis. The depletion or accumulation of sugars has further been shown to activate or repress, respectively, the expression of genes for photosynthetic components of a variety of C_3 species and ultimately influence photosynthesis itself (Krapp *et al*., 1993; Krapp and Stitt, 1995; Van Oosten and Besford, 1994, 1995; Basu *et al*., 1999). However, there are few reports describing the effects of sugar levels on the expression of genes encoding components of photosynthesis specific to C_4 plants, and results from C_3 studies may not always be pertinent to C_4 species. For example, in *Spinacia oleracea* (L.) the expression of rbcS, and consequently Rubisco protein activity, has been shown to be regulated by leaf sugar concentrations (Krapp *et al*., 1991). These results are not easily comparable to C_4 species, where Rubisco levels are typically only 50% of those of C_3 plants on a chlorophyll basis (Lunn and Furbank, 1999). Furthermore, in C_4 species sucrolytic and photosynthetic activities are localised in mesophyll and bundle sheath cells, respectively (Lunn and Furbank, 1997). Maize, in particular, shows a strong asymmetric distribution of activities, with cytosolic sucrose phosphate synthase (SPS; EC 2.4.1.14), sucrose phosphate phosphatase (SPP; EC 3.1.3.24) and fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) predominantly localized in the mesophyll (Downton and Hawker, 1973; Furbank *et al*., 1985), indicating that sucrose is synthesized almost exclusively in the mesophyll of maize source leaves.

In the current study, decreased levels of hexose were correlated with increased expression of several photosynthesis-related genes (Table 4.4). Drawing on information obtained from studies of maize it also appears likely that in sugarcane, it is a hexose-regulated signal originating primarily in mesophyll cells, which serves to regulate PEPC expression levels (Fig. 4.5). As PEPC has previously been shown to respond to sugars (Chollet *et al*., 1996; Sima and Desjardins, 2001), PEPC may influence signaling cascades that ultimately result in the up-regulation of C_4 photosynthesis under conditions of decreased cytosolic hexose. Of note is that hexoses have been implicated in the regulation of source metabolism via signal transduction pathways involving protein phosphorylation via MAPK activities (Ehness *et al*., 1997). In the present study, MAPK

expression was down-regulated, however, due to the wide variety of signaling pathways that are associated with MAPKs (Jonak *et al*., 1996; Lee *et al*., 2001; Zhang and Klessig, 2002), it is difficult to pinpoint the specific role of this enzyme during the source-sink perturbation (Fig. 4.5). Regulation of C_4 leaf photosynthesis has been suggested to involve phosphorylation of the PEPC enzyme (Duff and Chollet; 1995; Vidal and Chollet, 1997). Further metabolic and gene expression analyses in sugarcane will aim to examine the post-transcriptional regulation of PEPC by PEPC kinase (Jeanneau *et al*., 2002) and possible co-mediation by MAPKs and hexoses.

Increased photosynthetic activity was correlated with an increase in several transporter proteins, including a putative ATP/ADP transporter and two triose phosphate transporters (Table 4.3). Attempts to increase sucrose metabolism in transgenic C_3 *Oryza sativa* (Kitaake) by over-expression of maize PEPC have previously been shown to result in no change in leaf sucrose, but rather a decreased availability of P_i and increased consumption of cytosolic triose phosphate into malate (Agarie *et al*., 2002). In C_4 plants this phenomenon may be alleviated by a co-ordinated increase in the supply of cytosolic P_i and triose phosphate when photosynthetic activity increases. Sucrose produced in the mesophyll must however, additionally pass through the bundle sheath cells to be loaded into the phloem through either a symplastic or apoplastic, or both (Lunn and Furbank, 1999; Walsh *et al*., 2005). In sugarcane, the conducting cells of the phloem have been shown not to be connected to other cells of the leaf by plasmodesmata (Robinson-Beers and Evert, 1991). This suggests that phloem loading occurs from the apoplast in sugarcane leaves (Rae *et al*., 2005). Under conditions of increased photosynthesis and sucrose export, the observed increase in expression of sugar transporter proteins (Table 4.3) is not unexpected, and may be required to efficiently meet increased sink demand.

Hexokinase and FK were down-regulated during the shading treatments. It has been proposed that both enzymes participate in sugar sensing and signaling in plants (Pego and Smeekens, 2000; Rolland *et al*., 2006), particularly HXK, which showed positive correlation with decreasing hexose concentrations (Table 4.4). The role of HXK as a putative sensor of hexose signaling was examined by Jang *et al*. (1997) using sense and antisense constructs of the *Arabidopsis* HXK isoforms *Hxk1* and *Hxk2*. Those authors reported that plants over-expressing HXK genes exhibited glucose hypersensitive characteristics, whereas antisense plants were hyposensitive. The results of this study

together with those of Jang *et al.* (1997) support the hypothesis that HXK is a putative sensor for hexose signaling. More recently, Moore *et al.* (2003) demonstrated that point mutations in the catalytic domains of HXK resulted in an engineered protein that exhibited no phosphorylation activity, while still being capable of glucose signaling activity. This indicates that HXK may play two functionally distinct roles, at least in C_3 plants (Harrington and Bush, 2003). Two FK isoforms have been isolated and characterised in sugarcane (Hoepfner and Botha, 2004) but little is known about HXK. Further research will be required to clarify the nature of the relationship observed between HXK and hexose concentration (Table 4.4; Fig. 4.4).

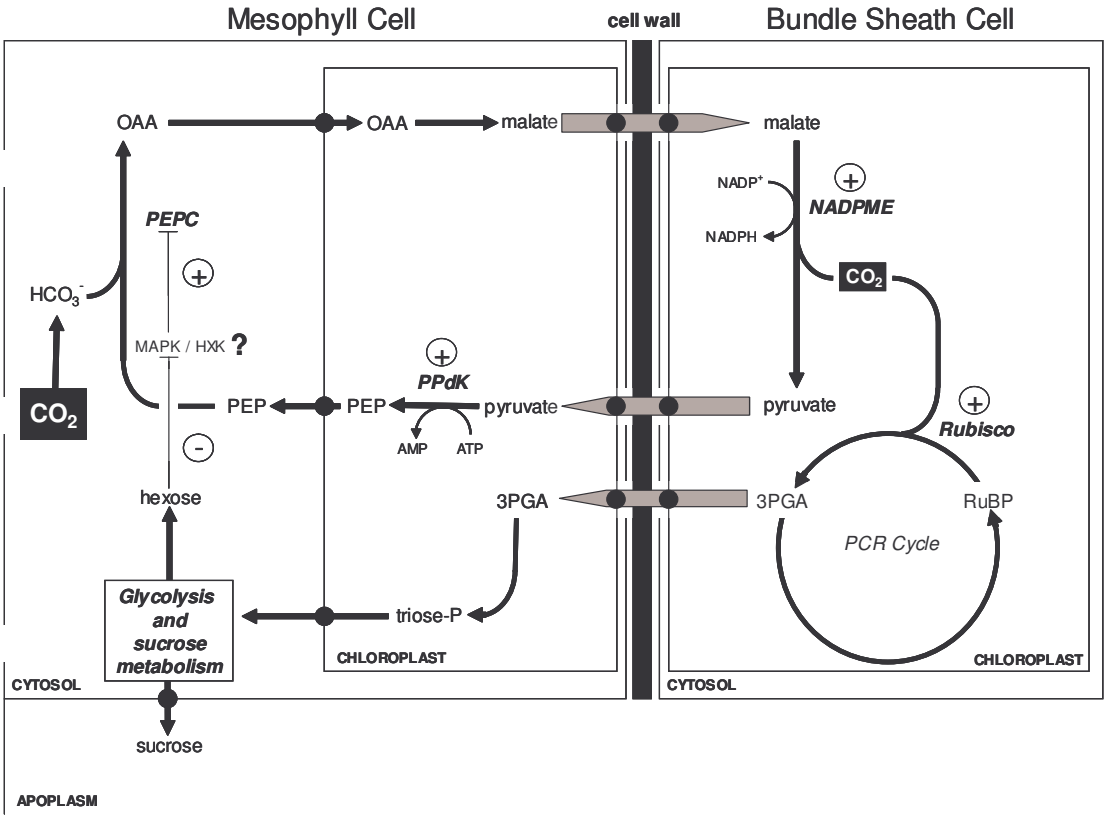


Fig. 4.5. The C_4 NADP-ME pathway of photosynthesis in sugarcane. Arrows within circles indicate the changes in metabolite and gene expression levels during a source-sink perturbation. The negative correlation observed between hexose and PEPC is indicated. Abbreviations: 3PGA – 3-phosphoglycerate; HXK – hexokinase (EC 2.7.1.1); MAPK – mitogen activated protein kinase (EC 2.7.1.37); NADP-ME – NADP-malic enzyme (EC 1.1.1.40); OAA – oxaloacetate; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase (EC 4.1.1.31); PPdK – pyruvate orthophosphate dikinase (EC 2.7.9.1); RuBP – ribulose bisphosphate; triose-P – triose phosphate.

The observed increases in photosynthetic rates (Table 4.1) were statistically correlated with a reduction in hexose content and changes in the expression of several genes (Table 4.4). Although changes in gene expression are indicative of coarse regulation, further study will be required to confirm whether expression patterns correlate to enzyme activity, which may change substantially as a result of post-translational control. Recent studies in *Arabidopsis* have indicated that changes in carbohydrates may initiate a significant gene signaling response that does not necessarily lead to long-term changes in plant behaviour (Stitt *et al*., 2006). Furthermore, analysis of whole tissue sugars levels does not necessarily relate to the precise sugar concentration in the cells actually responding to the signaling. However, the strong correspondence between photosynthesis, hexose and gene expression demonstrated in the present study and the similarity of these responses to those reported in C_3 plants (Krapp *et al*., 1991; Krapp *et al*., 1993; Franck *et al*., 2006) provides evidence for hexose as an important signaling molecule in C_4 sugarcane.

4.6 Conclusions

This is the first report for sugarcane in which physiological and metabolic changes during a source-sink perturbation have been examined in parallel to changes in leaf gene expression patterns. The work has revealed a strong relationship between source and sink tissues, where demand for carbon from sinks affects source leaf photosynthetic activity, metabolite levels and gene expression. Future research will include a closer examination of the expression patterns of several of the genes highlighted in the current study. This will include comparative expression analysis among different sugarcane cultivars to gauge the extent to which the changes in gene expression observed in this study pertain to other varieties. Clarification of how the sink acts to regulate source activity in sugarcane will provide researchers with additional potential targets for manipulation towards improving sucrose yield. The observation that sink demand limits source activity indicates that the signal feedback system reporting sink sufficiency and regulating source activity may be a potentially valuable for target for genetic manipulation. This study has demonstrated that increased carbon demand from the sink results in increased photosynthetic rates at the source. The communication of this relationship appears to correlate to a decrease in source hexose concentrations, and increased expression of genes involved in C_4 photosynthesis and metabolite transport.

4.7 References

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4.8 Supplementary information

Photosynthesis- and carbohydrate metabolism related ESTs selected for expression analysis. EST identity was established by sequence homology searches with known gene sequences in the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). The Expect (E) value is the statistical indicator of the significance of the match between query and database sequence. Gene categories are classified below.

General classification of gene product function	Gene category	Number of genes under analysis
Cell Wall Biosynthesis	A	9
Sugar Sensing and Signaling	B	12
Carbon (Starch) Metabolism	C	5
Sucrose Metabolism	D	23
Glycolysis	E	20
Triose-phosphate metabolism	F	13
Photosynthesis	G	16
Mitochondrial metabolism	H	13
Sugar Transport	I	17

Putative Identity	EC No.	Accession No.	Gene Category	Source	E Value	Protein Definition
Aconitate hydratase	4.2.1.3	AU161681	H	*Oryza sativa*	9.00E-22	Aconitase A [*Oryza sativa* (*japonica* cultivar-group)]
ADP-glucose pyrophosphorylase	2.7.7.33	AU172987	C	*Oryza sativa*	4.00E-05	ADP-glucose pyrophosphorylase [*Oryza sativa* (*japonica* cultivar-group)]
ADP-glucose pyrophosphorylase	2.7.7.27	AU174515	C	*Oryza sativa*	1.00E-20	ADP-glucose pyrophosphorylase [*Oryza sativa* (*japonica* cultivar-group)]
Alcohol dehydrogenase	1.1.1.1	AU091741	E	*Oryza sativa*	5.00E-52	alcohol dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Alcohol dehydrogenase	1.1.1.1	AU161221	E	*Oryza sativa*	6.00E-26	alcohol dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Alcohol dehydrogenase	1.1.1.1	AU093048	E	*Oryza sativa*	8.00E-12	alcohol dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Alcohol dehydrogenase	1.1.1.1	AA269289	E	*Saccharum officinarum*	1.00E-37	alcohol dehydrogenase [*Saccharum officinarum*]
Alcohol dehydrogenase	1.1.1.1	CN149616	E	*Sorghum bicolor*	2.00E-86	alcohol dehydrogenases [*Oryza sativa* (*japonica* cultivar-group)]
Aldolase	4.1.2.13	AU092513	F	*Oryza sativa*	2.00E-41	aldolase C-1 [*Oryza sativa*]
Aldolase	4.1.2.13	*not published*	F	*Saccharum officinarum*	*n/a*	*n/a*
ATP/ADP transporter	2.7.1.40	AU057209	I	*Oryza sativa*	3.00E-81	putative plastidic ATP/ADP transporter [*Oryza sativa* (*japonica* cultivar-group)]
Beta glycanase	3.1.2.6	BM330896	A	*Sorghum bicolor*	2.00E-52	glucan endo-1,3-beta-glucosidase [*Zea mays*]
Cellulase	3.2.1.4	CD230179	A	*Sorghum bicolor*	3.00E-36	cellulase [*Sorghum bicolor*]
Cellulose synthase	2.4.1.12	AI216932	A	*Saccharum officinarum*	5.00E-52	cellulose synthase [*Zea mays*].
Cellulose Synthase	2.4.1..12	CN147740	A	*Sorghum bicolor*	0.14	cellulose synthase [*Sorghum bicolor*]

Chlorophyll a/b binding protein	3.6.3.52	CN148954	G	*Sorghum bicolor*	5.00E-138	chlorophyll a-b binding protein [*Zea mays*]
Chlorophyll a/b binding protein	3.6.3.52	CN136692	G	*Sorghum bicolor*	6.00E-138	chlorophyll a-b binding protein [*Zea mays*]
Citrate Lyase	4.1.3.6	AU093499	H	*Oryza sativa*	3.00E-42	ATP citrate-lyase beta [*Oryza sativa* (*japonica* cultivar-group)]
Citrate synthase	2.3.3.1	C25436	H	*Oryza sativa*	1.00E-37	Citrate synthase, glyoxysomal precursor (GCS) [*Oryza sativa* (*japonica* cultivar-group)]
Citrate synthase	2.3.3.1	BE363510	H	*Sorghum bicolor*	7.00E-66	citrate synthase [*Oryza sativa* (*japonica* cultivar-group)]
Enolase	4.2.1.11	AA080586	F	*Saccharum officinarum*	2.00E-52	Enolase 2 (2-phosphoglycerate dehydratase 2) [*Zea mays*]
Enolase	4.2.1.11	AA080586	F	*Saccharum officinarum*	2.00E-52	enolase (2-phosphoglycerate dehydratase) [*Zea mays*]
Enolase	4.2.1.11	CN130620	F	*Sorghum bicolor*	1.00E-147	enolase [*Zea mays*]
Enolase	4.2.1.11	AU085839	F	*Oryza sativa*	1.20E+00	enolase [*Oryza sativa* (*japonica* cultivar-group)]
Enolase	4.2.1.11	AU063290	F	*Oryza sativa*	2.00E-51	enolase [*Oryza sativa* (*japonica* cultivar-group)]
Fructokinase	2.7.1.4	CN140006	B	*Sorghum bicolor*	8.00E-99	fructokinase 2 [*Zea mays*]
Fructose bisphosphatase	3.1.3.11	AU095636	C	*Oryza sativa*	6.00E-85	sedoheptulose-1,7-bisphosphatase precursor [*Oryza sativa* (*indica* cultivar-group)]
Fructose bisphosphatase	3.1.3.11	J04197	C	*Rattus norvegicus*	0.0	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 [*Rattus norvegicus]*
Fructose bisphosphatase	3.1.3.11	BG159258	C	*Sorghum bicolor*	6.00E-77	fructose-1,6-bisphosphatase [Oryza sativa (*japonica* cultivar-group)]
Fructose bisphosphate aldolase	4.1.2.13	AW745533	F	*Sorghum bicolor*	2.00E-31	Fructose-1,6-bisphosphate aldolase [*Oryza sativa* (*japonica* cultivar-group)]
Glucokinase	2.7.1.1	CD427137	B	*Sorghum bicolor*	1.60E-01	glycosyl hydrolase [Oryza sativa (*japonica* cultivar-group)]
Glucose phosphate adenyltransferase	2.7.7.27	CN133916	A	*Sorghum bicolor*	7.00E-65	Glucose-1-phosphate adenylyltransferase [*Triticum aestivum*]
Glyceraldehyde phosphate dehydrogenase	1.2.1.12	AU085851	F	*Oryza sativa*	2.00E-104	NAD-dependent aldehyde dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Glyceraldehyde phosphate dehydrogenase	1.2.1.12	PQ0178	F	*Saccharum officinarum*	1.00E-05	glyceraldehyde phosphate dehydrogenase [*Zea mays*]
Glyceraldehyde phosphate dehydrogenase	1.2.1.12	BG947834	F	*Sorghum bicolor*	7.00E-86	NAD-dependent aldehyde dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Hexokinase	2.7.1.1	AU057562	B	*Oryza sativa*	3.00E-55	hexokinase 1 [*Oryza sativa* (japonica cultivar-group)]
Hexokinase	2.7.1.1	AW286992	B	*Sorghum bicolor*	8.00E-36	hexokinase [*Zea mays*]
Hexose phosphate isomerase	5.3.1.9	AU174802	E	*Oryza sativa*	4.00E-51	Glucose-6-phosphate isomerase [*Oryza sativa* (*japonica* cultivar-group)]
Invertase	3.2.1.26	CD425062	D	*Sorghum bicolor*	1.00E-41	cell wall invertase [*Sorghum bicolor*]
Invertase	3.2.1.26	CD211376	D	*Sorghum bicolor*	0.14	beta-fructofuranosidase [*Sorghum bicolor*]
Invertase	3.2.1.26	CD230086	D	*Sorghum bicolor*	0.074	vacuolar acid invertase [*Oryza sativa* (*japonica* cultivar-group)]
Invertase	3.2.1.26	BG933362	D	*Sorghum bicolor*	1.2	soluble acid invertase [*Saccharum robustum*]
Invertase	3.2.1.26	CN149463	D	*Sorghum bicolor*	0.63	acid beta-fructofuranosidase [*Sorghum bicolor*]
Invertase	3.2.1.26	AU063804	D	*Oryza sativa*	5.00E-47	apoplastic invertase [*Oryza sativa* (*japonica* cultivar-group)]
Invertase	3.2.1.26	C71989	D	*Oryza sativa*	2.00E-46	vacuolar acid invertase [*Oryza sativa* (*japonica* cultivar-group)]

Invertase	3.2.1.26	AU058270	D	*Oryza sativa*	2.00E-72	cell wall invertase 1 [Oryza sativa (*japonica* cultivar-group)]
Invertase	3.2.1.26	D46056	D	*Oryza sativa*	5.00E-06	soluble acid invertase mRNA, partial cds [Oryza sativa (*japonica* cultivar-group)]
Invertase	3.2.1.26	AU056057	D	*Oryza sativa*	9.00E-74	cell wall invertase [*Oryza sativa* (*japonica* cultivar-group)]
Malate dehydrogenase	1.1.1.82	AU093809	H	*Oryza sativa*	8.00E-65	malate dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Malate dehydrogenase	1.1.1.82	AU093830	H	*Oryza sativa*	2.00E-60	cytosolic malate dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Malate dehydrogenase	1.1.1.38	AU091557	H	*Oryza sativa*	1.00E-64	cytosolic malate dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Mitogen-activated protein kinase	2.7.1.37	BM322441	B	*Sorghum bicolor*	1.00E-75	Mitogen-activated protein kinase [*Zea mays*]
Mitogen-activated protein kinase	2.7.1.37	BF588090	B	*Sorghum bicolor*	2.00E-96	mitogen-activated protein kinase [*Triticum aestivum*]
Mitogen-activated protein kinase	2.7.1.37	CN132740	B	*Sorghum bicolor*	1.00E-104	mitogen-activated protein kinase [*Triticum aestivum*]
NADP-dependent malic enzyme	1.1.1.40	CN136258	G	*Sorghum bicolor*	3.00E-141	NADP-dependent malic enzyme [*Sorghum bicolor*]
NADP-dependent malic enzyme	1.1.1.40	CN146318	G	*Sorghum bicolor*	1.00E-128	NADP-dependent malic enzyme [*Sorghum bicolor*]
Neomycin phosphotransferase	*n/a*	U43611	-	-	3.00E-159	neomycin resistance protein [synthetic construct]
Neutral invertase	3.2.1.26	*not published*	D	*Saccharum officinarum*	*n/a*	*n/a*
Neutral invertase	3.2.1.26	AJ003114	D	*Lolium perenne*	0.0	neutral/alkaline invertase [*Lolium perenne*]
Phenol*enol*pyruvate carboxylase	4.1.1.31	AU095289	G	*Oryza sativa*	5.00E-19	phosphoenolpyruvate carboxylase kinase 4 [*Oryza sativa* (*japonica* cultivar-group)]
Phenol*enol*pyruvate carboxylase	4.1.1.31	AU174895	G	*Oryza sativa*	3.00E-67	Phosphoenolpyruvate carboxykinase [*Oryza sativa* (*japonica* cultivar-group)]
Phenol*enol*pyruvate carboxylase	4.1.1.31	AU088696	G	*Oryza sativa*	1.30E-01	phosphoenolpyruvate carboxylase [*Oryza sativa* (*japonica* cultivar-group)]
Phenol*enol*pyruvate carboxylase	4.1.1.31	CD211795	G	*Sorghum bicolor*	4.00E-101	phosphoenolpyruvate carboxylase [*Saccharum* spp.]
Phenol*enol*pyruvate carboxylase	4.1.1.31	BG158755	G	*Sorghum bicolor*	1.00E-57	phosphoenolpyruvate carboxylase [*Oryza sativa* (*japonica* cultivar-group)]
Phosphofructokinase	2.7.1.11	D40756	E	*Oryza sativa*	5.00E-51	putative diphosphate-fructose-6-phosphate 1-phosphotransferase [*Oryza sativa* (*japonica* cultivar-group)]
Phosphofructokinase	2.7.1.11	AU057478	E	*Oryza sativa*	2.00E-74	putative pyrophosphate-dependent phosphofructo-1-kinase [*Oryza sativa* (*japonica* cultivar-group)]
Phosphofructokinase	2.7.1.11	J04197	E	*Rattus norvegicus*	0.0	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 [*Rattus norvegicus*]
Phosphoglucokinase	2.7.1.10	AU070573	E	*Oryza sativa*	5.00E-51	glucokinase [*Escherichia coli* CFT073]
Phosphoglucomutase	5.4.2.2	AU085880	E	*Oryza sativa*	6.00E-57	phosphoglucomutase [*Oryza sativa*]
Phosphoglucomutase	5.4.2.2	AU091730	E	*Oryza sativa*	1.00E-05	phosphoglucomutase [*Oryza sativa*]
Phosphoglucomutase	5.4.2.2	CN145013	E	*Sorghum bicolor*	6.00E-98	phosphoglucomutase [*Zea mays*]
psbA chloroplast protein	3.6.3.52	CD212978	G	*Sorghum bicolor*	3.00E-06	choroplastD1:SII Q binding protein (psbA) [*Gagea pomeranica*]
Pyrophosphate-dependent phosphofructokinase	2.7.1.90	AU092378	E	*Oryza sativa*	5.00E-84	Pyrophosphate_PFK [*Oryza sativa* (*japonica* cultivar-group)]
Pyrophosphate-dependent	2.7.1.90	AU092937	E	*Oryza sativa*	2.00E-10	Pyrophosphate_PFK [*Oryza sativa* (*japonica* cultivar-group)]

phosphofructokinase						
Pyrophosphate-dependent phosphofructokinase	2.7.1.90	U12337	E	*Giardia lamblia*	0.0	diphosphate-fructose-6-phosphate 1-phosphotransferase [*Giardia lamblia* ATCC 50803]
Pyrophosphate-dependent phosphofructokinase	2.7.1.90	M67447	E	*Propionibacterium*	0.0	PFP [*Propionibacterium freudenreichii* subsp.]
Pyrophosphate-dependent phosphofructokinase	2.7.1.90	*not published*	E	*Saccharum officinarum*		
Pyruvate carboxylase	6.4.1.1	C97133	H	*Oryza sativa*	5.00E-43	Pyruvate carboxylase [*Oryza sativa* (*japonica* cultivar-group)]
Pyruvate dehydrogenase	1.2.1.51	AA269174	H	*Saccharum officinarum*	3.00E-44	pyruvate dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
Pyruvate kinase	2.7.1.40	AU092584	H	*Oryza sativa*	7.00E-04	pyruvate kinase [*Arabidopsis thaliana*]
Pyruvate kinase	2.7.1.40	AU093283	H	*Oryza sativa*	1.00E-76	cytosolic pyruvate kinase [*Oryza sativa* (*japonica* cultivar-group)]
Pyruvate orthophosphate dikinase	2.7.9.1	CF071996	H	*Sorghum bicolor*	3.00E-76	pyruvate phosphate dikinase [*Sorghum bicolor*]
Rubisco	4.1.1.39	AW678375	G	*Sorghum bicolor*	1.00E-102	RuBisCO large subunit [*Saccharum* hybrid cultivar SP-80-3280]
Rubisco	4.1.1.39	CN150664	G	*Sorghum bicolor*	2.00E-85	RuBisCO small subunit [*Saccharum* sp.]
Rubisco	4.1.1.39	BG556089	G	*Sorghum bicolor*	7E-65	rubisco small subunit [*Avena clauda*]
Rubisco	4.1.1.39	BE593723	G	*Sorghum bicolor*	3.00E-140	RuBisCO large subunit [*Saccharum* sp.]
Rubisco activase	6.3.4.-	BM318446	G	*Sorghum bicolor*	7.00E-92	RuBisCO chloroplast precursor (RuBisCO activase) [*Zea mays*]
Rubisco transition peptide	*n/a*	CN142383	G	*Sorghum bicolor*	6.00E-84	ribulose-1,5-bisphosphate carboxylase (RuBPC) precursor [*Zea mays*]
Succinate dehydrogenase	1.3.5.1	AU174356	H	*Oryza sativa*	2.00E-34	succinate dehydrogenase subunit 3 [*Oryza sativa* (*japonica* cultivar-group)]
Sucrose phosphate phosphatase	3.1.3.24	AU095442	D	*Oryza sativa*	1.00E-126	sucrose-phosphate phosphatase [*Oryza sativa* (*japonica* cultivar-group)]
Sucrose phosphate phosphatase	3.1.3.24	CD424766	D	*Sorghum bicolor*	0.32	sucrose-phosphatase [*Medicago truncatula*]
Sucrose phosphate synthase	2.4.1.14	AU094286	D	*Oryza sativa*	2.00E-51	sucrose phosphate synthase [*Oryza sativa* (*japonica* cultivar-group)]
Sucrose synthase	2.4.1.13	AA080610	D	*Saccharum officinarum*	2.00E-82	sucrose synthase 3 [*Zea mays*]
Sucrose synthase	2.4.1.13	AA080610	D	*Saccharum officinarum*	2.00E-82	sucrose synthase [*Zea mays*].
Sucrose synthase	2.4.1.13	AA080634	D	*Saccharum officinarum*	4.00E-29	sucrose synthase [*Oryza sativa* (japonica cultivar-group)]
Sucrose synthase	2.7.1.40	CD235994	D	*Sorghum bicolor*	2.00E-85	sucrose synthase-2 [*Saccharum officinarum*].
Sucrose synthase	2.4.1.13	AU094024	D	*Oryza sativa*	5.00E-70	sucrose synthase [*Oryza sativa* (*japonica* cultivar-group)]
Sucrose synthase	2.4.1.13	AU175062	D	*Oryza sativa*	8.00E-82	sucrose synthase [*Oryza sativa* (japonica cultivar-group)]
Sucrose synthase	2.4.1.13	AU173014	D	*Oryza sativa*	2.00E-82	sucrose synthase 2 (Sucrose-UDP glucosyltransferase 2) [*Zea mays*]
Sugar transporter	2.7.1.40	AU163425	I	*Oryza sativa*	2.00E-53	sucrose transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	AU094600	I	*Oryza sativa*	4.00E-45	monosaccharide transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	AU163471	I	*Oryza sativa*	3.00E-47	monosaccharide transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	AU070945	I	*Oryza sativa*	5.00E-09	sucrose transporter [*Oryza sativa* (*indica* cultivar-group)]

Sugar transporter	2.7.1.40	AU093407	I	*Oryza sativa*	7.00E-87	putative alpha-glucosidase [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	AU056954	I	*Oryza sativa*	8.00E-44	monosaccharide transporter 3 [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	CD424204	I	*Sorghum bicolor*	5.00E-45	sucrose transporter [*Zea mays*]
Sugar transporter	2.7.1.40	CD423751	I	*Sorghum bicolor*	3.00E-130	putative plastidic ATP/ADP transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	CD204613	I	*Sorghum bicolor*	1.00E-41	sugar transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	BG054361	I	*Sorghum bicolor*	6.00E-67	putative high pI alpha-glucosidase [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	CD231617	I	*Sorghum bicolor*	8.00E-84	monosaccharide transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	AW680302	I	*Sorghum bicolor*	0.35	sucrose transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	3.2.1.26	BE597399	I	*Sorghum bicolor*	2.00E-63	sugar transporter [*Oryza sativa* (*japonica* cultivar-group)]
Sugar transporter	2.7.1.40	CF482398	I	*Sorghum bicolor*	3.00E-04	sucrose transporter [*Oryza sativa* (*japonica* cultivar-group)]
Trehalose phosphate phosphatase	3.1.3.12	AU101936	B	*Oryza sativa*	5.00E-64	trehalose phosphate phosphatase [*Oryza sativa* (*japonica* cultivar-group)]
Trehalose phosphate phosphatase	3.1.3.12	AU166371	B	*Oryza sativa*	3.00E-78	trehalose phosphate phosphatase [*Oryza sativa* (*japonica* cultivar-group)]
Trehalose phosphate synthase	2.4.1.15	AU092582	B	*Oryza sativa*	1.00E-19	trehalose-6-phosphate synthase [*Oryza sativa* (*japonic*a cultivar-group)]
Trehalose phosphate synthase	2.4.1.15	AF007778	B	*Saccharum officinarum*	3.00E-28	trehalose phosphate synthase [*Arabidopsis thaliana*]
Trehalose phosphate synthase	2.4.1.15	BI643732	B	*Sorghum bicolor*	1.00E-105	putative trehalose-6-phosphate synthase/phosphatase [*Oryza sativa* (*japonica* cultivar-group)]
Triose phosphate isomerase	5.3.1.1	AU164627	F	*Oryza sativa*	5.00E-58	triosephosphate isomerase [*Oryza sativa* (*japonica* cultivar-group)]
Triose phosphate isomerase	5.3.1.1	AA577653	F	*Saccharum officinarum*	2.00E-27	triose phosphate-isomerase [*Triticum aestivum*]
Triose phosphate translocator	2.7.1.40	CN149774	I	*Sorghum bicolor*	4.00E-128	triose phosphate/phosphate translocator [*Zea mays*]
Triose phosphate translocator	2.7.1.40	CN149403	I	*Sorghum bicolor*	2.00E-117	triose phosphate/phosphate translocator [*Zea mays*]
UDP glucose dehydrogenase	1.1.1.22	*not published*	A	*Saccharum officinarum*		
UDP-glucose dehydrogenase	1.1.1.22	D39326	A	*Oryza sativa*	2.00E-32	UDP-glucose 6-dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
UDP-glucose dehydrogenase	1.1.1.22	AA525658	A	*Saccharum officinarum*	1.00E-39	UDP-glucose 6-dehydrogenase [*Oryza sativa* (*japonica* cultivar-group)]
UDP-glucose glucosyltransferase	2.4.1.35	AW745467	E	*Sorghum bicolor*	7.00E-09	UDP-glucose glucosyltransferase [*Sorghum bicolor*]
UDP-glucose pyrophosphorylase	2.7.7.9	AU032651	E	*Oryza sativa*	6.00E-05	UDP-glucose pyrophosphorylase [*Oryza sativa* (*japonica* cultivar-group)]
UTP-glucose phosphate uridyltransferase	2.7.7.23	CD220905	A	*Sorghum bicolor*	5.00E-84	UTP-glucose-1-phosphate uridylyltransferase [*Oryza sativa* (*japonica* cultivar-group)]
Vacuolar pyrophosphorylase	2.7.7.27	D13472	D	*Hordeum vulgare*	0.0	pyrophosphate pyrophosphatase [*Hordeum vulgare*]

Chapter 5:
Regulation of photosynthesis by sugars in sugarcane leaves

5.1 Abstract

In sugarcane increased sink demand has previously been shown to result in increased photosynthetic rates that are correlated with a reduction in leaf hexose concentrations. To establish whether sink-limitation of photosynthesis is a result of sugar accumulation in the leaf, cold-girdling and excision techniques were used to modify leaf sugar concentrations in pot-grown sugarcane. Cold-girdling (5^{o}C) increased sucrose and hexose levels and resulted in a decline of photosynthetic rates over 5 d (48% and 35% decline in *A* and ETR, respectively). In excised leaves that were preincubated in darkness for 3 h, sucrose accumulation was reduced but accumulated again upon transfer to the light, while hexose concentrations remained lower than in controls (7.7 μmol mg^{-1} FW vs 18.6 μmol mg^{-1} FW hexose in controls). Furthermore, a 66% and 59% increase in *A* and ETR, respectively, was observed compared to controls maintained in the light. Sugar-induced changes in photosynthesis were independent of changes in stomatal conductance. This study demonstrated similarities in the effects on photosynthesis of altered leaf sucrose concentrations and those previously reported for the culm. In addition, this work supports the contention that hexoses, rather than sucrose, are responsible for the modulation of photosynthetic activity.

Keywords: hexose, leaf, photosynthesis, source, sucrose, sugarcane

5.2 Introduction

Although sugarcane is the primary source of the world's sugar, little is known about the regulation of leaf (source) photosynthetic rates by and the sugar-status of the culm (sink) (McCormick *et al*., 2006 [Chapter 3]). However, in other species, the carbon demands of sinks have been shown to govern the overall photosynthetic rates (Sheen, 1994; Basu *et al*., 1999; Iglesias *et al*., 2002; Paul & Foyer, 2001). Recent improvements in sugarcane yields have been almost entirely through increased cane yield, rather than increases in culm sucrose content. Sugarcane sucrose content has not increased significantly for several decades (Jackson, 2005). Attempts to increase sugar content through the

modification of plant genes involved in sugar metabolism have also been unsuccessful (Lakshmanan *et al*., 2005), indicating the possibility that inappropriate gene targets were selected for modification or that there is sufficient metabolic control redundancy to buffer against increases in stored sucrose through the manipulation of single genes (Wu & Birch, 2007). The underlying mechanisms that govern feedback regulation between source and sink tissues are thus likely to be important in attempts to manipulate stalk sucrose content.

Pammenter and Allison (2002) have demonstrated through measurement of structural and non-structural (i.e. sucrose) dry mass components that both partial defoliation and shading result in a bias towards sugarcane culm sucrose accumulation, at the expense of structural growth of the stem. The sucrose accumulating processes within the maturing sugarcane stem result in a strong sink-demand for photoassimilate (Marcelis, 1996; McCormick *et al*., 2006 [Chapter 3]). Significant differences in photosynthetic rates have been reported for individual sugarcane leaves related to the age of the plant, with young plants typically assimilating at significantly higher rates than older plants (Hartt & Burr, 1967; Bull & Tovey, 1974). Young leaves of three-month-old sugarcane photosynthesised faster (45 µmol m^{-2} s^{-1}) than similar young leaves on ten-month-old plants (25 µmol m^{-2} s^{-1}; Amaya *et al*., 1995). Whole plant photosynthetic rates were also lower in eight-month-old sugarcane plants than in four-month-old plants, regardless of the light intensity (Allison *et al*., 1997). The reason that the photosynthetic rate is dependent on plant age is probably due to the accumulation of sucrose in the culms of older plants (McCormick *et al*., 2006 [Chapter 3]). Partial defoliation of sugarcane was not effective in reducing culm sucrose concentration (Gutiérrez-Miceli *et al*., 2004). This indicates that the remaining photosynthetic capacity was sufficient to respond to sink demand.

Co-ordination between sink demand and source activity is the result of feedback mediated through the carbohydrate concentration in mature source leaves (Roitsch, 1999; Paul & Foyer, 2001; Paul & Pellny 2003). Although the molecular control mechanisms in sugarcane are still not fully known, studies in C_3 crops have indicated that that photosynthetic activity is strongly linked to a sugar sensing/signaling mechanism based on the local status of the primary transport sugar, sucrose, and/or its constituent hexoses (Abdin *et al*., 1998; Rolland *et al*., 2002; Gibson, 2005; Franck *et al*., 2006). The correlation between carbon assimilation and electron transport observed in

both C_3 and C_4 plants (Edwards & Walker, 1983; Earl & Tollenaar, 1998; Flexas *et al*., 2002) indicates that both the 'light' and 'dark' reactions of photosynthesis are mutually dependent on such regulation. The regulatory roles of several photosynthetic enzymes have been studied using knockout mutants and antisense transformants of the C_4 species *Flaveria bidentis* (L.) (Furbank & Taylor, 1995); however, there are as yet no reports documenting the control of sucrose synthesis in the leaves of C_4 plants (Lunn & Furbank, 1999; Rolland *et al*., 2006). Furthermore, the majority of C_4 studies have focused on maize. Due to the localisation of sucrose biosynthesis in the mesophyll cells, which is uncommon in other C_4 species, maize cannot be used as a universal model for understanding the regulatory role of sugars in C_4 leaves (Lunn & Furbank, 1997a). Further investigation is thus required to properly understand sugar-related regulation in other C_4 crop species such as sugarcane.

Cold-girdling of plant tissues, such as stem or leaf, has previously been reported to decrease or inhibit phloem transport, thus causing an accumulation of carbohydrate in tissues above the girdle (Michin *et al*. 1983; Krapp *et al*., 1993). Previous studies have successfully used cold-girdling to examine the relationship between carbohydrate accumulation and a subsequent decrease in leaf photosynthesis on a variety of C_3 plants, including spinach, tobacco and potato (Krapp *et al*., 1993; Krapp & Stitt, 1994; Koch, 1996). However, Lunn & Furbank (1997a) showed that increasing the sucrose content in C_4 maize leaves to levels found at the end of the light period had little or no effect on partitioning, and concluded that feedback inhibition by sucrose itself does not play a significant role in maize leaves. Conversely in *Amaranthus edulis* (L.), blocking the export of sucrose by cooling the stems led to large increases in sucrose and an inhibition of photosynthesis, suggesting that mechanisms do exist in some C_4 species for feedback inhibition by sucrose (Blechschmidt-Schneider *et al*., 1989).

Modification of culm sugar concentrations by leaf shading demonstrated that sink demand for carbon strongly influenced leaf photosynthetic rates and carbon partitioning, possibly through leaf hexose depletion (McCormick *et al*., 2006 [Chapter 3]). Whole plant shading has complex effects including possible influences on transpiration and thus transport of nutrients and hormones within the plant. In the research reported here, leaf hexose and sucrose concentrations were manipulated by exposure of excised leaves to darkness, loading of excised leaves with sucrose and hexoses and by cold-girdling intact leaves in order to characterise the regulation of photosynthesis by leaf

sugar levels. It was concluded that sugarcane photosynthesis was strongly controlled by the concentrations of hexoses in the leaf tissue and that culm sucrose concentration was likely to moderate photosynthesis by influencing leaf hexose concentrations through modification of the rate phloem transport from leaf to culm.

5.3 Materials and methods

5.3.1 Plant material

Nine-month-old pot-grown *Saccharum* spp. (L.) hybrid *cv.* N19 (N19) cultivated at Mount Edgecombe, KwaZulu-Natal (SASRI) was selected for leaf analyses (May 2006). Plants (4-5 stools per pot) were grown in 15 L pots containing mixed soil (composted bagasse, river sand, vermiculite, in a ratio of 3:1:0.1). Plants were irrigated using a drip irrigation system twice daily and initially grown outside in a semi-enclosed area that provided shelter from extreme wind conditions. Plants were then moved to a temperature controlled greenhouse (maintained at 28^{o}C) and allowed to acclimate for 1 week. The 2nd and 3rd fully expanded leaves (McCormick *et al*., 2006 [Chapter 3]) were used for experiments which were carried out within the glasshouse.

5.3.2 Leaf treatments

Cold-girdles were attached to leaves in the morning (8h00) and maintained at 5^{o}C for a period of 5 d. The girdle consisted of 0.75 cm diameter soft PVC tubing, firmly clamped around each leaf 30 cm from the leaf base. Cooled water (5^{o}C) was then pumped through the tubing using a Grant LTD6G cooling bath (Grant Instruments, Barrington, Cambridge, UK). Both untreated (n=4) and girdled (n=4) leaves were harvested simultaneously and immediately frozen in liquid nitrogen at intervals over the treatment period. Photosynthetic rates and sugar concentrations of the leaves were routinely measured (see below).

Leaves were cut under deionised water (pH 7.0) and then re-cut again under water approximately 5 cm above the previous cut. Leaves were placed in clear 50 ml centrifuge tubes containing 20 ml water and some of the leaves moved into a 95% shade cloth chamber (1–10 µmol m^{-2} s^{-1}) for 3 h (9h00–12h00). Following removal from

the shade-chamber into sunlight, each set was placed in either a 20 ml solution of 5 mM sucrose, 5 mM sorbitol or H_2O. The leaves were sampled to allow measurement of sugar concentrations and the photosynthetic rates of the leaves measured (n=4 per treatment).

To increase leaf sucrose concentrations to those found at 15h00 under field conditions (50–60 µmol g^{-1} FW), excised leaves were pre-fed a 167 mM sucrose solution for 1 h (8h00–9h00). The sugar concentration [*C*] required for the leaf to accumulate the necessary sucrose levels during the 1 h period was estimated from $[C]\,(mol\ L^{-1}) = \frac{Leaf\ Weight\ (g) \times [Final\ sucrose]\,(mol\ g^{-1}) \times time\ (h)}{Leaf\ area\ (m^2) \times Transpiration\ (L\ m^{-2}\ h^{-1})}$. All variables were calculated empirically using data gathered from leaves. To account for endogenous sucrose accumulation rates, the measured sucrose accumulation for the leaf during a 1 h photoperiod (*ca.* 10 µmol g^{-1} FW h^{-1}) were subtracted from [*C*]. The supplied sucrose solution contained ^{14}C sucrose label (5 kBq ml^{-1}; specific activity = 601 mCi $mmol^{-1}$; Amersham Biosciences, Buckinghamshire, UK). Following loading, the leaves (n=4 per treatment) were placed in darkness for 3 h and then transferred to light, following which photosynthetic variables and sugars levels were measured. Changes in distribution of the ^{14}C label between sucrose and hexoses over time were determined using thin layer chromatography. Similarly, leaves were also loaded with either glucose or fructose solution (50 mM) to increase hexose levels to measured peak diurnal concentrations (15 µmol g^{-1} FW). The glucose or fructose solution contained ^{14}C glucose (7.4 kBq ml^{-1}; specific activity = 317 mCi $mmol^{-1}$; Amersham Biosciences) or ^{14}C fructose, respectively (7.4 kBq ml^{-1}; specific activity = 306 mCi $mmol^{-1}$; Amersham Biosciences).

5.3.3 Sugar determination

Leaf samples were milled in an A11 Basic Analysis Mill (IKA®, Staufen, Germany) and stored at -80°C prior to analysis. Approximately 100 mg powdered tissue was incubated overnight in 10 volumes of sugar extraction buffer containing 30 mM HEPES (pH 7. 8), 6 mM $MgCl_2$ and ethanol 70% (v/v) at 70°C. Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic coupling assay used previously (McCormick *et al.*, 2006 [Chapter 3]). The phosphorylation of glucose by hexokinase/glucose-6-

phosphate dehydrogenase (EC 1.1.1.49) (Roche, Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5.3.1.9) (Roche) was quantified by following the reduction of $NADP^+$ to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader using KC4 software (Biotek Instrument, Inc., Vermont, USA).

5.3.4 Labelled sugar analysis

Labelled sugar containing extracts (150 µl) were evaporated to dryness in an Eppendorf Concentrator 5301 (Eppendorf AG, Hamburg, Germany) and resuspended in 15 µl H_2O. Sub-samples (5 µl) were spotted onto 10 x 20 cm silica gel plates (Merck, Darmstadt, Germany) using a semi-automatic Thin Layer Chromatography (TLC) sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland) and fractionated using a mobile phase consisting of 50% ethyl acetate (v/v), 25% acetic acid (v/v) in filtered water (0.45 micron pore size membrane filter) over 3 h. Silica plates were dried at 70°C for 10 min, sealed in polyethylene film and exposed to high-resolution phosphor screens (Type: SR; Packard, Canberra Company, Japan). After 24 h exposure, the images on the phosphor screens were captured and analysed by means of a Cyclone Storage Phosphor Screen imaging system using Optiquant Ver. 03.10 (Packard).

5.3.5 Gas exchange and fluorescence determinations

Gas exchange measurements were made on 2 cm^2 portions of leaf tissue using a portable gas exchange system (LI-6400, LI-COR Biosciences Inc., Nebraska, USA). Light was provided by a red/blue LED light source (LI-COR Biosciences Inc.) at photon irradiance of 1500 µmol m^{-2} s^{-1}. All leaf measurements were done under ambient CO_2 conditions (370 µmol mol^{-1}) at a maintained leaf temperature of 28°C. Gas exchange variables measured include photosynthetic assimilation (A), transpiration rate (E), stomatal conductance (G_s), and intercellular CO_2 (C_i).

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0.8 s, 6 000 µmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm'). The electron transport rate (ETR), defined as the actual flux of photons driving photosystem II (Maxwell & Johnson, 2000) was calculated

as $ETR = \left(\frac{Fm' - Fs}{Fm'} \right) fI\alpha_{leaf}$, where *Fs* is "steady-state" fluorescence (at 2 000 µmol m^{-2} s^{-1}), *Fm'* is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by photosystem II, typically assumed to be 0.4 for C_4 plant species (Edwards & Baker, 1993), *I* is incident photon flux density and α_{leaf} is leaf absorptance (0.85, LI-COR manual).

5.3.6 Statistical analysis

Results were subjected to analysis of variance (ANOVA) or Student's *t* tests to determine the significance of difference between responses to treatments. When ANOVA was performed, Tukey's honest significant difference (HSD) *post-hoc* tests were conducted to determine the differences between the individual treatments (SPSS Ver. 11.5, SPSS Inc., Illinois, USA).

5.4 Results

5.4.1 Effects of dark treatment and subsequent leaf feeding

Sugarcane leaves maintained in light accumulated high concentrations of sucrose (*ca*. 55 µmol g^{-1} FW) during the measured period (Fig. 5.1). Hexose concentrations also increased throughout the morning, reaching a plateau at midday and then decreased after 14h00. Both photosynthetic assimilation (*A*) and photosynthetic electron transport (ETR) of control plants decreased from midday onwards. The dark-treated leaves had reduced sucrose (13.4 ± 0.4 µmol g^{-1} FW) and hexose (12.6 ± 0.1 µmol g^{-1} FW) concentrations compared to control leaves (44.3 ± 4.4 µmol g^{-1} FW sucrose; 22 ± 1 µmol g^{-1} FW hexose) at 12h00. Following return to the light, the rate of sucrose accumulation of dark-treated leaves was higher than the initial sucrose accumulation rates of controls and 3-fold higher than sucrose accumulation for the controls during the same time period (data not shown). Hexose concentrations were lower in dark-treated leaves than in control leaves at 12h00 (Fig. 5.1). During the 3 h following the dark treatment, the hexose concentrations in leaves supplied with H_2O or 5 mM sorbitol decreased compared to control leaves and leaves that received 5 mM sucrose.

Dark-treated leaves that received either H_2O or 5 mM sorbitol had significantly increased A and ETR compared to control leaves maintained in light (Fig. 5.1; Table 5.1). In contrast, photosynthetic rates of dark-treated leaves that received 5 mM sucrose were not different from those of controls. The changes in photosynthetic rate induced by darkness, sorbitol and sucrose supply were not associated with significant changes in stomatal conductance, transpiration or intercellular CO_2 concentration (Table 5.1).

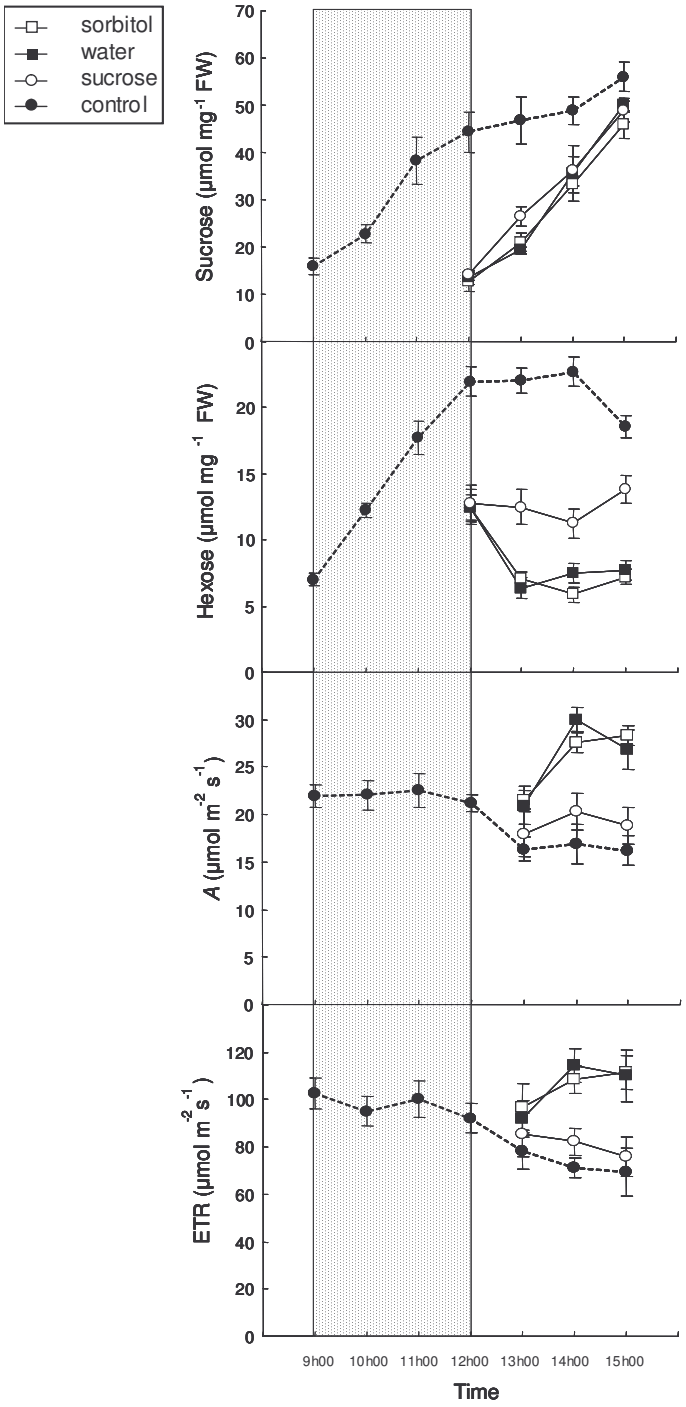

Fig. 5.1. Changes in sucrose (µmol g^{-1} FW), hexose (µmol g^{-1} FW), assimilation rate (*A*) (µmol m^{-2} s^{-1}) and electron transport rate (ETR) (µmol m^{-2} s^{-1}) in excised sugarcane leaves (n=4) over time. Samples include a control maintained in daylight (●) (9h00–15h00) and three dark-treatments (9h00–12h00). Following dark-treatment, leaves were placed in sucrose (5 mM) (○), sorbitol (5 mM) (■) or water (□). Dark-treated leaves were allowed 1 h to adapt to light conditions before photosynthetic readings were taken.

Table 5.1. Variables based on gas exchange analysis and leaf fluorescence of excised leaves from untreated (control) and dark-treated (9h00–12h00) at 15h00: photosynthetic rate (*A*), and electron transport rate (ETR) stomatal conductance (G_s), transpiration rate (*E*) and intercellular CO_2 concentration CO_2 (C_i). Dark-treated leaves were either maintained in H_2O or placed in sucrose (5 mM) or sorbitol (5 mM) at 12h00. Measurements were taken at an ambient RH of 55.6% ± 0.9 (mean ± SE) and an irradiance of 1500 µmol m^{-2} s^{-1} and CO_2 concentration of 370 µmol mol^{-1}. The shade treatment values are the mean ± SE (n=4) and are followed by letters indicating whether treatments had a significant influence (P<0.05), as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests.

	Control	Dark-Treated (sucrose)	Dark-Treated (sorbitol)	Dark-Treated (H_20)
A (µmol m^{-2} s^{-1})	16.2 ± 1.6 a	18.9 ± 1.9 a	28.35 ± 1.9 b	26.9 ± 2.1 b
ETR (µmol m^{-2} s^{-1})	69.3 ± 4.3 a	76.18 ± 8.3 a	111.6 ± 7.3 b	110.3 ± 11 b
G_s (mol m^{-2} s^{-1})	157 ± 22 a	168 ± 23 a	198 ± 26 a	251 ± 47 a
E (mmol m^{-2} s^{-1})	3.56 ± 0.41 a	3.9 ± 0.4 a	4.4 ± 0.58 a	4.8 ± 0.7 a
C_i (µmol mol^{-1})	172.6 ± 18.4 a	161.5 ± 11.5 a	124.3 ± 17.2 a	161.3 ± 14.1 a

5.4.2 Sugar loading prior to darkness

Pre-feeding leaves with sucrose successfully elevated sucrose concentrations to 46.36 ± 2.8 µmol g^{-1} FW within 1 h (Fig. 5.2). During the 3 h incubation in darkness, sucrose decreased slightly (*ca*. 5 µmol g^{-1} FW), while the concentration of hexoses in the sucrose-loaded leaves increased. After the dark treatment, sucrose concentrations of sucrose-loaded leaves increased only slightly in the light to 50.1 ± 2.1 µmol g^{-1} FW at 15h00. Hexose concentrations in sucrose-loaded leaves remained higher than controls for the remainder of the experiment, even though hexose in fed leaves decreased upon return to light. The sucrose concentration in the dark-treated leaves supplied with water remained low until being returned to light, whereupon sucrose accumulation increased markedly. Hexose concentrations of plants suppled with water remained lower than those of controls or sucrose-loaded leaves during the light period (12h00–15h00).

The ^{14}C-sucrose derived from ^{14}C-sucrose supplied to leaves decreased after the feeding period, while the ^{14}C-hexose pool derived from ^{14}C-sucrose increased (Fig. 5.3). Labelled hexoses were increased significantly by 12h00, indicating conversion of ^{14}C-sucrose during the dark treatment to ^{14}C-hexose. The increase of unlabelled hexose

(Fig. 5.2) in sucrose-loaded leaves was thus likely due to the conversion of sucrose to hexose during the dark period.

The photosynthetic rates (*A* and ETR) of dark-treated leaves supplied with water were significantly higher than those of controls upon return to light (Fig. 5.2; Table 5.2). The decreases in photosynthetic rates observed in these leaves were smaller than the previous experiment (Fig. 5.1), possibly due to the accumulation of photosynthate during the initial loading period (8h00–9h00) (Fig. 5.2). Hexoses and sucrose concentrations were increased in sucrose-loaded leaves. In these leaves the increased photosynthesis as a consequence of the dark treatment was eliminated.

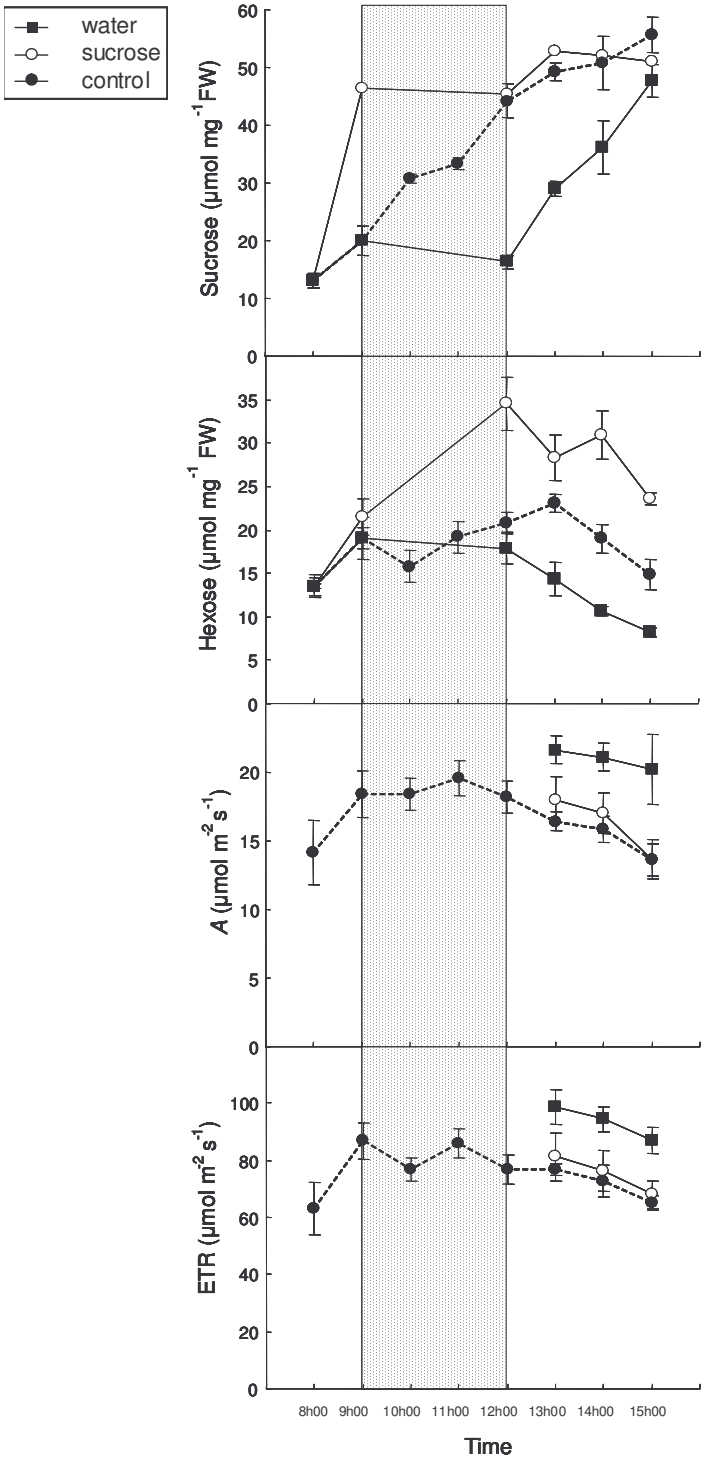

Fig. 5.2. Effects of pre-feeding sugarcane leaves with sucrose on sucrose (µmol g^{-1} FW), hexose (µmol g^{-1} FW), assimilation rate (*A*) (µmol m^{-2} s^{-1}) and electron transport rate (ETR) (µmol m^{-2} s^{-1}). After 1 h feeding (8h00–9h00), fed (○) and non-fed (■) samples (n=4) were placed in darkness for 3 h. A control was maintained in daylight (●) for the duration of the experiment. Dark-treated leaves were allowed 1 h to adapt to light conditions before photosynthetic readings were taken.

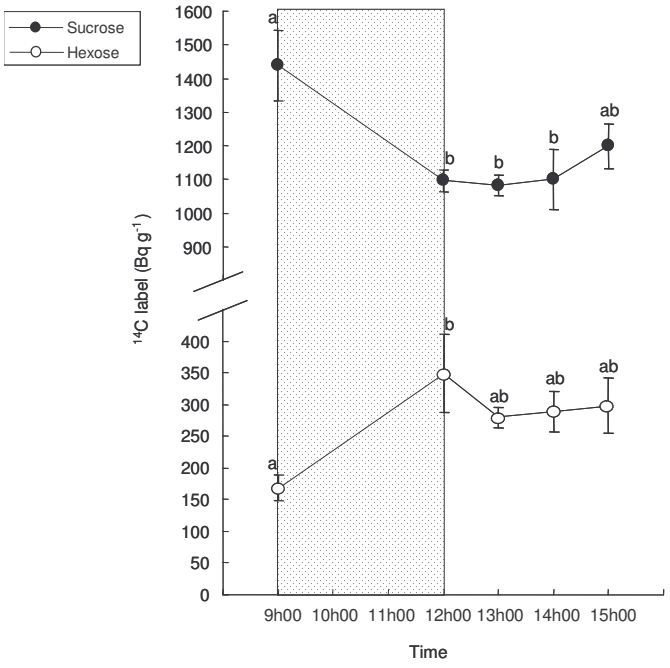

Fig. 5.3. Allocation of ^{14}C label (Bg g^{-1}) to sucrose (●) and hexose (○) pools of excised leaves (pot-grown sugarcane plants) (n=4) following feeding with ^{14}C sucrose (1 h). Immediately after feeding, leaves were placed in darkness for 3 h. Letters above SE bars indicate whether the treatment had a significant ($P<0.05$) influence as determined by ANOVA followed by post-hoc Tukey's honest significant difference (HSD) tests.

Table 5.2. Gas exchange analysis and leaf fluorescence variables for excised leaves of untreated (control) and dark-treated (9h00–12h00) at 15h00. See Table 5.1 for variable details. Dark-treated leaves were either maintained in H_2O or loaded with sucrose (167 mM) for 1 h prior to dark treatment. Measurements were taken at an ambient RH of 54.4% ± 2.64 (mean ± SE) and an irradiance of 1500 µmol m^{-2} s^{-1} and CO_2 concentration of 370 µmol mol^{-1}. The shade treatment values are the mean ± SE (n=4) and are followed by letters indicating whether treatments had a significant influence (P<0.05), as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests.

	Control	Dark-Treated (sucrose-loaded)	Dark-Treated (H_2O)
A (µmol m^{-2} s^{-1})	13.64 ± 1.16 a	13.67 ± 1.44 a	20.2 ± 2.5 b
ETR (µmol m^{-2} s^{-1})	64.9 ± 2.7 a	67.9 ± 6.8 a	87 ± 6 b
G_s (mmol m^{-2} s^{-1})	166 ± 31 a	170 ± 24 a	202 ± 11 a
E (mmol m^{-2} s^{-1})	3.55 ± 0.76 a	4.06 ± 0.46 a	4.21 ± 0.16 a
C_i (µmol mol^{-1})	201 ± 17.6 a	218.3 ± 9.9 a	186 ± 23 a

Hexose-loading suppressed *A* and ETR similarly to sucrose-loading (Fig. 5.4). However, changes in the tissue hexose concentrations in both the glucose- and fructose-loaded leaf tissue were not readily observable due to the interconversion of hexose and sucrose (Fig. 5.4; Fig. 5.5). More than half of supplied ^{14}C glucose and ^{14}C fructose was in the form of ^{14}C sucrose after the initial 1 h of feeding (53% and 60% for glucose- and fructose-loaded, respectively). During the dark treatment, the production of sucrose from ^{14}C hexose of leaves pre-loaded with either glucose or fructose was further increased at the expense of the hexose pool.

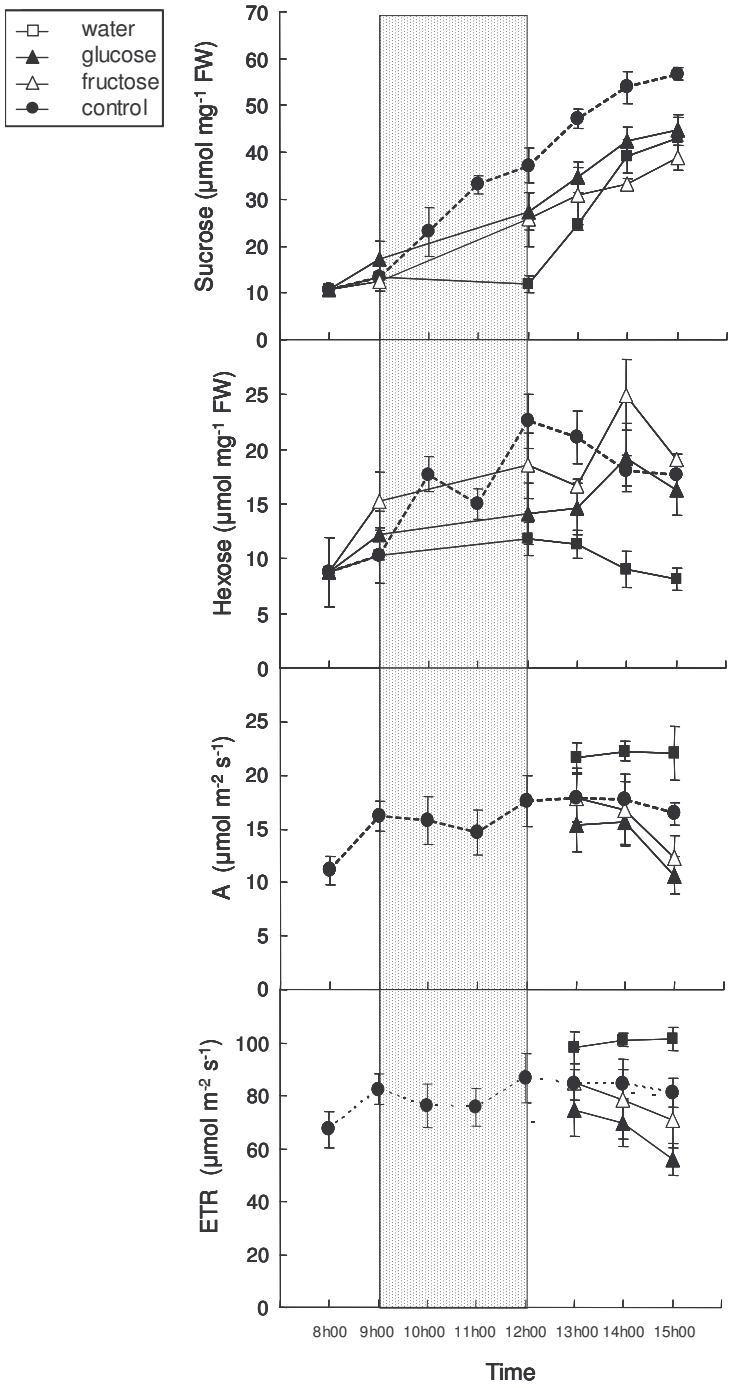

Fig. 5.4. Effects of pre-feeding sugarcane leaves with glucose or fructose on sucrose (µmol g^{-1} FW), hexose (µmol g^{-1} FW), assimilation rate (*A*) (µmol m^{-2} s^{-1}) and electron transport rate (ETR) (µmol m^{-2} s^{-1}). After 1 h feeding (8h00–9h00), samples pre-fed with glucose (Δ) or fructose (▲) and non-fed (■) samples (n=4) were placed in darkness for 3 h. A control was maintained in daylight (●) for the duration of the experiment. Dark-treated leaves were allowed 1 h to adapt to light conditions before photosynthetic readings were taken.

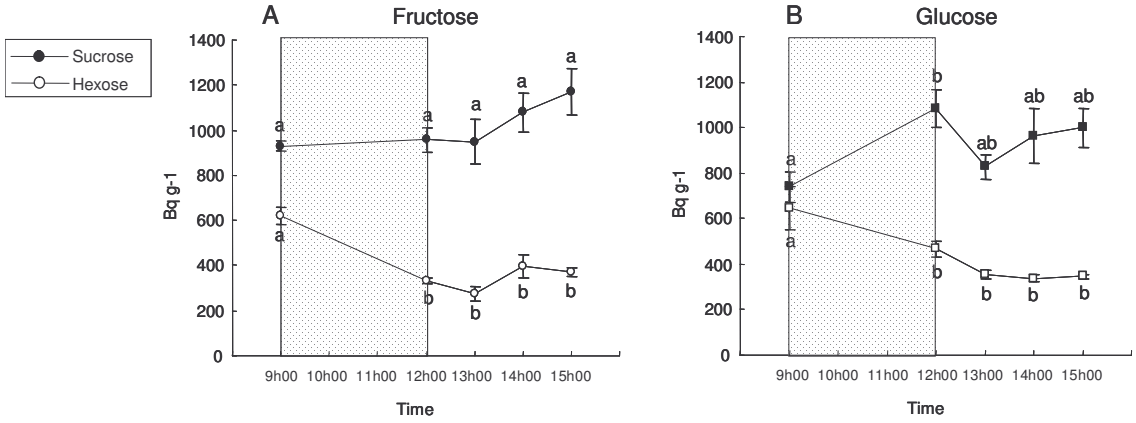

Fig. 5.5. Allocation of ^{14}C label (Bg g^{-1}) supplied in the form of ^{14}C fructose (left) or ^{14}C glucose (right) to the sucrose (●,■) and hexose (○,□) pools of excised sugarcane leaves over time. Following feeding (1 h), leaf samples (n=4) were placed in darkness for 3 h. Letters above SE bars indicate whether the treatment had a significant ($P<0.05$) influence as determined by ANOVA followed by post-hoc Tukey's honest significant difference (HSD) tests.

5.4.3 Effects of a cold-girdle on whole leaf

Cold-girdling of intact leaves induced an overall increase in both sucrose and hexose concentrations above the girdle compared to ungirdled (control) leaves during the treatment (Fig. 5.6). Sucrose concentrations were increased in the girdled leaves 7 h after girdling (15h00). To account for the time of day, leaves were sampled for sugar analysis consistently at this time of day for the remainder of the experiment. After 55 h cold-girdling sucrose concentrations were 2-fold higher than those of ungirdled leaves (114 ± 3.5 µmol g^{-1} FW). Hexose levels also rose significantly in cold-girdled leaves, reaching peak concentrations 3-fold higher after 79 h (30.3 ± 1.9 µmol g^{-1} FW). After 103 h, however, sucrose and hexose had decreased to similar levels observed in ungirdled leaves.

Photosynthetic rates were measured daily at 13h00 (Fig. 5.7). Ungirdled leaves had an average *A* of 21.8 ± 0.7 µmol m^2 s^{-1} and ETR of 84.7 ± 0.9 µmol m^2 s^{-1} which did not change significantly over the duration of the experiment. In cold-girdled leaves, *A* and ETR declined progressively over time, reaching minima of 11.6 ± 0.7 µmol m^2 s^{-1} and

56.8 ± 3.3 µmol m^2 s^{-1}, respectively after 79 h. Cold-girdled leaf G_s and transpiration rates did not differ from the ungirdled control until 79 h, after which they showed a significant decrease.

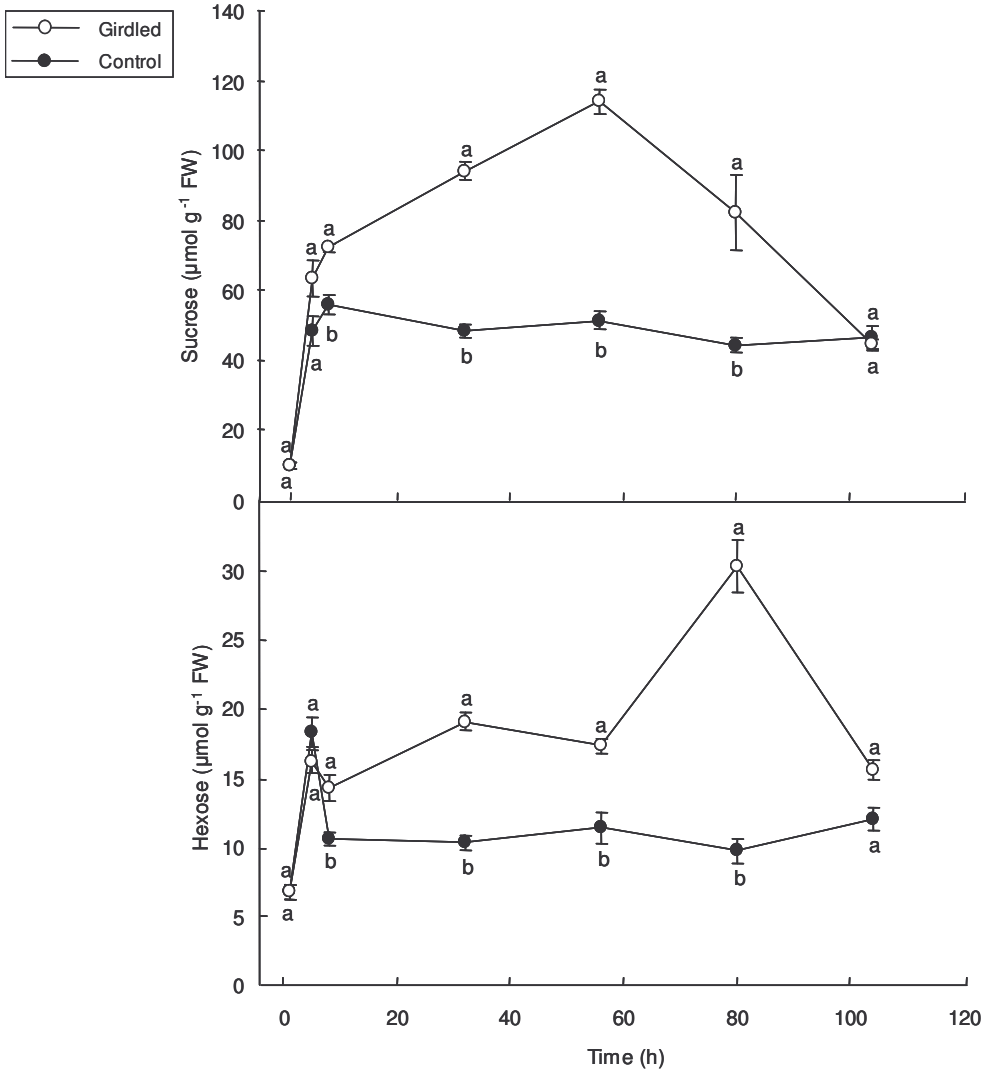

Fig. 5.6. Sucrose and hexose concentrations for cold-girdled (5°C) (○) and ungirdled (●) leaves of sugarcane plants over time. Letters above SE bars indicate whether the cold-girdling treatment (n=4) was significantly different from ungirdled controls ($P<0.05$) as determined by Student's *t* tests.

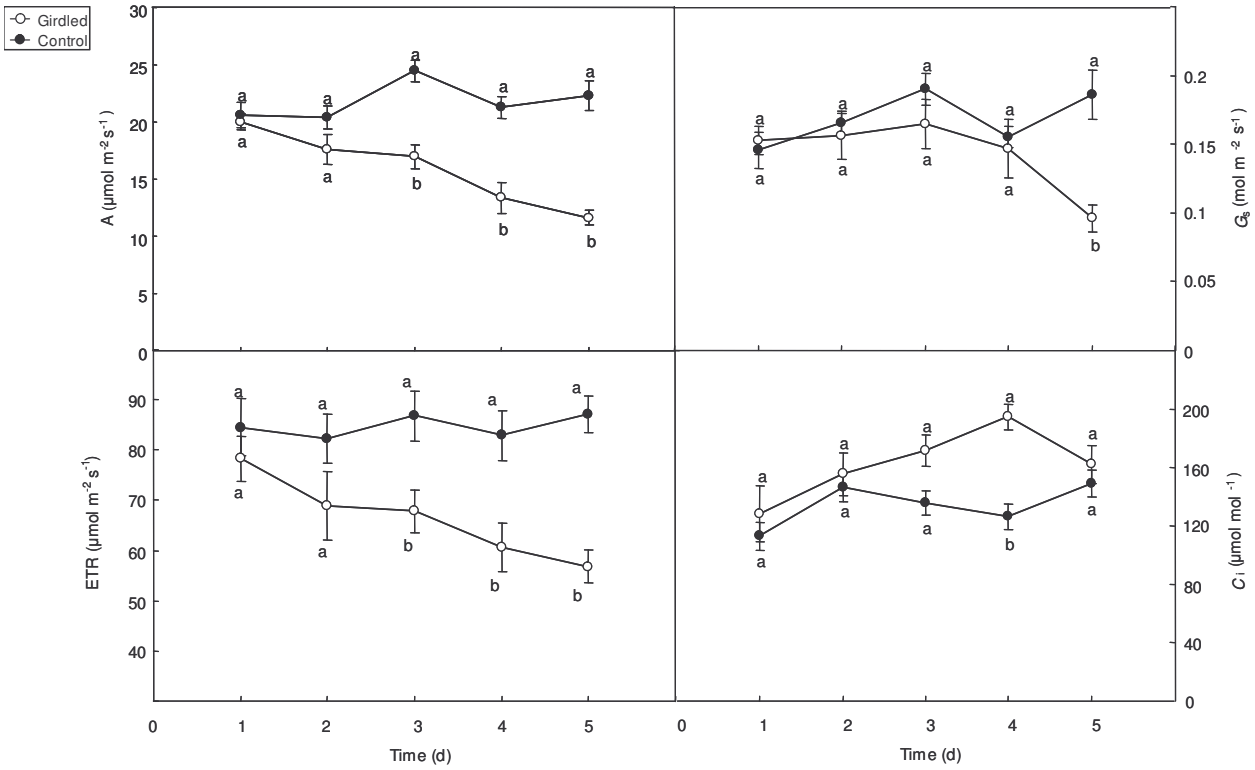

Fig. 5.7. Gas exchange analysis and leaf fluorescence variables of cold-girdled and ungirdled (control) leaves taken at 13h00, over a 5 day period. See Table 5.1 for variable details. Measurements were taken at an ambient RH of 56.3% ± 1.6 (mean ± SE) and an irradiance of 1500 µmol m^{-2} s^{-1} and CO_2 concentration of 370 µmol mol^{-1}. Values are the mean ± SE (n=4) and are followed by letters indicating whether cold-girdling treatments had a significant influence ($P<0.05$) as determined by Student's *t* tests.

5.5 Discussion

Sugar concentrations in sugarcane leaves increased rapidly on exposure to light. Sugarcane leaves have been shown to primarily accumulate and also export sucrose during the photoperiod (Hartt & Kortchack 1963; Lunn & Hatch, 1995; Du *et al*., 2000). Sucrose is readily exported from sugarcane leaves, with up to 80% of assimilate reportedly being exported immediately at midday (Du *et al*., 2000). The accumulation of sucrose in intact leaves represents a balance between the rate of synthesis and the rate of export. Sugar depletion by dark incubation subsequently stimulated photosynthesis. The effects of dark-depletion of sugars on photosynthesis were compared with the effects of pre-loading leaves with sucrose, glucose or fructose. Pre-loading with sugars negated the stimulatory effects of darkness on photosynthetic activity. When leaves were dark-treated to deplete leaf sugar concentrations, the photosynthetic activity in those pre-loaded with 5 mM sucrose was similar to that of leaves continuously supplied with light (Fig. 5.4). In contrast, photosynthesis of dark-treated leaves supplied with 5 mM sorbitol was increased compared to leaves continuously supplied with light (Fig. 5.1). This indicated that the observed suppression of photosynthesis by 5 mM sucrose was not due to an osmotic signal, but rather a possible consequence of sugar-sensing (Gibson, 2005).

Conversion of ^{14}C-sucrose into ^{14}C-hexoses occurred rapidly and thus differentiation between photosynthetic responses to hexoses and sucrose was not possible (Fig. 5.3). Leaves pre-loaded with glucose also rapidly converted ^{14}C-glucose to ^{14}C-sucrose (Fig. 5.5). Photosynthetic inhibition due to increased sucrose may thus be a result of sensitivity to the increased abundance of the hydrolytic products of sucrose. The localised activity of the sucrose-hexose interconversion enzyme, sucrose synthase (SuSy; EC 2.4.1.13), within vascular tissues has been discussed previously (Claussen *et al*., 1985; Wachter *et al*., 2003). SuSy may play an important signaling role by regulating the concentrations of apoplastic sugars. These results cannot effectively discriminate between hexose and sucrose-signaling, however they do substantiate the role of these sugars as signaling molecules that act to regulate sugarcane source activity. Although much progress has been made, the molecular bases of sugar-based signaling systems are still not clearly understood (for recent review see Rolland *et al*., 2006). Similarly, the signaling mechanisms which link phloematic, apoplastic and intracellular sugar concentrations remain to be fully elucidated (Gibson, 2005).

In addition to leaf excision experiments, cold-girdling was used to manipulate leaf sugar concentrations since this retains an intact xylem and nutrient supply system. Cold-girdling resulted in a significant increase in sucrose and hexose concentrations in sugarcane leaves and a concurrent decrease in both *A* and ETR rates over time (Fig. 5.6; 5.7). The inhibition of photosynthetic activity, observed in previous cold-girdling work, has been attributed to carbohydrate-induced changes in the expression of genes associated with the light-harvesting apparatus and enzymes in the pathway of CO_2 assimilation (Koch, 1996). Krapp & Stitt (1994) observed a decline in *rbcS* expression that ultimately led to significant decreases in Rubisco activity. In *Chenopodium rubrum* (L.) cell cultures fed with glucose, the repression of *rbcS* was observed only until the glucose supply was exhausted, whereupon *rbcS* transcript levels began to recover (Krapp *et al*. 1993). This recovery preceded any significant decrease in the internal carbohydrate content, indicating that processes related to sugar transport or the metabolism of carbohydrate were acting as the trigger (Krapp *et al*. 1993). As sugarcane is known to cycle hexose and sucrose (Whittaker & Botha, 1997), it is difficult to attribute these regulatory effects to any particular sucrolytic enzyme. In addition, sugar transporters specific to either hexose or sucrose may prove interesting candidates for future study of source-sink regulation.

Although sugar levels remained high during the initial 55 h days of cold-girdling, both sucrose and hexose levels dropped after 103 h of cold-girdling (Fig.5.6). The rates of *A* and ETR declined over the period of cold-girdling (Fig. 5.7). The decline in sucrose and hexose concentrations after 79 h may have been due to severely restricted photosynthesis and consequent remobilization of vacuolar sucrose for respiration. However, the mechanical/biochemical effects of cold-girdling, are still contentious, and cold-girdling effectiveness seems to vary between species (Hannah *et al*., 2001). Peuke *et al*. (2006) concluded that long-term cold-girdling may cause carbohydrate accumulation by leaching sucrose from the phloem and inhibiting normal retrieval by sucrose transporters, such as the recently identified sucrose carrier *RcSCR1* (Eisenbarth & Weig, 2005), instead of simply inhibiting mass flow. Long-term adaptation to cold-girdling treatment also cannot be excluded (Peuke *et al*., 2006; Hannah *et al*., 2001). Although *A* and ETR decreased soon after application of the cold-girdle, *E* and G_s only decreased significantly after 103 h of cold-girdling. This indicates that the initial influence of the cold-girdle was through biochemical modulation of photosynthesis, rather than simply through control of stomata.

Stomata have long been known to play an important role in regulating the capacity of mesophyll tissue to fix carbon (Wong *et al*., 1979). The correlation between G_s and A has been well studied under conditions of water stress (Sharkey *et al*., 1990; Ort et al., 1994; Lawlor, 1995), however, the degree to which other processes, such as metabolic damage or down regulation, co-limit photosynthesis are widely debated (Flexas & Medrano, 2002). In transgenic *Nicotiana tabacum* (L.) with reduced levels of Rubisco no discernible change in G_s was observed even though assimilation rates were significantly reduced (von Caemmerer *et al*., 2004). In the current study, sugar-induced changes in A were not dependent on G_s. This suggests that sugars may affect sugarcane leaf photosynthetic activity through metabolic signaling processes, such as hexokinase-mediated regulation (Rolland & Sheen, 2005).

In model C_3 species, such as *Arabidopsis thaliana*, sucrose, glucose and fructose are known to play pivotal roles in regulating source activities (Rolland *et al*., 2006). Photosynthesis and export appear to be up-regulated under low sugar conditions, while a co-ordinate increase in sink activity has been observed when source carbon is abundantly available (Bläsing *et al*., 2005; Roitsch *et al*., 1999). Much less is known about the role of these sugars in determining photosynthesis in the leaves of C_4 plants (Lunn & Furbank, 1999). In the present study, sugarcane leaves appeared to typically operate below their maximum achievable photosynthetic rates, which were readily increased by limiting leaf sucrose accumulation through a relatively short period of darkness. Dark treatment led to an increase in leaf A and ETR (Table 5.1; 5.2), which may be indicative of a robust regulatory link between photosynthesis and leaf sucrose status. Furthermore, this supports previous evidence that the photosynthetic activity of sugarcane leaves is highly adaptive to the perceived demand for photosynthate from sink tissues (Gutiérrez-Miceli *et al*., 2004; McCormick *et al*., 2006 [Chapter 3]). The observed increases in the rate of leaf sucrose accumulation in dark-treated leaves may be due partly to the light dependent-activation of sucrose phosphate synthase (EC 2.4.1.14), which is reported to have increased affinity for UDP-glucose in monocotyledonous species, and an additional lack of inhibition by P_i due to depleted triose-P concentrations following low light conditions (Lunn & Furbank, 1997b).

Hexose concentrations in untreated sugarcane leaves generally followed the ambient glasshouse light conditions, reaching a peak at midday, and then declining (Fig 5.1; 5.2; 5.4). A similar diurnal change in hexose sugars has been previously observed in C_4

maize leaves (Kalt-Torres *et al*., 1987), indicating that hexose may play an important role in regulating diurnal daily photosynthetic activity in leaves of C_4 species. Conversely, hexose levels decreased over time in dark-treated leaves with elevated photosynthetic activities. These results are similar to those of previous source-sink experiments using partial shading, where sugarcane leaves with increased photosynthetic capacity exhibited a corresponding reduction in hexose (McCormick *et al*., 2006 [Chapter 3]). Although sucrose is the primary product of photosynthesis, many sugar-signaling effects on growth and metabolism can be attributed to its hydrolytic hexose products (Rolland *et al*., 2006). Along with the activity of hexokinase (EC 2.7.1.1), hexoses have previously been implicated as key components in the regulation of photosynthesis and leaf development (Ehness *et al*., 1997; Paul & Pellny, 2003). In mature leaves of *Spinacia oleracea* (L.), glucose has been shown to repress both expression and translation of genes and proteins associated with photosynthesis (Krapp *et al*., 1991; Kilb *et al*., 1995). Furthermore, over-expression of cell wall invertase (EC 3.2.1.26) in mature leaves tobacco has been demonstrated to result in an accumulation of leaf carbohydrates and a consequent decrease in *rbsS* transcript levels (Von Schaewen, 1990). We therefore suggest that hexose concentrations may constitute a signal that, as in other species (Krapp *et al*., 1991; Kilb et al., 1995; Roitsch *et al*., 1995; Dekkers *et al*., 2004), modulates leaf photosynthetic activity in sugarcane.

5.6 Concluding remarks

Results from cold girdling, dark treatments, sucrose- and hexose-loading experiments have demonstrated the existence of a significant relationship between sugar concentrations and photosynthesis in sugarcane leaf tissue, indicating that sugar signaling is important in regulating leaf metabolism. Attempts to increase sucrose accumulation in sugarcane must take into account the fact that source-sink feedback mechanisms control photosynthesis in sugarcane. The ability of sugarcane to accumulate large concentrations of sucrose in the culm may be due to a relative insensitivity of photosynthesis to culm sucrose concentrations.

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Chapter 6:
Sugar accumulation induces differential expression of genes related to carbohydrate metabolism, photosynthesis and sugar-sensing: evidence for a trehalose-related signaling mechanism in sugarcane leaves

6.1 Summary

- In sugarcane, photosynthetic activity has been shown to be regulated by the demand for carbon from sink tissues. There is evidence, from other plant species, that sink-limitation of photosynthesis is facilitated by sugar-signaling mechanisms in the leaf that affect photosynthesis through regulation of gene expression.
- To manipulate leaf sugar levels, field-grown sugarcane leaves were cold-girdled (5^{o}C) for 80 h. During this time, sucrose and hexose concentrations above the girdle increased by 77% and 81%, respectively. Conversely, leaf photosynthetic activity (*A*) and electron transport rates (ETR) decreased by 66% and 54%, respectively.
- Expression profiling by means of an Affymetrix GeneChip Sugarcane Genome Array was used to identify genes responsive to the cold-girdling treatment (56 h). A number of genes (73) involved in fundamental metabolic pathways, including photosynthesis, carbohydrate metabolism, and sugar-signaling, were identified as being differentially expressed.
- Decreased expression of genes related to photosynthesis and increased expression of genes involved in glycolytic carbon partitioning, cell wall synthesis, phosphate metabolism and stress were observed. Furthermore, trehalose phosphate phosphatase (TPP; EC 5.3.1.1) and trehalose 6-phosphate synthase (TPS; EC 2.4.1.15) were up- and down-regulated, respectively, indicating a role for trehalose 6-phosphate (T6P) as a putative sugar-sensor in sugarcane leaves.

Keywords: cold-girdling, expression profiling, genes, leaf, photosynthesis, sugar, sugarcane, trehalose.

6.2 Introduction

Sugarcane (*Saccharum* L. spp. hybrids) is the primary resource for world sucrose production (Lunn & Furbank, 1999; Wu & Birch, 2007) and, hence, considerable efforts are being made to improve sugarcane biomass production and sucrose yields (Jackson, 2005). Previously varietal improvement relied on crossing and selection, but now new molecular techniques are being used in concert with these more conventional approaches to increase crop yields (Huckett & Botha, 1995; Ma *et al*., 2000; Leibbrandt & Snyman, 2003; Wu & Birch, 2007). Research into the regulation of carbohydrate metabolism in model C_3 plants, such as *Arabidopsis thaliana* (L.), has advanced significantly in recent years (Bläsing *et al*., 2005; Franco-Zorrilla *et al*., 2005; Gibon *et al*., 2006; Müller *et al*., 2007). In contrast, the study of C_4 plants has lagged behind, with the majority of work focusing on maize (*Zea mays* L.) (Lunn & Furbank, 1999; Lee *et al*., 2002; Sawers *et al*., 2007). Consequently, the regulatory mechanisms involved in sugarcane sucrose metabolism and accumulation are still relatively uncharacterised (Grof & Campbell, 2001; Casu *et al*., 2004; Watt *et al*., 2005).

In a variety of species, the photosynthetic activity of source leaves appears to be dependent on the demand for carbon from sink tissues (Basu *et al*., 1999; Paul & Foyer, 2001; Minchin *et al*., 2002; Paul & Pellny, 2003). Previous work in C_3 plants, using cold-girdling and sugar feeding techniques, has demonstrated the limiting effects of increased leaf sugar concentrations on chlorophyll content, Rubisco expression and activity, and overall photosynthetic rates (Krapp *et al*., 1991; Schäfer *et al*., 1992; Krapp & Stitt, 1995; Iglesias *et al*., 2002). Evidence increasingly shows that the interactions between source and sink systems are regulated by levels of the major transport sugar, sucrose, and/or its constituent hexoses (Pego *et al*., 2000; Rolland *et al*., 2002; Franck *et al*., 2006).

Several sugar-sensing mechanisms are suggested to be involved in regulating photosynthesis (Rolland *et al*., 2006). Hexokinase (HXK; EC 2.7.1.1) is a glucose sensor that is believed to function for both glucose phosphorylation (i.e. in glycolysis) and glucose signaling (Harrington & Bush, 2003; Rolland *et al*., 2006). Analysis of the activity of two catalytically inactive constructs of the *Arabidopsis AtHXK1* gene has revealed that HXK plays an integral role in growth promotion, independent of its ATP-binding function (Moore *et al*., 2003). The activity of HXK has been detected in the cytosol, the chloroplast stroma and even the nucleus (Moore *et al*., 2003; Yanagisawa *et*

al., 2003; Giese *et al*. 2005), which suggests a prominent role for the enzyme in several intracellular sugar-sensing pathways. However, the characterisation of several transporter genes, such as a proton-sucrose symporter in sugar-beet whose expression is regulated by sucrose and not hexose (Vaughan *et al*., 2002), indicate the existence of a HXK-independent, sucrose-specific signaling pathway (Rolland *et al*., 2006). Furthermore, studies of trehalose sugar-signaling suggest that the phosphorylated product of trehalose 6-phosphate synthase (TPS; EC 2.4.1.15), trehalose 6-phosphate (T6P), mediates an essential, HXK-independent signaling process in higher plants (Eastmond *et al*., 2003; Schluepmann *et al*., 2003; Paul, 2007). Recently, changes in leaf sugar levels have been shown to lead to rapid changes in T6P content, indicating that T6P can act as a signal for the sugar status of source tissues (Lunn *et al*., 2006). The limited activity of TPS observed in *Arabidopsis* has suggested that control of the system could be exerted mainly at the level of T6P breakdown, by trehalose phosphate phosphatase (TPP; EC 5.3.1.1) (Ramon & Rolland, 2007). The modification of the trehalose pathway, in both C_3 and C_4 species, has since been shown to impact positively on several aspects of crop physiology, including gross photosynthetic activity, relative growth rates and drought tolerance (Pellny *et al*. 2004; Satoh-Nagasawa *et al*., 2006; Karim *et al*., 2007).

In addition to the role of sugar sensors, inorganic phosphate (P_i) could facilitate a regulatory link between sugars and photosynthetic source activity (Paul & Pellny, 2003). Several enzymes directly involved in photosynthesis, such as chloroplastic ATPase, are sensitive to P_i concentration and are inhibited if P_i availability decreases (Pammenter *et al*., 1993). Under conditions of increased leaf carbohydrate accumulation, the excessive accumulation of P_i in phosphorylated sugar intermediates, such as triose or hexose-phosphates, may limit the availability of P_i turnover between the chloroplast and cytosol, and thus restrict photosynthetic activity (Pieters *et al*., 2001; Paul & Pellny, 2003). Recent work has demonstrated that the expression of P_i-starvation responsive genes in *Arabidopsis* is stimulated by increased sucrose (Franco-Zorrilla *et al*., 2005; Müller *et al*., 2007). Conversely, several sugar responsive genes, including TPS, have been shown to respond to changes in P_i (Müller *et al*., 2007). Together, these studies indicate a strong link between P_i and sugar signaling mechanisms, at least in C_3 species. Transgenic *Arabidopsis* plants over-expressing inorganic pyrophosphatase (PPase; EC 3.6.1.1) exhibited significant increases in P_i, sugar concentrations and an associated decrease in photosynthetic rates, suggesting an important regulatory role for PPase in

maintaining an optimal PP_i: P_i ratio (Lee *et al*., 2005). However, the sensitivity of different species to negative photosynthetic feedback based on changes in leaf sugar status, and the extent of further interactions with P_i, may vary considerably (Krapp & Stitt, 1995). The regulatory roles of sugar-linked sensing mechanisms in C_4 species are even less well characterised, and require further investigation.

In sugarcane, large differences in photosynthetic rates have been previously observed for individual sugarcane leaves related to the age of the plant, with young plants typically assimilating at significantly higher rates than older plants (Hartt & Burr, 1967; Bull & Tovey, 1974; Amaya *et al*., 1995; Allison *et al*., 1997). The reason that the photosynthetic rate is dependent on plant age is probably due to the accumulation of sucrose in the culms of older plants (McCormick *et al*., 2006 [Chapter 3]). Gutiérrez-Miceli *et al*. (2004) have reported that partial defoliation of sugarcane has no significant effect in reducing culm sucrose concentration, indicating that the remaining photosynthetic capacity was sufficient to adapt to sink demand. The flexibility of photosynthetic activity in sugarcane leaves was recently investigated using a partial shading technique where all but one source leaf was covered in shade cloth (McCormick *et al*., 2006 [Chapter 3]). The sole leaf exhibited a significant increase in photosynthetic activity, while the observed partitioning patterns of carbon in the culm were indicative of increased sink demand. Furthermore, a strong negative correlation was revealed between leaf hexose concentrations and photosynthesis in the sole source leaf, suggesting that hexose is involved in regulating the source-sink dynamic (McCormick *et al*., 2006 [Chapter 3]). This suggests that HXK or HXK-independent mechanisms, such as trehalose metabolism, may play a role in mediating source photosynthetic rates in sugarcane.

Expressed sequence tag (EST) analysis has been widely used in attempts to identify genes associated with the regulation of sucrose accumulation during sugarcane culm development (Arruda, 2001; Carson *et al*., 2002; Casu *et al*., 2003; 2004). These collections have been brought together as an Affymetrix Genechip Sugarcane Genome Array bearing 255 964 ESTs (Casu *et al*., 2007). This system provides a powerful tool for the profiling of quantitative changes in sugarcane gene expression in response to developmental and environmental cues. One such application is the clarification of the roles of particular genes and gene networks in the feedback regulation of sugarcane photosynthesis. The current study has implemented a cold-girdling technique in field-

grown sugarcane leaves to examine the feedback relationship between source photosynthetic activity and the status of leaf sugar concentrations. In addition, associated changes in leaf transcript abundance were analysed using the Affymetrix GeneChip Sugarcane Genome Array. Cold-girdling induced accumulation of sugars in the leaf and the concurrent decline in photosynthetic activity was related to significant changes in the expression of several genes encoding products involved in carbohydrate metabolism, photosynthesis, sugar transport and stress response. Furthermore, TPP and TPS expression were up- and down-regulated, respectively, which provided evidence for the existence of a T6P sugar-signaling mechanism in sugarcane leaves. In addition, the array data indicated a potential mechanistic limitation imposed by excess source sugar accumulation on P_i availability and photosynthetic activity.

6.3 Materials and methods

6.3.1 Plant material and treatment

Twelve-month-old field-grown *Saccharum* spp. (L.) hybrid *cv.* N19 (N19) cultivated at Mount Edgecombe, KwaZulu-Natal (SASRI) were used in the study, which was conducted in November, 2006. Plants were grown on a 5 x 15 m plot located on a north-east facing slope with a gradient of *ca.* 10°. Two weeks prior to cold-girdling treatment, plants were irrigated 2–3 times per week to ensure adequate hydration. For all leaf studies, the second and third fully expanded leaves were used (McCormick *et al.*, 2006 [Chapter 3]). The experiment was staggered, so that cold-girdles were attached to leaves (n=10) every morning (8h00) for a period of 4 d, and all leaves were collected at 15h00 on the final day. The girdle consisted of 0.75 cm (diameter) soft plastic tubing, firmly clamped around each leaf, approximately 30 cm from the leaf base. Cooled water maintained at 5°C was then pumped through the tubing using a Grant LTD6G cooling bath (Grant Intruments, Barrington, Cambridge, UK). At harvest, leaf samples 10 cm above the cold-girdle were immediately frozen in liquid nitrogen (–196°C) and subsequently milled in an A11 Basic Analysis Mill (IKA®, Staufen, Germany). Ground leaf tissue was stored at –80°C in 50 ml centrifuge tubes prior to analysis.

6.3.2 Sugar determination

Approximately 100 mg powdered tissue was incubated overnight in 10 volumes of a buffer containing 30 mM HEPES (pH 7. 8), 6 mM $MgCl_2$ and ethanol 70% (v/v) at 70°C. Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic coupling assay described previously (McCormick *et al*., 2006 [Chapter 3]). The phosphorylation of glucose by hexokinase/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Roche, Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5.3.1.9) (Roche) was quantified by following the reduction of NADP to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader using KC4 software (Biotek Instrument, Inc., Vermont, USA).

6.3.3 Gas exchange and fluorescence determinations

Gas exchange measurements were made on 2 cm^2 portions of leaf tissue using a portable gas exchange system (LI-6400, LI-COR Biosciences Inc., Nebraska, USA). Light was provided by a red/blue LED light source (LI-COR Biosciences Inc.) at photon irradiance of 2 000 µmol m^{-2} s^{-1}. All leaf measurements were done under ambient CO_2 conditions (380 µmol mol^{-1}) at a leaf temperature of 28°C between 10h00 and 12h00. Gas exchange variables measured include photosynthetic assimilation (*A*), transpiration rate (*E*), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and leaf temperature. Comparative measurements (n=8) were performed on the day of harvest for leaves that were cold-girdled or untreated. Furthermore, readings were taken daily during the cold-girdling treatment to measure photosynthetic consistency in untreated leaves. To contrast photosynthetic rates in untreated leaves and leaves cold-girdled for 74 h (n=4), the response of *A* to C_i (*A*:C_i) was measured by varying the external CO_2 concentration from 0 to 1 000 µmol mol^{-1} under a constant photosynthetically active radiation (PAR) of 2 000 µmol m^{-2} s^{-1}. An equation $A = a\left(1 - e^{(-bC_i)}\right) - c$ was fitted to the *A*:C_i data using least squares. The portion of the curve where the slope approaches zero due to limitation in the supply of substrate (ribulose-1,5-bisphosphate), which is equivalent to the CO_2- and light-saturated photosynthetic rate (J_{max}) (Lawlor, 1987), was calculated from this equation (a, J_{max}; b, curvature parameter; c, dark respiration (R_d).

Linear regression was performed on the data between a C_i of 0 and 200 µmol mol^{-1} to determine the efficiency of carboxylation (*CE*; Lawlor, 1987).

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0.8 s, 6 000 µmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm') at varying external CO_2 concentrations (0 – 1 000 µmol mol^{-1}). The electron transport rate (ETR), defined as the actual flux of photons driving photosystem II (PSII) was calculated from $ETR = \left(\frac{Fm' - Fs}{Fm'} \right) fI\alpha_{leaf}$, where *Fs* is "steady-state" fluorescence (at 2 000 µmol m^{-2} s^{-1}), *Fm'* is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by PSII, typically assumed to be 0.4 for C_4 plant species (Edwards & Baker, 1993), *I* is incident photon flux density and α_{leaf} is leaf absorptance (0.85, LI-COR manual). The component fluorescence variables were derived as described by Maxwell & Johnson (2000).

6.3.4 RNA preparation

Total RNA was extracted from individual untreated leaves (n=4) and leaves cold-girdled for 56 h (n=4) using the extraction protocol of Bugos *et al.* (1995). RNA concentration and quality were calculated from ultra-violet (UV) spectrophotometric absorbance measurements at 260 nm and 260:280 nm, respectively (Beckman DU-7500 spectrophotometer, USA) and assessed for integrity via gel electrophoresis (Ingelbrecht *et al.*, 1998). All RNA samples were stored at –80°C until required.

6.3.5 GeneChip array hybridisations

Transcript abundances in each leaf RNA sample were analysed using Affymetrix Genechip Sugarcane Genome Arrays (Affymetrix, Inc., Santa Clara, CA, USA). All labelling, hybridisations of biotinylated cRNA to the array chips and data collection were performed by the Australian Genome Research Facility (AGRF, Melbourne, VIC, Australia). Total RNA was checked for quality and integrity using a Bioanalyser 2100 and the NanoChip protocol according to the manufacturer's directions (Agilent Technologies, Palo Alto, CA, USA). Labelled target used in hybridisations was generated from 15 µg of total RNA using the One-Cycle Eukaryotic Target Labeling

Assay. Hybridisations, washing staining and scanning were performed as specified in the manufacturer's protocol (Affymetrix, Inc.). The scanner operating software, GCOS, converted the signal on each array into a DAT file, which was then used to generate CEL and CHP files for analysis.

6.3.6 Statistical analysis

Gas exchange data and sugar measurements were subjected to analysis of variants (ANOVA) or Student's *t* tests to determine the significance of differences amongst responses to treatment. When ANOVA was performed, Tukey's honest significant difference (HSD) *post-hoc* tests were conducted to determine the differences between the individual treatments (SPSS Ver. 11.5, SPSS Inc., Illinois, USA).

Microarray statistical analysis was performed using GeneSpring GX 7.3 (Agilent Technologies, USA) at the Australian Genome Research Facility Ltd. (Queensland, Australia). Raw data files were analysed firstly by Robust Multiarray Analysis (RMA) normalisation (Irizarry *et al*., 2003), which was subsequently normalised, per gene, to the median. Normalised expression levels were then compared between treatment and control data sets (Supplementary Figure 6.1). A fold change (greater than two-fold) between treatment and control was used to identify biological significance. ANOVA was then performed to flag differentially expressed genes between control and treatment ($P<0.05$). The provided GenBank number corresponding to each differentially expressed probe set was assessed for putative protein identity using the BLASTX function (Altschul *et al*., 1997) within the National Centre of Biotechnological Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov). These results were further cross-checked and compared to supplementary tables provided by Casu *et al*. (2007). Genes were then categorised into their accepted metabolic pathways or a suitable metabolic category as defined by the Kyoto Encyclopedia of Gene and Genomes (KEGG - http://www.genome.ad.jp/kegg/) and EMBL-EBI protein database (Interpro - http://www.ebi.ac.uk/interpro/).

6.4 Results

6.4.1 Effects of cold-girdling on leaf sugar concentrations

In untreated sugarcane leaves, sucrose concentrations increased from 8.4 ± 0.9 µmol g^{-1} in the morning (9h00) to 53.7 ± 3.4 µmol g^{-1} in the afternoon (16h00) (Fig. 6.1). Hexose (glucose and fructose) concentrations also increased during the morning, reaching a plateau at midday and then decreasing thereafter. Afternoon measurements of sucrose and hexoses did not differ significantly during the experiment, indicating a consistent diurnal pattern amongst untreated leaves. Conversely, in cold-girdled leaves, sucrose concentrations continued to increase over time, reaching 99.4 ± 9.6 µmol g^{-1} after 56 h (Fig. 6.1). Following 8 h of cold-girdling treatment, leaf hexoses were significantly increased compared to untreated controls. The concentrations of hexose in cold-girdled leaves declined over time, but remained significantly higher than controls until 80 h.

6.4.2 Effects of cold-girdling on leaf photosynthetic activity

Gas exchange and fluorescent activity of untreated sugarcane leaves measured under ambient conditions did not differ significantly throughout the experiment, producing average *A* and ETR values of 24.4 ± 0.6 µmol CO_2 m^{-2} s^{-1} and 133 ± 14.3 µmol CO_2 m^{-2} s^{-1}, respectively (Table 6.1). In contrast, cold-girdled leaves exhibited a significant reduction in photosynthetic activity over the duration of the treatment; after 74 h of cold-girdling, *A* and *ETR* declined to levels of 66 % and 54 % below untreated leaves, respectively (Table 6.1). Cold-girdled leaves also showed reduced *E* and G_s over time, although the changes in *E* were not as pronounced as those of *A* and ETR.

The gas exchange and ETR variables derived from A:C_i and ETR:C_i curves of leaves cold-girdled for 74 h were substantially lower than control leaves (Fig. 6.2). Significant reductions in the substrate-limited photosynthetic rate ($J_{max,}$ 66%) and carboxylation efficiency (*CE*, 84%) were further observed in comparison to untreated controls.

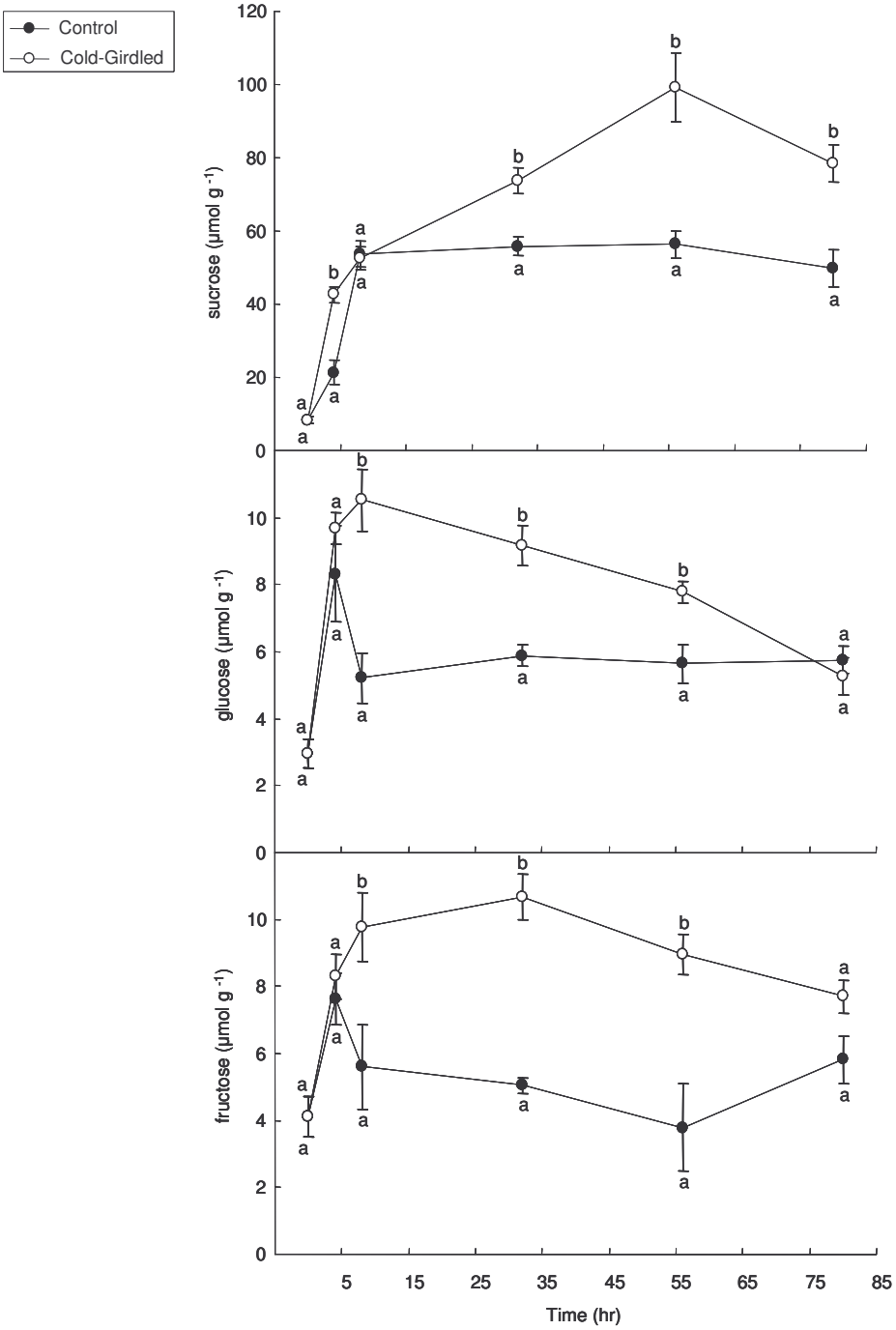

Fig. 6.1. Sucrose, glucose and fructose concentrations for cold-girdled (5°C) (○) and ungirdled (control) (●) leaves of sugarcane plants over time. Letters above SE bars indicate whether the cold-girdling treatment (n=5) was significantly different from controls ($P<0.05$) as determined by Student's *t*-tests.

Table 6.1. Gas exchange analysis and leaf fluorescence variables for untreated leaves (control) and leaves cold-girdled for different periods of time: photosynthetic rate (*A*), electron transport rate (ETR), stomatal conductance (G_s), transpiration rate (*E*) and intercellular CO_2 concentration CO_2 (C_i). Measurements were taken at an ambient RH of 37.4% ± 3.9 (mean ± SE) and an irradiance of 2000 µmol m^{-2} s^{-1} and CO_2 concentration of 370 µmol mol^{-1}. The values for cold-girdled leaves are the mean ± SE (n=8) and are followed by letters indicating whether treatments had a significant influence (P<0.05), as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests. For the duration of the cold-girdling treatment, untreated controls were measured daily, but as no significant differences were detected over time, the overall mean ± SE (n=32) is presented for this group.

	control	**cold-girdled leaves**			
		0 hr	**24 hr**	**48 hr**	**72 hr**
A (µmol m^{-2} s^{-1})	24.4 ± 0.6 a	24.2 ± 1.2 a	15.1 ± 0.9 ab	10.9 ± 1 bc	8.2 ± 0.85 c
ETR (µmol m^{-2} s^{-1})	133.13 ± 14 a	127 ± 10 a	73.6 ± 5.4 b	61.3 ± 4.4 b	60.1 ± 3.9 b
G_s (mmol m^{-2} s^{-1})	160 ± 22 a	162 ± 8 a	123 ± 7 b	107 ± 10 b	93 ± 8 b
E (mmol m^{-2} s^{-1})	4.3 ± 0.19 a	4.2 ± 0.21 a	3.2 ± 0.18 b	2.8 ± 0.2 b	2.6 ± 0.2 b
C_i (µmol mol^{-1})	121.6 ± 27 a	101.9 ± 4 a	151.1 ± 9 b	185.7 ± 12 bc	209.5 ± 10 c

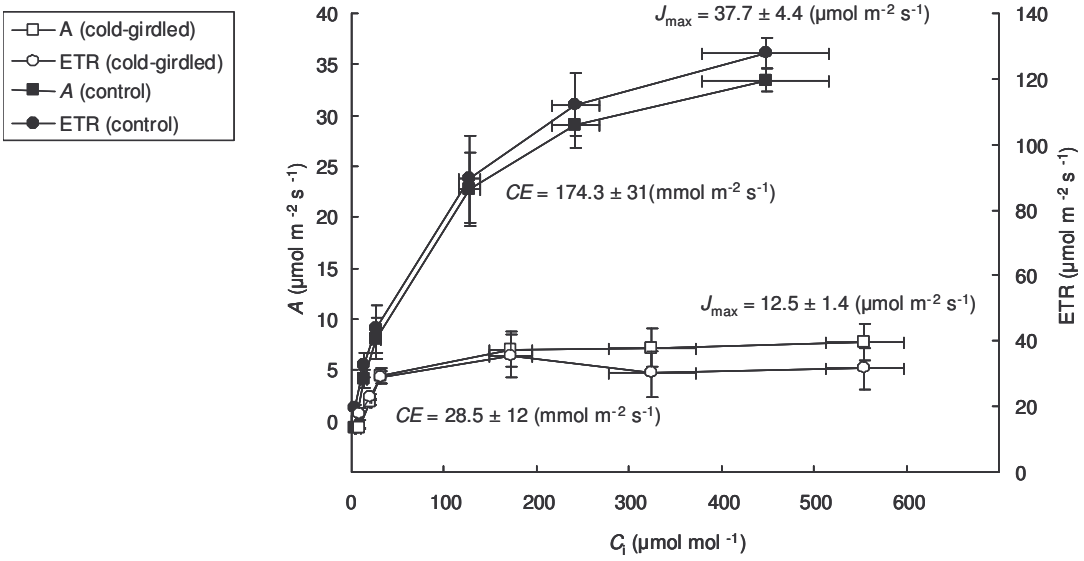

Fig. 6.2. Changes in photosynthetic CO_2 assimilation (µmol m-2 s-1) and photosynthetic electron transport rate (ETR) of leaves cold-girdled for 50 h and ungirdled (control) leaves versus intercellular CO_2 concentration (C_i, µmol mol-1) in twelve-month-old field-grown sugarcane (n=4). Substrate supply limited assimilation (J_{max}) and carboxylation efficiency (*CE*) for each treatment are indicated. Measurements were made at an average ambient RH of 35.3% ± 1 and an irradiance of 2000 µmol m^{-2} s^{-1}.

6.4.3 Analysis of differentially expressed transcripts between control and cold-girdled leaf tissue

Genechip Sugarcane Genome Array analysis was performed on total RNA derived from individual cold-girdled (56 h) and control leaf samples. Each treatment contained four replicates and each sample was hybridised separately to the array. Of the total 8 236 sugarcane probe sets compared between the cold-girdling treatment and control, 673 were significantly differentially expressed (Fig. 6.3). The variation between replicate leaf samples ('natural' variation) within these probe sets produced an average coefficient of variation (CV) of 17% and 18.3% for controls and cold-girdled samples, respectively (data not shown).

Probe set gene identities were confirmed by BLAST analysis and then, where appropriate, classified to a broad group of general metabolic categories (Table 6.2; Supplementary Table 6.1). A total of 246 probe sets were identified as either a 'hypothetical protein' by BLAST analysis or with 'unknown function' according to the

classification of Casu *et al*. (2007). A further 96 probe sets did not fit into the above categories and were thus grouped under 'miscellaneous metabolism'. In order to focus array results on events related to leaf carbohydrate and photosynthetic status, a total of 140 probe sets in six categories were specifically targeted for further analysis: i) carbohydrate metabolism, ii) photosynthesis (light and dark reactions); iii) sugar signaling, transport and P_i metabolism, iv) mitochondrial metabolism, v) cell wall metabolism and vi) stress response. Within these groups, a total of 73 gene identities were represented (Table 6.3).

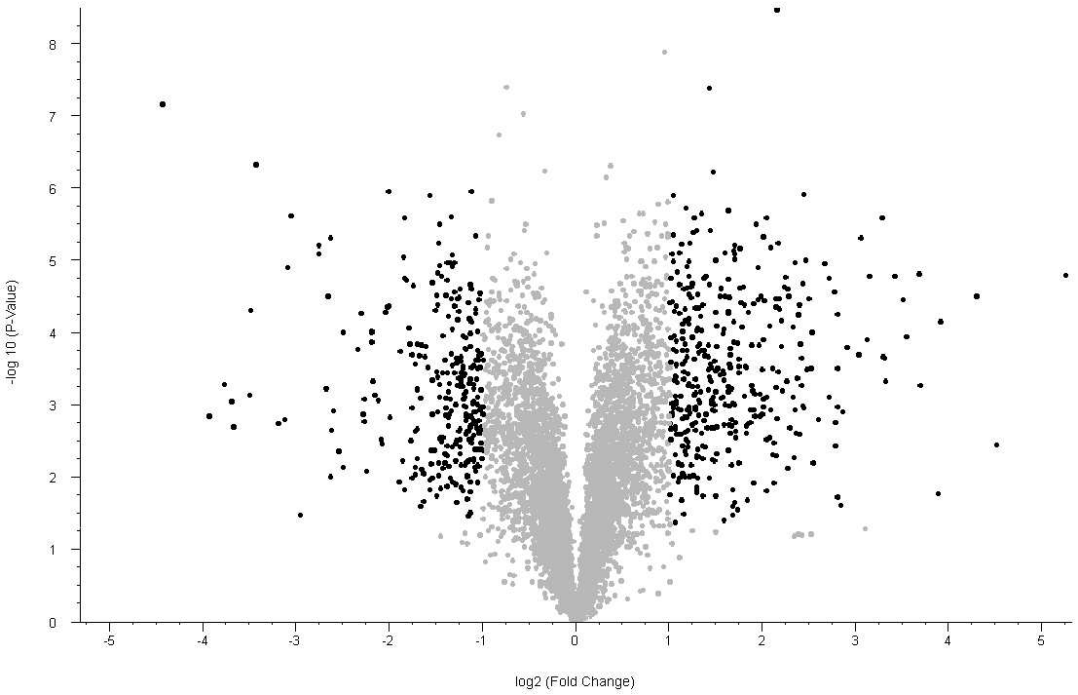

Fig. 6.3. Expression of genes during a cold-girdling treatment in sugarcane leaves. Genes that were significantly differentially expressed are indicated in black. Data is arranged along dimensions of biological (expression fold change between the controls and treatment) and statistical significance value ($P<0.05$) based on a Students *t*-test of differences between replicates (n=4). The x-axis indicates biological impact of the change, while the y-axis indicates the statistical evidence, or reliability of the change.

Table 6.2. General categorisation of putative gene function of 673 differentially expressed probe sets identified by microarray analysis in leaves (n=4) cold-girdled for 56 h. The number of genes cited reflects both different genes and variants of single genes.

General categories of gene function	Probe sets in category
Carbohydrate metabolism	13
Mitochondrial metabolism	5
C_4 photosynthesis (light and dark)	46
Sugar transport, signaling and P_i metabolism	11
Cell wall metabolism	23
Stress response	42
Hormone response	13
DNA, RNA transcription and binding	43
Lipid, phospholipid and fatty acid synthesis	22
Other transporters	15
Amino acid and protein metabolism	74
Heavy metal metabolism	11
Cytoskeletal metabolism	13
Miscellaneous metabolism	96
Unknown	246

6.4.4 Response of photosynthesis- and carbohydrate metabolism-related genes to cold-girdling

Probe sets associated with carbohydrate metabolism that were up-regulated included genes coding for three enzymes that regulate the fate of triose-phosphate, *viz*. fructose-1,6-bisphosphate aldolase (FBPase; EC 4.1.2.13), triose phosphate isomerase (TPI; EC 5.3.1.1) and glyceraldehyde-3-phosphate dehydrogenase (GPD; EC 1.2.1.12) (Table 6.3). Genes that were down-regulated include pyrophosphate-dependent phosphofructokinase (PFP; EC 2.7.1.90) and a cell wall invertase (CWI; EC 3.2.1.26) isoform, *Incw4*. Several genes related to starch metabolism were also up-regulated, including ADP-glucose pyrophosphorylase, (AGPase; EC 2.7.7.27), which exhibited a twelve-fold increase in expression.

All genes associated with the light and dark reactions of photosynthesis were down-regulated (Table 6.3). Notably, for the dark reactions this included both the large and small subunit of Rubisco (EC 4.1.1.39), and the C_4 photosynthesis-related genes NADP-dependent malate dehydrogenase (NADP-MD; EC 1.1.1.82) and NADP-dependent malic enzyme (NADP-ME; EC 1.1.1.40), located in the mesophyll and bundle sheath

chloroplasts, respectively. Genes related to the light reactions comprised the largest group of down-regulated genes, which included several subunits of chloroplastic ATPase, NADH dehydrogenase and photosystem I and II. Photosystem I subunit O was the most down-regulated (22-fold) of all identified differentially expressed probe sets.

The expression of three genes associated with mitochondrial processes were up-regulated in response to the cold-girdling treatment (Table 6.3). Tricarboxylic acid (TCA) cycle enzyme NADH-dependent malate dehydrogenase (NADH-MD; EC 1.1.1.37), which catalyses the production of oxaloacetate (OAA) from malate, was up-regulated, as were two isoforms of mitochondrial uncoupling protein (MUP). All genes identified as related to cell wall metabolism and synthesis displayed increased expression. These comprised 10 genes, including callose synthase and several isoforms of cellulose synthase.

6.4.5 Changes in expression of genes related to sugar transport and signaling, P_i metabolism and stress response

A total of eleven probe sets constituting seven genes with putative roles in sugar trans-membrane transport, sugar signaling mechanisms and P_i metabolism were identified as differentially expressed (Table 6.3). The sugar transporters encoded by these genes consisted of a sugar transporter of unknown function (PST Type 2a), a putative hexose transporter and a glucose 6-phosphate/phosphate translocator (G6PT). All transporters were up-regulated; however, G6PT was, on average, expressed at levels three-fold higher than hexose transporters. In addition, PPase and two probe sets identified as P_i translocators were found to be up-regulated. Two genes related to trehalose metabolism, TPS and TPP, were down- and up-regulated, respectively.

Numerous genes (22) with both known and putative involvement in stress response were identified during expression analysis, the majority of which were up-regulated (Table 6.3). These include alcohol dehydrogenase (ADH; EC 1.1.1.1), chalcone synthase (CS; EC 2.3.1.74) and a proteinase inhibitor. Notably, one of the highest up-regulated (23-fold) probe sets identified in this category was jacalin, a carbohydrate-binding lectin. Down-regulated genes include the beta subunit of SNF1-related kinase (SnRK1; EC 2.7.11.1). Furthermore, two probe sets representing a gene that encodes mitogen-activated protein kinase (MAPK; EC 2.7.11.24), were up- and down-regulated.

This contrasting change in expression was likely to be due to these probe sets representing distinct isogenes or gene family members particular to specific tissues.

Table 6.3. Putative gene identity and fold-change of 140 differentially regulated probe sets, constituting 73 genes, in sugarcane leaves cold-girdled for 56 h. Genes are classified into the following metabolic processes: i) carbohydrate metabolism (glycolysis and starch metabolism), ii) mitochondrial metabolism, iii) photosynthesis (light and dark reactions), iv) sugar signaling, transport and P_i metabolism, v) cell wall metabolism and vi) stress response. Arrows indicate the degree of up- or down-regulation for each probe set. The protein EC number is provided where appropriate.

No.	Putative identity	Probe set	EC	Genbank	Fold change	
Carbohydrate metabolism						
	Glycolysis					
1	fructose-1,6-bisphosphate aldolase	SofAffx.1015.1.S1_s_at	4.1.2.13	CF574067	2.4	↑
		Sof.3192.2.S1_at		CA206457	2.3	↑
2	glyceraldehyde-3-phosphate dehydrogenase	Sof.2554.1.S1_at	1.2.1.12	CA093039	2.1	↑
		Sof.3865.1.S1_at		CA196594	2.5	↑
3	cell wall invertase	SofAffx.1973.1.S1_at	3.2.1.26	BU925731	0.4	↓
4	pyrophosphate-dependent 6-phosphofructose-1-kinase	Sof.3023.2.S1_a_at	2.7.1.90	CA208800	0.4	↓
		Sof.3023.1.S1_s_at		CA070403	0.4	↓
5	triosphosphate isomerase	Sof.2883.1.S1_at	5.3.1.1	CA147454	3.1	↑
Starch metabolism						
6	1,4-alpha-glucan branching enzyme	Sof.2109.1.S1_a_at	2.4.1.18	CA202596	2.9	↑
7	ADP-glucose pyrophosphorylase	Sof.4578.1.S1_a_at	2.7.7.27	CA151666	12.8	↑↑↑
8	beta-amylase	Sof.1235.1.S1_at	3.2.1.2	CA169143	5.2	↑↑
9	isoamylase	Sof.4751.1.S1_at	3.2.1.68	CA289034	8.2	↑↑↑
10	phosphorylase	Sof.1216.1.S1_at	2.4.1.1	CA169622	8.8	↑↑↑
Mitochondrial metabolism						
1	mitochondrial malate dehydrogenase (NADH-dependent)	Sof.3353.1.S1_at	1.1.1.37	CA181748	3.0	↑
2	mitochodrial transcription termination factor	Sof.1026.1.S1_at	-	CA265014	0.5	↓
		Sof.1026.2.S1_at		CA161102	0.3	↓
3	mitochondrial uncoupling protein 1	SofAffx.14.1.S1_at	-	AY644460	2.4	↑
		Sof.2009.1.S1_at		CA170908	2.1	↑
C_4 photosynthesis and related metabolic components						
	Light reactions					
1	ATP synthase CF0 A chain gene	SofAffx.2196.1.S1_at	3.6.3.14	50198865-69	0.3	↓
	ATP synthase CF0 A chain gene	SofAffx.2171.1.S1_at		50198865-59	0.3	↓
	ATP synthase CF0 C chain gene	SofAffx.2188.1.S1_at		50198865-61	0.4	↓
	ATP synthase CF0 C chain gene	SofAffx.2186.1.S1_at		50198865-60	0.4	↓
	ATP synthase CF1 beta chain gene	SofAffx.2118.1.S1_at		50198865-70	0.2	↓↓
2	chlorophyll a/b-binding protein	Sof.4725.1.S1_at	-	CA186676	0.3	↓↓
		Sof.3564.1.S1_at		CA298559	0.5	↓

		Sof.3564.1.S1_a_at		CA298559	0.4	↓
3	cytochrome P450	Sof.2722.1.S1_at	1.14.14.1	CA174395	0.4	↓
		Sof.1713.2.S1_at		CA068881	0.1	↓↓↓
		Sof.1713.1.A1_at		CA068965	0.1	↓↓↓
4	cytochrome b6 f complex subunit VIII gene	SofAffx.2164.1.S1_at	-	50198865-54	0.3	↓
5	cytochrome biogenesis protein gene	SofAffx.2179.1.S1_at	-	50198865-22	0.4	↓
6	geranylgeranyl hydrogenase	Sof.2428.1.S1_at	-	CA223413	0.4	↓
		Sof.3048.1.A1_s_at		CA105372	0.4	↓
7	magnesium chelatase	SofAffx.56.1.S1_at	-	CF577098	0.3	↓
		SofAffx.1150.1.S1_at		CF573280	0.3	↓
		Sof.4426.1.A1_at		CA067637	0.3	↓↓
9	NADH dehydrogenase subunit 1	SofAffx.2184.1.S1_at	1.6.99.3	50198865-27	0.4	↓
	NADH dehydrogenase subunit 1	SofAffx.2185.1.S1_at		50198865-28	0.5	↓
	NADH dehydrogenase subunit 4	SofAffx.2180.1.S1_at		50198865-23	0.5	↓
	NADH dehydrogenase subunit 5	SofAffx.2177.1.S1_at		50198865-20	0.4	↓
	NADH dehydrogenase subunit 7	SofAffx.2187.1.S1_at		50198865-29	0.4	↓
9	photosystem I P700 apoprotein A1 gene	SofAffx.2191.1.S1_at	-	50198865-64	0.3	↓↓
10	photosystem I reaction center subunit XI	Sof.3556.1.S1_at	-	CA274385	0.2	↓↓
		SofAffx.748.1.S1_at		CF574993	0.4	↓
11	photosystem I subunit O	Sof.2003.2.S1_a_at	-	CA300310	0.05	↓↓↓
12	photosystem I subunit VII gene	SofAffx.2181.1.S1_at	-	50198865-24	0.4	↓
13	photosystem I subunit VIII gene	SofAffx.2122.1.S1_at	-	50198865-72	0.4	↓
14	photosystem II 47 kDa protein gene	SofAffx.2146.1.S1_at	-	50198865-86	0.5	↓
15	photosystem II oxygen evolving complex	Sof.3600.1.S1_at	-	CA280949	0.2	↓↓
		Sof.3585.1.S1_at	-	CA251032	0.3	↓↓
16	protochlorophyllide reductase	Sof.2965.1.S1_at	1.3.1.33	CA195783	0.2	↓↓
Dark reactions						
1	chloroplastic malate dehydrogenase (NADP-dependent)	SofAffx.151.1.S1_at	1.1.1.82	CF577213	0.4	↓
2	NADP-dependent malic enzyme	Sof.2259.3.S1_x_at	1.1.1.40	CA093639	0.2	↓↓
		Sof.2259.3.S1_at		CA093639	0.2	↓↓
		Sof.2259.2.A1_s_at		CA093726	0.4	↓
		Sof.2259.1.S1_x_at		CA192976	0.4	↓
3	phosphoglycerate kinase (chloroplastic)	Sof.3174.2.S1_at	2.7.2.3	CA085978	0.3	↓↓
		Sof.3174.1.S1_at		CA194701	0.4	↓
4	RuBisCO	SofAffx.93.1.S1_at	4.1.1.39	CF577137	0.1	↓↓↓
	RuBisCO large subunit-binding protein	Sof.4352.2.S1_a_at		CA247421	0.4	↓
	RuBisCO large subunit-binding protein	Sof.4352.1.S1_at		CA257279	0.5	↓
5	RuBisCO small subunit	Sof.3568.1.S1_at		CA275587	0.1	↓↓↓
	RuBisCo subunit binding-protein beta subunit	SofAffx.880.1.S1_s_at		CF574395	0.5	↓
	RuBisCo subunit binding-protein beta subunit	SofAffx.880.1.S1_at		CF574395	0.5	↓
Sugar signaling, transport, and P_i metabolism						
1	glucose-6-phosphate/phosphate- transporter	Sof.161.1.S1_at	-	CA109224	9.7	↑↑↑
		Sof.3931.2.A1_s_at	-	CA295728	8.7	↑↑↑
2	sugar transporter (putative hexose)	SofAffx.1960.1.S1_at	-	BU925715	2.4	↑
3	sugar transporter type 2a (putative)	SofAffx.8.1.S1_at	-	AY165599	3.0	↑
4	trehalose 6-phosphate synthase	Sof.4223.1.S1_at	2.4.1.15	CA176198	0.2	↓↓
		SofAffx.974.1.S1_at		CF573831	0.3	↓↓
5	trehalose-phosphatase	Sof.3105.2.A1_at	3.1.3.12	CA086449	3.6	↑

6	inorganic pyrophosphatase	Sof.3119.2.S1_at	3.6.1.1	CA111104	3.7	↑
		SofAffx.1668.1.S1_s_at		CF571565	4.4	↑↑
7	phosphate translocator	SofAffx.1399.1.S1_at		CF572284	4.4	↑↑
		Sof.4652.1.S1_at		CA227092	3.1	↑
Cell wall metabolism						
1	beta-expansin	Sof.3272.3.A1_a_at	-	CA248491	2.3	↑
2	caffeic acid 3-O-methyltransferase	SofAffx.314.1.S1_s_at	2.1.1.68	CF572353	2.4	↑
		Sof.3584.1.S1_at		AJ231133	2.3	↑
3	callose synthase	Sof.3815.1.S1_a_at	2.4.1.34	CA196999	2.2	↑
4	cellulose synthase	Sof.4824.2.S1_a_at	2.4.1.12	CA250044	2.6	↑
		Sof.3525.1.S1_at		CA254294	2.9	↑
		Sof.3822.1.S1_at		CA221011	2.8	↑
		Sof.2699.1.S1_a_at		CA244798	3.3	↑
		Sof.5033.1.S1_at		CA148942	4.9	↑↑
		SofAffx.1961.1.S1_s_at		BU925771	5.1	↑↑
		Sof.3361.1.S1_x_at		CA134516	5.0	↑↑
5	endo-1,4-beta-glucanase	Sof.1249.1.S1_at	3.2.1.4	CA105799	7.0	↑↑
		Sof.1249.2.S1_at		CA265758	3.1	↑
		Sof.4805.1.S1_at		CA218775	5.1	↑↑
		Sof.4805.2.S1_a_at		CA219099	2.4	↑
6	endo-1,3-beta-glucanase	Sof.3799.1.A1_a_at	3.2.1.6	CA085569	2.2	↑
7	chitinase	Sof.2983.1.S1_at	3.2.1.14	CA181562	5.8	↑↑
		Sof.2983.1.S1_a_at		CA181562	5.3	↑↑
		SofAffx.1726.1.S1_s_at		CF571539	6.8	↑↑
8	pectin acetylesterase	Sof.4115.1.S1_at	3.1.1.6	CA244283	3.9	↑
9	UDP-D-glucuronate decarboxylase	Sof.4204.3.S1_a_at	4.1.1.35	CA254458	2.1	↑
10	xyloglucan endo-beta-1,4-glucanase	SofAffx.1583.1.S1_at		CF571758	4.5	↑↑
		Sof.3569.1.S1_at		CA074575	2.1	↑
Stress response						
1	alcohol dehydrogenase	SofAffx.1881.1.S1_at	1.1.1.1	CF571023	3.3	↑
		Sof.3021.1.S1_at		CA065880	2.3	↑
2	aldehyde dehydrogenase	Sof.488.2.S1_at	1.2.1.3	CA148351	2.2	↑
		Sof.488.1.A1_at		CA204366	2.3	↑
3	chalcone synthase	Sof.702.1.S1_at	2.3.1.74	BU103687	5.4	↑↑
4	cold acclimation protein	SofAffx.400.1.S1_at	-	CF575714	2.2	↑
		Sof.3467.1.S1_s_at		CA267486	4.0	↑
		Sof.3467.1.S1_at		CA267486	3.5	↑
5	cold induced - low temperature and salt responsive protein	SofAffx.125.1.S1_s_at	-	CF575717	2.7	↑
		Sof.4293.1.S1_at		BQ535164	2.7	↑
		SofAffx.125.3.S1_x_at		CF574964	3.0	↑
		SofAffx.125.1.S1_x_at		CF575717	2.1	↑
		SofAffx.125.2.S1_at		CF572632	2.4	↑
6	flavodoxin	Sof.19.1.S1_at	1.19.6.1	CA093296	0.5	↓
7	glutamate decarboxylase	Sof.3466.1.A1_at	4.1.1.15	CA270299	2.7	↑
		Sof.3466.2.S1_at		CA171450	3.0	↑
8	heat shock like protein	Sof.2216.1.A1_at	-	CA093476	0.5	↓
9	jacalin	Sof.676.1.S1_at	-	CA135644	22.7	↑↑↑

10	leaf senescence related protein	Sof.4979.1.S1_at	-	CA122355	3.4	↑
11	lethal leaf spot	Sof.1.1.A1_at	-	CA218370	2.4	↑
		Sof.1.2.S1_at		CA286120	2.3	↑
12	mitogen-activated protein kinase	Sof.2621.2.S1_at	2.7.11.24	CA272048	5.1	↑↑
		Sof.1333.1.S1_at		CA164068	0.3	↓
13	pathogen related protein	Sof.1462.1.S1_at	-	CA275478	0.3	↓
14	peroxidase	Sof.820.1.S1_at	1.11.1.7	CA266080	2.1	↑
		SofAffx.2013.1.S1_s_at		CO373606	4.1	↑↑
15	phosphoethanolamine N-methyltransferase	Sof.4034.1.S1_at	2.1.1.103	CA239805	3.3	↑
16	proteinase inhibitor	SofAffx.1506.2.S1_at	-	CF571685	2.8	↑
		SofAffx.2049.1.S1_at		CO373862	6.5	↑↑
		SofAffx.1811.1.S1_at		CF570630	2.1	↑
17	remorin C	Sof.2845.1.A1_at	-	CA174984	3.3	↑
18	response regulator	SofAffx.947.1.S1_at	-	CF573861	0.1	↓↓↓
19	SNF1-related kinase (beta subunit 2)	SofAffx.135.1.S1_s_at	2.7.11.1	CF577254	0.2	↓↓
		Sof.1710.1.S1_at		CA093131	0.2	↓↓
20	stress responsive protein	Sof.2872.1.S1_at	-	CA071411	3.4	↑
		Sof.4297.1.S1_at		CA119563	4.7	↑↑
		Sof.3913.1.S1_s_at		CA253240	3.0	↑
		Sof.3913.1.S1_at		CA253240	3.9	↑
		Sof.3673.1.A1_at		CA106990	2.1	↑
		Sof.3456.1.S1_a_at		CA203963	0.2	↓↓↓
21	superoxide dismutase	Sof.703.2.S1_a_at	1.15.1.1	CA144287	2.1	↑
22	wound induced protein	SofAffx.162.1.S1_at	-	CF576603	2.8	↑

6.5 Discussion

During the photoperiod, sugarcane leaves primarily accumulate and export sucrose (Hartt & Kortchack 1963; Lunn & Hatch, 1995; Du *et al*., 2000). Sucrose is readily exported in daylight, with up to 80% of assimilate reportedly being exported from leaves immediately at midday (Du *et al*., 2000). The accumulation of sucrose in leaves of field-grown sugarcane during the day thus represents the balance between the rate of photosynthesis and the rate of export, which is, in turn, dependent on carbon demand from culm tissue (Du *et al*., 2000; McCormick *et al*., 2006 [Chapter 3]). In the current study, neither the photosynthetic rate nor the “end of day” leaf sucrose concentration significantly differed within the control plants during the experiment, indicating the presence of a similar diurnal supply and demand dynamic to that observed in other C_4 species, such as maize (Kalt-Torres *et al*., 1987).

6.5.1 Leaf sugar accumulation is associated with a decrease in photosynthesis

The application of a cold-girdle to leaves effectively disrupted the balance between export and fixation of carbon (Fig. 6.1). Similar to results from leaf cold-girdling experiments conducted in other species (Krapp *et al*., 1993; Krapp & Stitt, 1995; Iglesias *et al*., 2002), accumulation of sugars, in the form of hexose and sucrose, was linked to a severe depression in photosynthetic rates, including *A*, ETR, J_{max} and *CE*. A similar decline in photosynthesis has also been observed when glucose was fed to detached *Spinacia oleracea* (L.) leaves (Krapp *et al*., 1991). Similarly, cold-girdling of *Spinacia* leaves resulted in a decrease in *A* soon after the application of the cold-girdle, while no significant effect on E and G_s was observed (Krapp & Stitt, 1995). In the current study, E and G_s were significantly lower after 24 h, although this change was not as marked as that observed for *A* and ETR, which continued to decline until 72 h (Table 6.1). This suggests that the cold-girdle influenced photosynthesis through biochemical modulation, rather than simply through control of stomata.

6.5.2 Leaf sugar accumulation leads to a decrease in the expression of photosynthesis-related genes

Changes in cold-girdled leaf gene expression are a result of the accumulation of the end-products of photosynthesis and the consequent disruption in the homeostasis of the source-sink regulatory mechanisms (Smeekens, 2000; Paul & Foyer, 2001). The accumulation of leaf carbohydrates has previously been shown to repress the expression of genes involved in photosynthesis, and furthermore, to disturb the carbon: nitrogen balance through changes in the investment of nitrogen into the photosynthetic machinery (Paul & Driscoll, 1997; Paul & Pellny, 2003). In cold-girdled *Spinacia* leaves, the accumulation of sugars resulted in a concurrent decrease in the expression of transcripts coding for Rubisco (*rbcS*), chlorophyll-a-binding protein (*cab*) and the D-subunit of thylakoid ATP synthase (*atpD*) (Krapp & Stitt, 1995). Decreased Rubisco levels have been shown to limit photosynthesis (Quick *et al*., 1991), while an inverse correlation between Rubisco activity and glucose concentration has been reported (Krapp *et al*., 1991).

In the present study, the accumulation of leaf sugars resulted in the down-regulation of 21 photosynthesis-related genes, including those mentioned above, comprising genes

associated with the C_4 photosynthetic pathway, photosystems I and II, and the PCR cycle (Table 6.3). It is likely that sugar-mediated down-regulation of photosynthesis in C_3 species occurs via an intracellular mechanism, as both photosynthesis and sucrose synthesis occur in the same cell. However, in C_4 plants, sucrolytic and photosynthetic activities are localised in mesophyll and bundle sheath cells, respectively (Fig. 6.4), although the extent of this separation varies between species (Lunn & Furbank, 1997). Work in maize, a C_4 relative of sugarcane, has demonstrated that the sucrolysis and glycolysis-related enzymes, sucrose phosphate synthase (SPS; EC 2.4.1.14), sucrose phosphate phosphatase (SPP; EC 3.1.3.24) and FBPase, are predominantly localized in the cytosol of mesophyll cells (Downton and Hawker, 1973; Furbank *et al*., 1985), indicating that sucrose is synthesized almost exclusively in the mesophyll of maize source leaves. Thus the putative sugar-signaling pathways limiting photosynthesis in the current cold-girdling study are likely to be different to those observed in work on C_3 species (Krapp *et al*., 1993; Krapp & Stitt, 1995; Iglesias *et al*., 2002).

6.5.3 Leaf sugar accumulation disturbs glycolysis and sugar partitioning, and induces a stress-related response

The observed increases in FBPase, TPI and GPD, and co-ordinate decrease in PFP expression (Table 6.3), indicate a decrease in partitioning of imported chloroplastic triose-phosphate (triose-P) towards hexose and sucrose production with a shift towards alternative biosynthetic processes (Fig. 6.4). The overall increase in expression of all genes identified as involved in cell wall metabolism further suggests an increase in carbon partitioning to other activities under conditions of excess sugar abundance. In addition, the up-regulation of NADH-MD and MUP expression are indicative of an increase in glycolytic flux towards mitochondrial metabolism. Notably, increases in MUP activity are associated with increased conversion of pyruvate to citrate within the TCA cycle (Smith *et al*., 2004) and increased tolerance to oxidative stress, specifically from photorespiration (Sweetlove *et al*., 2006). This suggests that changes in mitochondria-related genes in the current study are likely a direct response to the cold-girdling treatment.

Numerous genes involved in stress response were also up-regulated. Davies and Robinson (2000) have suggested that the considerable changes in osmotic pressure and water potential that occur during grape berry ripening may result in transcription of genes

involved in stress management. Thus, it is possible that abiotic stresses accompanying high accumulation of leaf sugars observed in the current study resulted in the increased expression of stress-related genes, as has been seen during sugarcane culm maturation (Carson *et al*., 2002). However, past studies have demonstrated that the expression of several of the stress response genes, including ADH (Denis, 1987), CS (Tsukaya *et al*., 1991) and proteinase inhibitors (Johnson & Ryan, 1990), is regulated by sugars, in particular, glucose. Furthermore, expression of a jacalin gene was recently shown to be induced though glucose feeding in *Arabidopsis* (Masclaux-Daubresse *et al*., 2007). The up-regulation of these genes in the current study thus strongly suggests that stress- and sugar-sensing mechanisms may interact and operate within similar signaling pathways in sugarcane. It has been suggested that a common phosphorylating signal transduction pathway may exist for sugar and stress signaling (Ehness *et al*., 1997).

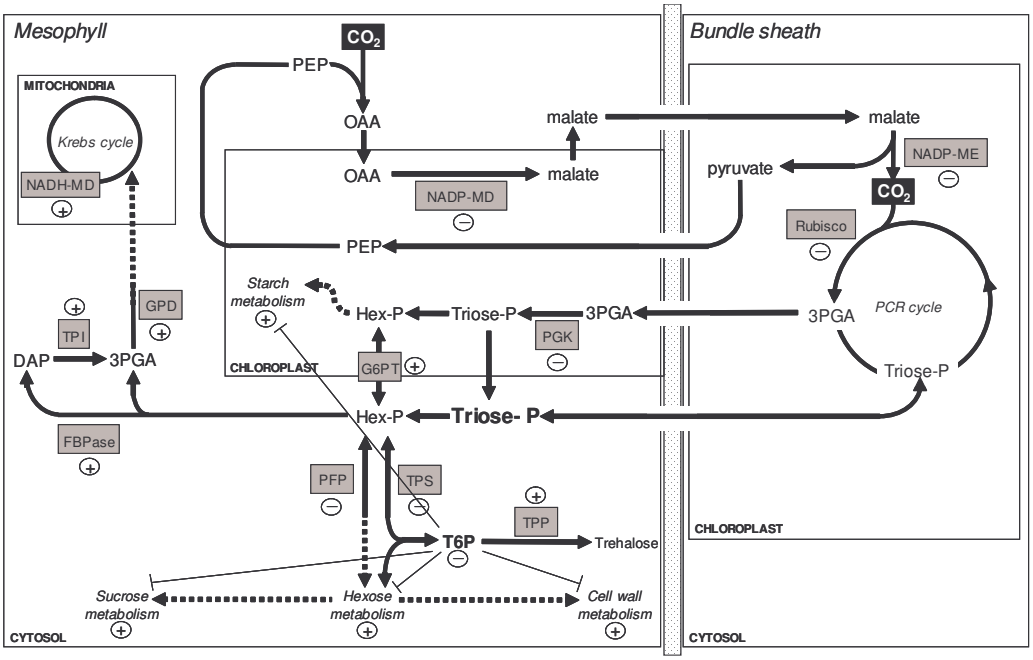

Fig. 6.4. The separation of glycolysis and photosynthesis in a C_4 system. A putative signaling role for trehalose 6-phosphate (T6P) is shown. Boxes in grey illustrate genes that were up (+) or down (-) regulated in sugarcane leaves cold-girdled for 56 h. Importantly, the availability of mesophyll triose-P for recycling P_i back to the PCR cycle in the bundle sheath is indicated. Abbreviations: 3PGA – 3-phosphoglycerate; hexose-P – hexose phosphates, DAP - dihydroxyacetone phosphate; GPD - glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); G6PT – glucose 6-phosphate/phosphate translocator; hex-P – hexose phosphate; NADH-MD – NADH-dependent malate dehydrogenase (EC 1.1.1.37); NADP-MD – NADP-dependent malate dehydrogenase (EC 1.1.1.82); NADP-ME – NADP-dependent malic enzyme (EC 1.1.1.40); OAA – oxaloacetate; PEP – phosphoenolpyruvate; PGK - phosphoglycerate kinase (EC 2.7.2.3); PPdK – pyruvate orthophosphate dikinase (EC 2.7.9.1); RuBP – ribulose bisphosphate; TPS - trehalose phosphate synthase (EC 2.4.1.15); TPP - trehalose phosphate phosphatase (EC 5.3.1.1); triose-P – triose phosphate; triosphosphate isomerase (EC 5.3.1.1).

The CWI isoform *Incw4*, a putatively unbound apoplastic CWI (Kim *et al*., 2000), was significantly down-regulated (Table 6.3). The expression of CWI has previously been linked to a variety of tissue specific, sugar sensing-mechanisms and stress related stimuli (Sinha *et al*., 2002). *Nicotiana tabacum* (L.) over-expressing CWI reportedly exhibits an accumulation of leaf carbohydrates and a consequent decrease in *rbsS* transcript levels (Von Schaewen, 1990). In the present study, down-regulation of *Incw4* was likely a response to the accumulation of apoplastic hexose. The corresponding up-regulation of a sugar transporter suggests the presence of an additional 'mopping up' mechanism for hexose (and sucrose), perhaps to assist in re-partitioning excess carbon into different pathways.

The locations of the identified sugar transport genes and associated mechanisms of regulation remain unclear. However, the expression of putative sugar transporter (PST type 2a) transcripts has previously been identified within phloem companion cells and primarily linked to the maintenance of sugar fluxes (Casu *et al*., 2003). In yeast, membrane bound receptors closely related to sugar transporters are used to sense external sugar concentrations and activate a signal transduction pathway leading to the regulation of transporter gene expression (Lalonde *et al*., 1999). As sugar transporters are known to play a key role in sugar partitioning and are linked to intracellular sugar signaling mechanisms regulating photosynthesis in higher plants (Lalonde *et al*., 1999; Williams *et al*., 2000), the up-regulation of sugar transporters in the current study may facilitate a dual role; firstly, to partition excess sugars to the phloem or intracellular storage compartments, and secondly, to initiate a signal to down-regulate photosynthetic carbon accumulation. Notably, the signal tranduction kinases, MAPK and SnRK1, also exhibited differential expression in response to the cold-girdling treatment. Whereas SnRK1 has frequently been proposed to be involved in sugar responses leading to transcriptional regulation of genes involved in carbohydrate metabolism (Toroser *et al*., 2000; Tiessen *et al*., 2003), MAPKs are associated with the transduction of intracellular signals by extracellular targets (Roitsch *et al*., 2003). Thus, the changes in the expression of these genes observed in this work could be indicative of an associated sugar-stress induced signaling cascade between apoplast and cytosol (Ehness *et al*., 1997; Rolland *et al*., 2002).

6.5.4 Trehalose metabolism – a potential sugar-signaling mechanism in sugarcane

A novel sugar-signaling system in sugarcane leaves is evident from the observed changes in two genes related to trehalose metabolism, *viz*. TPS and TPP (Table 6.3). The trehalose pathway has previously been characterised in sugarcane (Glasziou & Gayler, 1969; Bosch, 2005; Glassop *et al*., 2007), although those studies examined the effects of trehalose on culm sucrose accumulation. After initially being identified as playing a role in sensing carbon status in yeast (Blásquez *et al*., 1993), T6P, and not trehalose, is now implicated as a key sugar-sensing component involved in several aspects of plant growth and development (Eastmond *et al*., 2003; Paul, 2007; Ramon & Rolland, 2007). T6P does not appear to interact with HXK in higher plant species, including sugarcane (Bosch, 2005), suggesting that trehalose metabolism is a HXK-independent signaling pathway (Eastmond *et al*., 2002). Notably, the present microarray data did not indicate any significant response from genes related to HXK or fructokinase (EC 2.7.1.4). It is possible that HXK signaling does not play as prominent a role in sugar sensing in sugarcane, as in C_3 species (Graham *et al*., 1994; Jang & Sheen, 1994; Moore *et al*., 2003). However, the present work has examined regulation only at the transcript level, and further enzymatic analysis will be required to identify whether allosteric regulation of HXK activity occurs. Based on the abundance of work demonstrating the role of HXK in glucose signaling (for review see Rolland *et al*., 2006), a sensing function in sugarcane is not unlikely.

In the current study, the observed changes in TPS and TPP may have led to a decrease in T6P concentrations. Based on results from several recent studies, changes in T6P may have significant effects on sugar partitioning and the regulation of photosynthesis in sugarcane leaves (Fig. 6.4). Whereas increased T6P levels obtained through over-expression of TPS in transgenic tobacco, has resulted in enhanced photosynthetic capacity per unit leaf area (Paul *et al*., 2001); in transgenic *Arabidopsis* and tobacco, increased expression of TPP has revealed that T6P depletion is associated with an accumulation of sugar phosphates, decreased ATP levels and reduced photosynthetic rates (Paul *et al*., 2001; Schluepmann *et al*., 2003). The precise mechanisms which link T6P signaling to changes in photosynthetic activity are still unresolved, however, it is suggested that T6P is a conduit for communicating sugar status in the cytosol to the chloroplast (Pellny *et al*., 2005; Paul, 2007). Nevertheless, as sucrose metabolism and photosynthesis are, to some extent, separated in C_4 plants (Lunn & Furbank, 1997;

1999), the signaling pathway involving T6P in sugarcane may differ considerably from C_3 species.

Down-regulation of TPS, with an associated reduction in T6P concentrations, has been shown to result in an accumulation of sugars and starch, and increased cell wall deposition in *Arabidopsis* embryos (Gomez *et al*., 2006). In particular, the direct redox activation of AGPase by T6P has confirmed a role for T6P in regulating starch synthesis (Kolbe *et al*., 2005). In the current work, the expression of AGPase and other starch-related genes were significantly increased, demonstrating that T6P may be involved in regulating source partitioning between sugars and starch in sugarcane leaves (Fig. 6.4). The accumulation of starch, as opposed to sucrose, is not well documented in sugarcane, and only constitutes a small fraction (17%) of daily leaf carbon accumulation (Du *et al*., 2000). As such, the observed changes in starch-related genes were unexpected. However, the putative involvement of T6P in regulating sugar, starch and photosynthetic activity, as seen in other species (Paul *et al*., 2001; Schluepmann *et al*., 2003), identifies trehalose genes as strong potential targets for manipulating the availability of sucrose for export from leaves (Fig. 6.4).

6.5.5 Evidence for P_i limitation and possible interactions with trehalose

The array data obtained in this study provided evidence of a further P_i–related, negative feedback effect on photosynthetic capacity in sugarcane. In leaves, the rate of end-product accumulation largely determines the rate at which P_i is recycled back to the reactions of photosynthesis (Pieters *et al*., 2001; Paul & Pellny, 2003). However, under conditions of excess carbon abundance, the accumulation of P_i within phosphorylated hexose and triose pools can act to suppress photosynthetic activity (Stitt & Quick, 1989) (Fig. 6.4). The G6PT gene is not typically expressed in photosynthetic tissue (Lloyd & Zakhleniuk, 2004); however, in the current cold-girdling experiment, large increases in G6PT expression, in addition to increases in P_i translocator expression, were observed (Table 6.3). Similar increases in G6PT were previously found in sucrose accumulating *Arabidopsis pho3* mutants, suggesting that the regulation of P_i transport may represent an alternative mechanism for balancing the chloroplastic and cytosolic P_i pools (Lloyd & Zakhleniuk, 2004). Alternatively, up-regulation of G6PT expression may be a means to alleviate hexose accumulation in the chloroplast, resulting from sugar-mediated feedback limitation of triose-P export. However, the increase in expression of PPase in

the present study does indicate a possible supplementation of cytosolic P_i supply at the expense of pyrophosphate (PP_i) (Table 6.3) (Lee *et al*., 2005). Notably, during the cold-girdling treatment, the initial accumulation of leaf hexose declined after 8 h over the duration of the experiment, while sucrose declined after 56 h (Fig. 6.1). This may be indicative of adaptation by cold-girdled leaves to changes in carbon balance through the partitioning excess sugars into other pathways (Peuke *et al*., 2006), possibly as an additional mechanism to increase P_i availability.

Evidence for close interactions between P_i and sugar-signaling pathways now exists (Müller *et al*. 2007). P_i status has been shown to affect the sugar-mediated, transcriptional regulation of genes related to carbohydrate metabolism and photosynthesis, including AGPase and the small subunit of Rubisco (Nielsen *et al*., 1998; Ciereszko *et al*., 2001). Similar to results from the present study, alterations in gene expression associated with P_i-related metabolism have been linked to changes in TPS/TPP gene expression in sucrose accumulating *Arabidopsis* mutants (Lloyd & Zakhleniuk, 2004). Low T6P concentrations are associated with high levels of phosphorylated sugar intermediates and low ATP levels, whereas high T6P is associated with the opposite (Paul, 2007; Schluepmann *et al*., 2007). Müller *et al*. (2007) have recently provided further evidence for a positive correlation between T6P and P_i levels in *Arabidopsis* plants, as TPS expression was shown to increase under conditions of P_i abundance. Although the regulatory mechanisms that link P_i and sugar sensors are likely different in C_4 plants compared to model C_3 species, the current work provides evidence for a transcript-related interaction between P_i- and trehalose-metabolism in sugarcane. However, to confirm this relationship additional work will be required, at both the enzymatic and metabolite levels.

6.6 Concluding remarks

Photosynthetic rates in sugarcane leaves are regulated by the demand for carbon from sink tissues. Sink demand, in turn, determines the sugar status of the source. The observed accumulation of sugars, particularly hexoses, in cold-girdled leaves has provided strong evidence for a sink-dependent, sugar-based negative feedback effect on photosynthetic activity. Concurrent analyses of changes in gene expression indicate a link between sugar status and the regulation of transcripts involved in a variety of

different metabolic process, including stress response, carbohydrate metabolism and photosynthesis. These data indicate that feedback regulation may operate through several routes. In particular, T6P, P_i and sugar transporters appear to play key roles in regulating sugarcane source leaf production and export. Future metabolic work should concentrate on the individual, and possibly integrated, roles of these targets. As export rates in sugarcane leaves are reportedly high, preliminary results from the current study suggest that appropriate increases in T6P levels could result in an improvement in source photosynthetic rates and overall sink sucrose accumulation.

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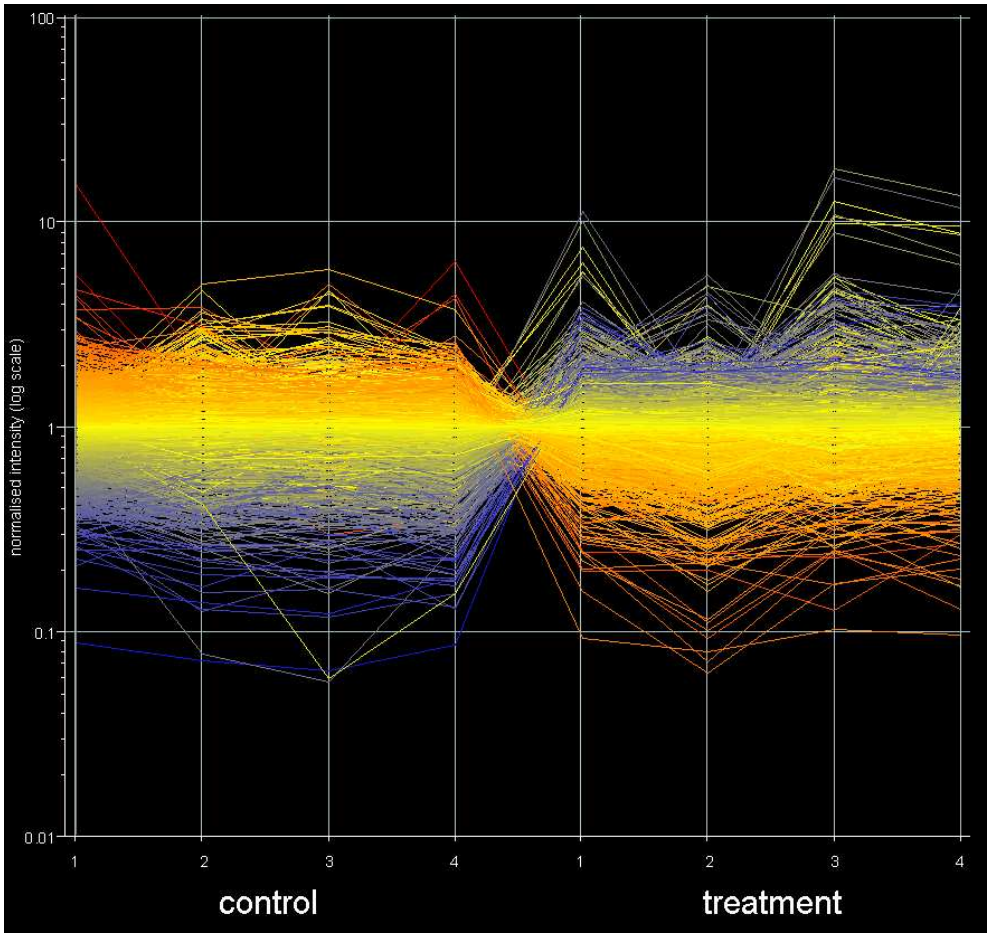
6.8 Supplementary material



Supplementary Figure 6.1. Normalised gene expression profile comparison between cold-girdled (56 h) and control leaves. Up- and down-regulation of genes in controls (n=4) is seen in red and blue, respectively, whereas the reverse applies for the cold-girdled leaves. Genes that remain unaffected by the treatment are depicted in yellow.

Supplementary Table 6.1. List of probe sets differentially expressed during the cold-girdling treatment. Putative identity was assigned using the BLASTX function within the National Centre of Biotechnological Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov). Where E values are absent, probe sets homology was matched to those assigned by Casu *et al.* (2007). Fold changes indicate statistical significance values (P<0.05) as determined by ANOVA (n=4).

No.	Gene Name	Fold change	EC	p-value	Genbank	Putative Identity	Homology	E value
Carbohydrate metabolism (cytosolic)								
1	SofAffx.1015.1.S1_s_at	2.439	4.1.2.13	0.014	CF574067	fructose-1,6-bisphosphate aldolase	*Oryza sativa* (*japonica* cultivar-group)	0.069
2	Sof.3192.2.S1_at	2.314	4.1.2.13	0.001	CA206457	fructose-1,6-bisphosphate aldolase	*Oryza* sativa (*japonica* cultivar-group)	6.00E-11
3	Sof.2554.1.S1_at	2.084	1.2.1.12	0.002	CA093039	glyceraldehyde-3-phosphate dehydrogenase	*Oryza sativa* (*japonica* cultivar-group)	7.00E-58
4	Sof.3865.1.S1_at	2.534	1.2.1.12	0.000	CA196594	glyceraldehyde-3-phosphate dehydrogenase	*Zea mays*	6.00E-55
5	SofAffx.1973.1.S1_at	0.44	3.2.1.26	0.003	BU925731	invertase (cell wall - Incw4)	*Zea mays*	9.00E-26
6	Sof.3023.2.S1_a_at	0.358	2.7.1.90	0.009	CA208800	pyrophosphate-dependent 6-phosphofructose-1-kinase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-121
7	Sof.3023.1.S1_s_at	0.416	2.7.1.90	0.002	CA070403	pyrophosphate-dependent 6-phosphofructose-1-kinase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-71
8	Sof.2883.1.S1_at	3.14	5.3.1.1	0.000	CA147454	triosphosphate isomerase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-17
Starch metabolism								
1	Sof.2109.1.S1_a_at	2.927	2.4.1.18	0.001	CA202596	1,4-alpha-glucan branching enzyme	*Zea mays*	1.00E-49
2	Sof.4578.1.S1_a_at	12.78	2.7.7.27	0.000	CA151666	ADP-glucose pyrophosphorylase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-18
3	Sof.1235.1.S1_at	5.236	3.2.1.2	0.003	CA169143	beta-amylase	*Zea mays*	8.00E-53
4	Sof.4751.1.S1_at	8.18	3.2.1.68	0.000	CA289034	isoamylase	*Arabidopsis thaliana*	3.00E-16
5	Sof.1216.1.S1_at	8.834	2.4.1.1	0.000	CA169622	phosphorylase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-42
Mitochondrial metabolism								
1	Sof.3353.1.S1_at	3.025	1.1.1.37	0.001	CA181748	malate dehydrogenase(NADH+ dependent)	*Arabidopsis thaliana*	----
2	Sof.1026.1.S1_at	0.454	-	0.000	CA265014	mitochodrial transcription termination factor	*Oryza sativa* (*japonica* cultivar-group)	7.00E-61
3	Sof.1026.2.S1_at	0.317	-	0.008	CA161102	mitochondrial transcription terminator factor	*Arabidopsis thaliana*	----
4	SofAffx.14.1.S1_at	2.39	-	0.010	AY644460	mitochondrial uncoupling protein 1	*Saccharum officinarum*	7.00E-144

5	Sof.2009.1.S1_at	2.066	-	0.000	CA170908	mitochondrial uncoupling protein 2	*Saccharum officinarum*	5.00E-24
C_4 photosynthesis and related metabolic components								
Light reactions								
1	SofAffx.2196.1.S1_at	0.305	3.6.3.14	0.007	50198865-69	ATP synthase CF0 A chain gene	*Saccharum* hybrid cultivar	----
2	SofAffx.2171.1.S1_at	0.332	3.6.3.14	0.000	50198865-59	ATP synthase CF0 A chain gene	*Saccharum* hybrid cultivar	----
3	SofAffx.2188.1.S1_at	0.396	3.6.3.14	0.001	50198865-61	ATP synthase CF0 C chain gene	*Saccharum* hybrid cultivar	----
4	SofAffx.2186.1.S1_at	0.415	3.6.3.14	0.001	50198865-60	ATP synthase CF0 C chain gene	*Saccharum* hybrid cultivar	----
5	SofAffx.2118.1.S1_at	0.229	3.6.3.14	0.001	50198865-70	ATP synthase CF1 beta chain gene	*Saccharum* hybrid cultivar	----
6	Sof.4725.1.S1_at	0.287	-	0.000	CA186676	chlorophyll a/b-binding protein	*Zea mays*	8.00E-60
7	Sof.3564.1.S1_at	0.456	-	0.000	CA298559	chlorophyll a/b binding protein LHCII type III	*Hordeum vulgare*	7.00E-26
8	Sof.3564.1.S1_a_at	0.42	-	0.000	CA298559	chlorophyll a/b binding protein LHCII type III	*Hordeum vulgare*	7.00E-26
9	Sof.2722.1.S1_at	0.36	1.14.14.1	0.000	CA174395	cytochrome P450	*Arabidopsis thaliana*	2.00E-04
10	Sof.1713.2.S1_at	0.0924	1.14.14.1	0.000	CA068881	cytochrome P450	*Triticum monococcum*	2.00E-45
11	Sof.1713.1.A1_at	0.119	1.14.14.1	0.000	CA068965	cytochrome P450	*Arabidopsis thaliana*	----
12	SofAffx.2164.1.S1_at	0.301	-	0.002	50198865-54	cytochrome b6 f complex subunit VIII gene	*Saccharum* hybrid cultivar	----
13	SofAffx.2179.1.S1_at	0.448	-	0.002	50198865-22	cytochrome biogenesis protein gene	*Saccharum* hybrid cultivar	----
14	Sof.2428.1.S1_at	0.35	-	0.004	CA223413	geranylgeranyl hydrogenase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-54
15	Sof.3048.1.A1_s_at	0.435	-	0.001	CA105372	geranylgeranyl hydrogenase	*Triticum aestivum*	2.00E-29
16	SofAffx.56.1.S1_at	0.307	-	0.006	CF577098	magnesium chelatase	*Saccharum* hybrid cultivar	----
17	SofAffx.1150.1.S1_at	0.315	-	0.001	CF573280	magnesium chelatase	*Saccharum* hybrid cultivar	----
18	Sof.4426.1.A1_at	0.276	-	0.006	CA067637	magnesium chelatase	*Saccharum* hybrid cultivar	----
19	SofAffx.2184.1.S1_at	0.39	1.6.99.3	0.001	50198865-27	NADH dehydrogenase subunit 1	*Saccharum* hybrid cultivar	----
20	SofAffx.2185.1.S1_at	0.453	1.6.99.3	0.000	50198865-28	NADH dehydrogenase subunit 1	*Saccharum* hybrid cultivar	----
21	SofAffx.2180.1.S1_at	0.459	1.6.99.3	0.000	50198865-23	NADH dehydrogenase subunit 4	*Saccharum* hybrid cultivar	----
22	SofAffx.2177.1.S1_at	0.366	1.6.99.3	0.014	50198865-20	NADH dehydrogenase subunit 5	*Saccharum* hybrid cultivar	----
23	SofAffx.2187.1.S1_at	0.445	1.6.99.3	0.010	50198865-29	NADH dehydrogenase subunit 7	*Saccharum* hybrid cultivar	----
24	SofAffx.2191.1.S1_at	0.292	-	0.000	50198865-64	photosystem I P700 apoprotein A1 gene	*Saccharum* hybrid cultivar	----
25	Sof.3556.1.S1_at	0.249	-	0.000	CA274385	photosystem I reaction center subunit XI	*Hordeum vulgare*	6.00E-54
26	SofAffx.748.1.S1_at	0.398	-	0.000	CF574993	photosystem I reaction center subunit XI,	*Oryza sativa* (*japonica* cultivar-	4.00E-07

						hloroplast precursor	group)	
27	Sof.2003.2.S1_a_at	0.0461	-	0.000	CA300310	photosystem I subunit O gene	*Arabidopsis thaliana*	----
28	SofAffx.2181.1.S1_at	0.358	-	0.001	50198865-24	photosystem I subunit VII gene	*Saccharum* hybrid cultivar	----
29	SofAffx.2122.1.S1_at	0.379	-	0.001	50198865-72	photosystem I subunit VIII gene	*Saccharum* hybrid cultivar	----
30	SofAffx.2146.1.S1_at	0.453	-	0.001	50198865-86	photosystem II 47 kDa protein gene	*Saccharum* hybrid cultivar	----
31	Sof.3600.1.S1_at	0.224	-	0.000	CA280949	photosystem II oxygen evolving complex	*Arabidopsis thaliana*	----
32	Sof.3585.1.S1_at	0.269	-	0.012	CA251032	photosystem II oxygen evolving complex	*Arabidopsis thaliana*	----
33	Sof.2965.1.S1_at	0.205		0.000	CA195783	Protochlorophyllide reductase	*Zea mays*	3.00E-22
Dark reactions								
1	SofAffx.151.1.S1_at	0.354	1.1.1.82	0.000	CF577213	chloroplastic malate dehydrogenase (NADP-dependent)	*Saccharum spontaneum*	0.014
2	Sof.2259.3.S1_x_at	0.22	1.1.1.40	0.000	CA093639	NADP-dependent malic enzyme	*Sorghum bicolor*	1.00E-41
3	Sof.2259.3.S1_at	0.207	1.1.1.40	0.001	CA093639	NADP-dependent malic enzyme	*Sorghum bicolor*	1.00E-41
4	Sof.2259.2.A1_s_at	0.38	1.1.1.40	0.001	CA093726	NADP-dependent malic enzyme	*Sorghum bicolor*	2.00E-38
5	Sof.2259.1.S1_x_at	0.397	1.1.1.40	0.000	CA192976	NADP-dependent malic enzyme	*Zea mays*	7.00E-38
6	Sof.3174.2.S1_at	0.297	2.7.2.3	0.000	CA085978	phosphoglycerate kinase (chloroplastic)	*Spinacia oleracea*	2.00E-103
7	Sof.3174.1.S1_at	0.397	2.7.2.3	0.012	CA194701	phosphoglycerate kinase (chloroplastic)	*Oryza sativa* (*japonica* cultivar-group)	7.00E-71
8	SofAffx.93.1.S1_at	0.114	4.1.1.39	0.002	CF577137	RuBisCO	*Saccharum* hybrid cultivar	----
9	Sof.4352.2.S1_a_at	0.402	4.1.1.39	0.000	CA247421	RuBisCO large subunit-binding protein	*Zea mays*	5.00E-107
10	Sof.4352.1.S1_at	0.465	4.1.1.39	0.005	CA257279	RuBisCO large subunit-binding protein	*Zea mays*	2.00E-71
11	Sof.3568.1.S1_at	0.078	4.1.1.39	0.001	CA275587	RuBisCO small subunit	*Zea mays*	5.00E-30
12	SofAffx.880.1.S1_s_at	0.5	4.1.1.39	0.001	CF574395	RuBisCo binding-protein beta subunit	*Zea mays*	3.00E-15
13	SofAffx.880.1.S1_at	0.48	4.1.1.39	0.002	CF574395	RuBisCo binding-protein beta subunit	*Zea mays*	3.00E-15
Sugar signaling, transport, and P_i metabolism								
1	Sof.161.1.S1_at	9.716	-	0.000	CA109224	glucose-6-phosphate/hosphate-transporter	*Oryza sativa* (*japonica* cultivar-group)	2.00E-55
2	Sof.3931.2.A1_s_at	8.656	-	0.000	CA295728	glucose-6-phosphate/phosphate-translocator	*Oryza sativa* (*japonica* cultivar-group)	5.00E-13
3	SofAffx.1960.1.S1_at	2.399	-	0.000	BU925715	sugar transporter (putative hexose)	*Oryza sativa* (*japonica* cultivar-group)	1.00E-42
4	SofAffx.8.1.S1_at	3.002	-	0.002	AY165599	sugar transporter type 2a (putative)	*Saccharum* hybrid cultivar	0.00E+00
5	Sof.4223.1.S1_at	0.218	2.4.1.15	0.008	CA176198	trehalose 6-phosphate synthase	*Oryza sativa*	1.00E-27
6	SofAffx.974.1.S1_at	0.278	2.4.1.15	0.000	CF573831	trehalose 6-phosphate synthase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-24
7	Sof.3105.2.A1_at	3.645	3.1.3.12	0.002	CA086449	trehalose-phosphatase	*Oryza sativa* (*japonica* cultivar-	2.00E-67

							group)	
8	Sof.3119.2.S1_at	3.722	3.6.1.1	0.001	CA111104	inorganic pyrophosphatase	*Beta vulgaris*	0.55
9	SofAffx.1668.1.S1_s_at	4.392	3.6.1.1	0.003	CF571565	inorganic pyrophosphatase	*Saccharum* hybrid cultivar	----
10	SofAffx.1399.1.S1_at	4.43		0.000	CF572284	phosphate translocator	*Saccharum* hybrid cultivar	----
11	Sof.4652.1.S1_at	3.1		0.000	CA227092	phosphate translocator	*Oryza sativa* (*japonica* cultivar-group)	3.00E-25
Cell wall metabolism								
1	Sof.3272.3.A1_a_at	2.335	-	0.000	CA248491	beta-expansin	*Triticum aestivum*	0.002
2	SofAffx.314.1.S1_s_at	2.37	2.1.1.68	0.000	CF572353	caffeic acid 3-O-methyltransferase	*Saccharum* hybrid cultivar	----
3	Sof.3584.1.S1_at	2.314	2.1.1.68	0.001	AJ231133	caffeic acid 3-O-methyltransferase	*Saccharum officinarum*	0.0
4	Sof.3815.1.S1_a_at	2.239	2.4.1.34	0.002	CA196999	callose synthase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-13
5	Sof.4824.2.S1_a_at	2.591	2.4.1.12	0.000	CA250044	cellulose synthase	*Solanum tuberosum*	8.00E-46
6	Sof.3525.1.S1_at	2.866	2.4.1.12	0.005	CA254294	cellulose synthase	*Arabidopsis thaliana*	6.00E-63
7	Sof.3822.1.S1_at	2.788	2.4.1.12	0.001	CA221011	cellulose synthase catalytic subunit 11	*Zea mays*	1.00E-88
8	Sof.2699.1.S1_a_at	3.273	2.4.1.12	0.003	CA244798	cellulose synthase catalytic subunit 12	*Zea mays*	2.00E-56
9	Sof.5033.1.S1_at	4.892	2.4.1.12	0.002	CA148942	cellulose synthase-2	*Zea mays*	5.00E-42
10	SofAffx.1961.1.S1_s_at	5.116	2.4.1.12	0.002	BU925771	cellulose synthase-4	*Zea mays*	8.00E-52
11	Sof.3361.1.S1_x_at	5.044	2.4.1.12	0.005	CA134516	cellulose synthase-6	*Zea mays*	8.00E-05
12	Sof.1249.1.S1_at	6.954	3.2.1.4	0.000	CA105799	endo-1,4-beta-glucanase	*Triticum aestivum*	6.00E-19
13	Sof.1249.2.S1_at	3.12	3.2.1.4	0.000	CA265758	endo-1,4-beta-glucanase	*Hordeum vulgare*	5.00E-54
14	Sof.4805.1.S1_at	5.091	3.2.1.4	0.001	CA218775	endo-1,4-beta-glucanase	*Oryza sativa* (*japonica* cultivar-group)	6.00E-08
15	Sof.4805.2.S1_a_at	2.415	3.2.1.4	0.000	CA219099	endo-1,4-beta-glucanase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-115
16	Sof.3799.1.A1_a_at	2.186	3.2.1.6	0.000	CA085569	endo-1,3-beta-glucanase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-80
17	Sof.2983.1.S1_at	5.779	3.2.1.14	0.000	CA181562	chitinase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-15
18	Sof.2983.1.S1_a_at	5.295	3.2.1.14	0.000	CA181562	chitinase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-15
19	SofAffx.1726.1.S1_s_at	6.816	3.2.1.14	0.000	CF571539	chitinase	*Saccharum* hybrid cultivar	----
20	Sof.4115.1.S1_at	3.868	3.1.1.6	0.000	CA244283	pectin acetylesterase	*Oryza sativa* (*japonica* cultivar-group)	5.00E-42
21	Sof.4204.3.S1_a_at	2.063	4.1.1.35	0.001	CA254458	UDP-D-glucuronate decarboxylase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-71
22	SofAffx.1583.1.S1_at	4.515	3.2.1.151	0.000	CF571758	xyloglucan endo-beta-1,4-glucanase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-40
23	Sof.3569.1.S1_at	2.124	3.2.1.151	0.000	CA074575	xyloglucan endo-beta-1,4-glucanase	*Oryza sativa* (*japonica* cultivar-	1.00E-17

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Stress response								
1	SofAffx.1881.1.S1_at	3.343	1.1.1.1	0.000	CF571023	alcohol dehydrogenase	*Saccharum* hybrid cultivar	----
2	Sof.3021.1.S1_at	2.313	1.1.1.1	0.000	CA065880	alcohol dehydrogenase	*Miscanthus sinensis*	4.00E-65
3	Sof.488.2.S1_at	2.203	1.2.1.3	0.000	CA148351	aldehyde dehydrogenase	*Zea mays*	9.00E-68
4	Sof.488.1.A1_at	2.28	1.2.1.3	0.000	CA204366	aldehyde dehydrogenase	*Zea mays*	5.00E-62
5	Sof.702.1.S1_at	5.366	2.3.1.74	0.001	BU103687	chalcone synthase	*Triticum aestivum*	0
6	SofAffx.400.1.S1_at	2.151		0.000	CF575714	cold acclimation protein	*Triticum aestivum*	2.00E-19
7	Sof.3467.1.S1_s_at	3.991		0.000	CA267486	cold acclimation protein	*Triticum aestivum*	6.00E-28
8	Sof.3467.1.S1_at	3.532		0.000	CA267486	cold acclimation protein	*Triticum aestivum*	6.00E-28
9	SofAffx.125.1.S1_s_at	2.713		0.000	CF575717	cold induced, low temperature and salt responsive protein	*Pennisetum glaucum*	3.00E-21
10	Sof.4293.1.S1_at	2.726		0.002	BQ535164	cold induced, low temperature and salt responsive protein	*Arabidopsis thaliana*	7.00E-17
11	SofAffx.125.3.S1_x_at	2.965		0.000	CF574964	cold induced, low temperature and salt responsive protein	*Pennisetum glaucum*	3.00E-21
12	SofAffx.125.1.S1_x_at	2.082		0.000	CF575717	cold induced, low temperature and salt responsive protein	*Arabidopsis thaliana*	1.00E-15
13	SofAffx.125.2.S1_at	2.441		0.005	CF572632	cold induced, low temperature and salt responsive protein	*Solanum tuberosum*	8.00E-16
14	Sof.19.1.S1_at	0.482	1.19.6.1	0.012	CA093296	flavodoxin	*Medicago truncatula*	6.00E-30
15	Sof.3466.1.A1_at	2.687	4.1.1.15	0.000	CA270299	glutamate decarboxylase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-31
16	Sof.3466.2.S1_at	2.966	4.1.1.15	0.001	CA171450	glutamate decarboxylase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-107
17	Sof.2216.1.A1_at	0.478		0.002	CA093476	heat shock like protein	*Arabidopsis thaliana*	6.00E-47
18	Sof.676.1.S1_at	22.67		0.004	CA135644	jacalin	*Oryza sativa* (*japonica* cultivar-group)	1.00E-14
19	Sof.4979.1.S1_at	3.405		0.000	CA122355	leaf senescence related protein	*Oryza sativa* (*japonica* cultivar-group)	1.00E-18
20	Sof.1.1.A1_at	2.439		0.000	CA218370	lethal leaf spot	*Zea mays*	7.00E-27
21	Sof.1.2.S1_at	2.317		0.000	CA286120	lethal leaf-spot	*Zea mays*	7.00E-137
22	Sof.2621.2.S1_at	5.089	2.7.11.24	0.000	CA272048	mitogen-activated protein kinase	*Saccharum officinarum*	1.00E-13
23	Sof.1333.1.S1_at	0.315	2.7.11.24	0.025	CA164068	mitogen-activated protein kinase	*Saccharum officinarum*	1.00E-08
24	Sof.1462.1.S1_at	0.306		0.002	CA275478	pathogen related protein	*Arabidopsis thaliana*	---
25	Sof.820.1.S1_at	2.132	1.11.1.7	0.006	CA266080	peroxidase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-30
26	SofAffx.2013.1.S1_s_at	4.104	1.11.1.7	0.003	CO373606	peroxidase	*Zea mays*	1.00E-20
27	Sof.4034.1.S1_at	3.316	2.1.1.103	0.000	CA239805	phosphoethanolamine N-	*Zea mays*	2.00E-38

						methyltransferase		
28	SofAffx.1506.2.S1_at	2.786		0.001	CF571685	proteinase inhibitor	*Zea mays*	2.00E-25
29	SofAffx.2049.1.S1_at	6.531		0.001	CO373862	proteinase inhibitor	*Zea mays*	6.00E-14
30	SofAffx.1811.1.S1_at	2.096		0.003	CF570630	proteinase inhibitor	*Medicago truncatula*	1.00E-15
31	Sof.2845.1.A1_at	3.305		0.002	CA174984	remorin C	*Oryza sativa* (*japonica* cultivar-group)	3.00E-05
32	SofAffx.947.1.S1_at	0.117		0.002	CF573861	response regulator	*Zea mays*	7.00E-44
33	SofAffx.135.1.S1_s_at	0.247	2.7.11.1	0.000	CF577254	SNF1-related kinase complex (beta subunit 2)	*Saccharum* hybrid cultivar	---
34	Sof.1710.1.S1_at	0.243	2.7.11.1	0.004	CA093131	SNF1-related kinase complex (beta subunit 2)	*Arabidopsis thaliana*	---
35	Sof.2872.1.S1_at	3.422		0.000	CA071411	stress induced protein	*Oryza sativa* (*japonica* cultivar-group)	4.00E-55
36	Sof.4297.1.S1_at	4.737		0.000	CA119563	stress inducible protein	*Arabidopsis thaliana*	---
37	Sof.3913.1.S1_s_at	3.024		0.000	CA253240	stress responsive protein	*Triticum aestivum*	1.00E-59
38	Sof.3913.1.S1_at	3.902		0.000	CA253240	stress responsive protein	*Triticum aestivum*	1.00E-59
39	Sof.3673.1.A1_at	2.141		0.001	CA106990	stress responsive protein	*Triticum aestivum*	1.00E-34
40	Sof.3456.1.S1_a_at	0.171		0.001	CA203963	stress responsive protein	*Triticum aestivum*	6.00E-32
41	Sof.703.2.S1_a_at	2.102	1.15.1.1	0.000	CA144287	superoxide dismutase	*Zea mays*	5.00E-82
42	SofAffx.162.1.S1_at	2.799		0.002	CF576603	wound induced protein	*Solanum lycopersicum*	2.00E-12
Miscellaneous Pathways								
Hormone response								
1	Sof.3801.1.S1_at	5.197		0.000	AY521566	1-aminocyclopropane-1-carboxylate oxidase	*Saccharum officinarum*	3.00E-160
2	SofAffx.573.1.S1_at	4.24		0.001	CF571282	abscisic acid and stress inducible protein	*Oryza sativa* (*japonica* cultivar-group)	9.00E-20
3	SofAffx.260.1.S1_at	4.499		0.000	CF570774	abscisic acid responsive elements-binding factor	*Oryza sativa* (*japonica* cultivar-group)	1.00E-04
4	SofAffx.1449.1.S1_s_at	4.837		0.000	CF572515	abscisic acid-induced protein	*Saccharum* hybrid cultivar	----
5	Sof.1305.1.A1_at	2.226		0.000	CA109060	auxin efflux carrier	*Zea mays*	1.00E-15
6	SofAffx.1348.1.S1_at	3.669		0.001	CF572342	auxin response factor 7a	*Oryza sativa* (*japonica* cultivar-group)	4.00E-25
7	Sof.3691.1.S1_at	0.384		0.000	CA067840	auxin-induced protein	*Saccharum* hybrid cultivar	1.00E-31
8	Sof.4481.2.S1_at	2.384		0.002	BQ533069	ethylene responsive element binding protein	*Oryza sativa* (*japonica* cultivar-group)	2.00E-07
9	Sof.1227.2.S1_a_at	3.766		0.012	CA263872	ethylene responsive element binding protein	*Oryza sativa* (*japonica* cultivar-group)	3.00E-14
10	Sof.5220.1.S1_at	0.418		0.000	CA109495	ethylene responsive transcription factor	*Saccharum officinarum*	7.00E-35

11	Sof.2770.2.S1_at	2.412	0.000	CA151741	F-box protein	*Arabidopsis thaliana*	2.00E-23
12	Sof.774.1.S1_at	2.256	0.000	CA264879	squalene monooxygenase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-29
13	Sof.1176.1.A1_at	0.421	0.001	CA168983	sterol-4-methyl-oxidase	*Arabidopsis thaliana*	3.00E-22
DNA, RNA translation and binding							
1	Sof.2740.1.S1_a_at	2.05	0.000	CA241080	adenosine kinase	*Zea mays*	2.00E-62
2	Sof.677.1.S1_at	2.367	0.000	CA145110	cytidine deaminase	*Zea mays*	3.00E-76
3	Sof.2480.1.A1_at	3.851	0.000	CA257103	DNA binding protein / transcription factor	*Arabidopsis thaliana*	2.00E-41
4	SofAffx.1392.1.S1_at	2.014	0.000	CF572343	DNA binding protein / transcription factor	*Oryza sativa*	0.019
5	Sof.2220.2.S1_at	2.197	0.002	CA093730	DNA binding protein / transcription factor	*Oryza sativa* (*japonica* cultivar-group)	5.00E-08
6	Sof.4509.1.S1_at	2.436	0.005	CA131481	endonuclease	*Oryza sativa* (*japonica* cultivar-group)	9.00E-10
7	Sof.4028.2.S1_at	0.437	0.007	CA122909	histone H2A	*Arabidopsis thaliana*	---
8	Sof.4028.1.S1_a_at	0.361	0.001	CA243079	histone H2A	*Zea mays*	1.00E-18
9	Sof.4025.1.S1_s_at	0.383	0.000	CA077455	histone H2A	*Oryza sativa* (*japonica* cultivar-group)	8.00E-39
10	Sof.3707.1.S1_at	0.293	0.003	CA133751	histone H3	*Arabidopsis thaliana*	---
11	Sof.4148.1.S1_s_at	0.414	0.000	CA128434	histone H4	*Citrus jambhiri*	7.00E-40
12	SofAffx.294.1.S1_s_at	2.125	0.007	CF576446	methyltransferase (cycloartenol 24-C)	*Oryza sativa* (*japonica* cultivar-group)	4.00E-19
13	Sof.2550.1.A1_at	0.407	0.012	CA250420	methyltransferase (SAM-dependent)	*Oryza sativa* (*japonica* cultivar-group)	1.00E-34
14	Sof.190.2.S1_a_at	2.087	0.001	CA105999	methyltransferase (sterol 24-C)	*Zea mays*	2.00E-54
15	Sof.190.1.S1_at	2.296	0.000	CA106090	methyltransferase (sterol 24-C)	*Zea mays*	2.00E-53
16	Sof.2519.2.S1_a_at	0.48	0.001	CA188257	nucleasome/chromatin assembly factor	*Zea mays*	2.00E-52
17	Sof.1127.1.A1_at	0.361	0.006	CA109222	nucleoside phosphatase	*Oryza sativa* (*japonica* cultivar-group)	5.00E-40
18	Sof.3246.1.S1_at	0.488	0.002	CA294015	ribosomal 40S protein S1	*Arabidopsis thaliana*	---
19	Sof.1180.1.S1_at	2.168	0.001	CA297203	ribosomal 60S protein L3	*Arabidopsis thaliana*	---
20	SofAffx.774.1.S1_at	0.444	0.002	CF574872	ribosomal 30S protein (plastid-specific)	*Oryza sativa* (*japonica* cultivar-group)	3.00E-14
21	SofAffx.2108.1.S1_s_at	0.454	0.016	50198865-8	ribosomal 50S protein	*Saccharum* hybrid cultivar	---
22	SofAffx.765.1.S1_s_at	0.439	0.001	CF574890	ribosomal 50S protein	*Arabidopsis thaliana*	----
23	Sof.1465.1.A1_s_at	0.301	0.000	CA296516	ribosomal 50S protein L12	*Arabidopsis thaliana*	----
24	Sof.2851.1.S1_at	0.307	0.001	CA122843	ribosomal 50S protein L17	*Arabidopsis thaliana*	----

25	SofAffx.50.1.S1_s_at	0.407	0.000	CF577168	ribosomal 50s protein L9, chloroplast	*Oryza sativa* (*japonica* cultivar-group)	2.00E-14
26	SofAffx.2101.1.S1_at	0.423	0.000	50198865-1	ribosomal protein L14 gene	*Saccharum* hybrid cultivar	---
27	SofAffx.2133.1.S1_at	0.409	0.000	50198865-43	ribosomal protein L2 gene	*Saccharum* hybrid cultivar	---
28	SofAffx.2142.1.S1_at	0.405	0.000	50198865-82	ribosomal protein L33 gene	*Saccharum* hybrid cultivar	---
29	SofAffx.2109.1.S1_s_at	0.44	0.000	50198865-30	ribosomal protein S15 gene	*Saccharum* hybrid cultivar	---
30	SofAffx.2169.1.S1_at	0.436	0.000	50198865-58	ribosomal protein S2 gene	*Saccharum* hybrid cultivar	---
31	SofAffx.2192.1.S1_at	0.365	0.001	50198865-65	ribosomal protein S4 gene	*Saccharum* hybrid cultivar	---
32	SofAffx.2189.1.S1_at	0.453	0.012	50198865-62	ribosomal S14 gene	*Saccharum* hybrid cultivar	---
33	SofAffx.886.1.S1_s_at	0.147	0.000	CF574293	transcription factor	*Saccharum* hybrid cultivar	---
34	SofAffx.886.1.S1_at	0.161	0.010	CF574293	transcription factor	*Saccharum* hybrid cultivar	---
35	SofAffx.578.1.S1_at	0.129	0.000	CF575469	transcription factor	*Saccharum* hybrid cultivar	---
36	SofAffx.331.1.S1_s_at	4.999	0.001	CF576113	transcription factor	*Saccharum* hybrid cultivar	---
37	Sof.4439.1.A1_at	0.274	0.000	CA273789	transcription factor	*Arabidopsis thaliana*	----
38	Sof.3562.2.S1_at	0.147	0.033	CA206226	transcription factor	*Arabidopsis thaliana*	----
39	Sof.1709.1.S1_at	0.418	0.000	CA090536	transcription factor	*Triticum aestivum*	5.00E-07
40	Sof.308.1.S1_at	2.698	0.000	CA123026	transformer-SR ribonucleoprotein	*Arabidopsis thaliana*	----
41	Sof.1330.1.A1_at	2.102	0.000	CA093269	zinc finger protein	*Oryza sativa* (*japonica* cultivar-group)	7.00E-44
42	Sof.3479.1.A1_at	2.142	0.000	CA183562	zinc finger protein	*Oryza sativa (*japonica cultivar-group)	3.00E-61
43	Sof.1494.1.A1_at	0.218	0.000	CA106996	zinc finger protein	*Oryza sativa*	5.00E-17
Lipid, phospholipid and fatty acid (mitochondrial beta-oxidation pathway) metabolism							
1	SofAffx.864.1.S1_s_at	2.267	0.000	CF574204	acyl carrier protein	*Zea mays*	9.00E-26
2	SofAffx.411.1.S1_at	0.165	0.002	CF575893	acyl carrier protein	*Saccharum* hybrid cultivar	---
3	Sof.2976.1.S1_at	2.243	0.004	CA194677	acyl carrier protein	*Zea mays*	2.00E-32
4	Sof.4346.1.A1_at	0.342	0.010	CA186650	Acyl-coenzyme A oxidase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-64
5	Sof.4349.1.S1_at	0.481	0.007	CA065884	acyltransferase	*Arabidopsis thaliana*	5.00E-59
6	Sof.3883.1.S1_at	2.808	0.001	CA233387	annexin	*Medicago truncatula*	3.00E-06
7	Sof.3504.1.S1_at	2.449	0.015	CA208201	annexin p33	*Zea mays*	5.00E-32
8	Sof.1329.1.S1_at	2.586	0.000	CA269127	enoyl-[acyl-carrier protein] reductase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-44

9	Sof.4079.1.S1_at	2.295	0.004	CA123071	enoyl-CoA hydratase	*Oryza sativa* (*japonica* cultivar-group)	6.00E-52
10	Sof.3291.1.S1_at	0.464	0.001	CA291308	esterase/lipase	*Oryza sativa* (*japonica* cultivar-group)	8.00E-18
11	Sof.3284.1.S1_s_at	3.346	0.000	CA163837	lipid binding protein	*Arabidopsis thaliana*	3.00E-12
12	Sof.3284.1.S1_at	3.19	0.015	CA163837	lipid binding protein	*Arabidopsis thaliana*	3.00E-12
13	Sof.1025.1.S1_a_at	3.878	0.000	CA160989	lipid binding protein	*Arabidopsis thaliana*	1.00E-12
14	Sof.3597.1.S1_at	4.765	0.000	CA242934	lipid-transfer protein	*Oryza sativa* (*japonica* cultivar-group)	2.00E-31
15	Sof.3427.2.S1_at	5.801	0.006	CA085551	myo-inositol 1-phosphate synthase	*Zea mays*	1.00E-70
16	Sof.3427.1.S1_at	4.332	0.005	CA256860	myo-inositol 1-phosphate synthase	*Zea mays*	2.00E-69
17	SofAffx.699.1.S1_s_at	6.966	0.001	CF574629	Myo-inositol-1-phosphate synthase	*Zea mays*	6.00E-18
18	SofAffx.699.1.S1_at	4.306	0.000	CF574629	myo-inositol-1-phosphate synthase	*Oryza sativa* (*japonica* cultivar-group)	6.00E-18
19	SofAffx.1050.1.S1_at	3.089	0.004	CF573411	plant lipase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-37
20	Sof.3444.2.S1_a_at	3.165	0.002	CA271402	plant lipase	*Oryza sativa* (*japonica* cultivar-group)	7.00E-80
21	Sof.1440.1.S1_at	3.231	0.000	CA284296	plant lipase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-67
22	Sof.450.1.S1_at	5.409	0.001	CA293588	Undecaprenyl pyrophosphate synthetase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-45
Miscellaneous. transporters							
1	Sof.4946.1.S1_a_at	8.281	0.000	CA200693	ADP-ribosylation factor	*Arabidopsis thaliana*	1.00E-15
2	Sof.1342.1.S1_at	5.503	0.000	CA275595	amino acid transporter	*Arabidopsis thaliana*	---
3	SofAffx.221.1.S1_at	4.212	0.003	CF575182	amino acid transporter	*Arabidopsis thaliana*	3.00E-20
4	Sof.4831.1.S1_at	2.673	0.001	CA286162	anion transporter	*Arabidopsis thaliana*	7.00E-24
5	Sof.110.1.A1_at	2.774	0.000	CA128659	ATPase (kinesin-organelle transport)	*Oryza sativa* (*japonica* cultivar-group)	0.031
6	Sof.131.1.S1_at	0.429	0.000	CA125461	ATPase-like protein	*Sorghum bicolor*	3.00E-10
7	SofAffx.597.1.S1_at	2.101	0.000	CF575528	cation transporter	*Arabidopsis thaliana*	6.00E-14
8	SofAffx.1947.1.S1_at	0.155	0.000	CF570474	efflux transporter	*Saccharum* hybrid cultivar	---
9	Sof.5080.1.A1_at	4.567	0.000	CA277004	heavy metal transport	*Oryza sativa* (*japonica* cultivar-group)	4.00E-05
10	Sof.3268.2.S1_x_at	6.021	0.002	CA285541	heavy metal transport	*Oryza sativa* (*japonica* cultivar-group)	4.00E-10
11	Sof.1082.1.S1_at	2.226	0.000	CA148697	nitrate transporter	*Oryza sativa* (*japonica* cultivar-group)	4.00E-44
12	SofAffx.766.1.S1_at	2.433	0.001	CF574742	potassium transporter	*Oryza sativa* (*japonica* cultivar-group)	0.12

13	Sof.3602.1.S1_at	0.451	0.001	CA295007	protein translocase/ protein transporter	*Arabidopsis thaliana*	3.00E-18
14	Sof.234.1.S1_at	6.969	0.019	CA085096	protein transport protein	*Arabidopsis thaliana*	**---**
15	Sof.1342.1.S1_s_at	5.2	0.000	CA275595	transmembrane amino acid transporter	*Oryza sativa* (*japonica* cultivar-group)	1.00E-38
Amino acid and protein metabolism							
1	Sof.2944.1.S1_at	10.64	0.000	CA122418	3-deoxy-D-arabino-heptolosonate-7-phosphate synthetase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-40
2	Sof.521.1.S1_at	2.28	0.002	CA067649	3-phosphoshikimate 1-carboxyvinyltransferase	*Zea mays*	2.00E-51
3	SofAffx.704.1.S1_at	0.468	0.000	CF574659	amino acid permease	*Arabidopsis thaliana*	1.00E-08
4	Sof.4252.1.S1_a_at	11.6	0.000	CA133575	aminotransferase (alanine)	*Arabidopsis thaliana*	**---**
5	Sof.4252.2.S1_a_at	5.001	0.000	CA137135	aminotransferase (alanine)	*Zea mays*	6.00E-86
6	Sof.1326.2.S1_at	0.493	0.001	CA145855	aminotransferase (alanine)	*Oryza sativa* (*japonica* cultivar-group)	9.00E-100
7	Sof.1288.2.S1_at	2.075	0.000	CA266448	aminotransferase (ornithine)	*Oryza sativa* (*japonica* cultivar-group)	2.00E-81
8	Sof.1288.2.S1_a_at	2.516	0.001	CA266448	aminotransferase (ornithine)	*Oryza sativa* (*japonica* cultivar-group)	2.00E-81
9	Sof.1981.1.S1_at	3.382	0.000	CA135406	aminotransferase (tyrosine)	*Oryza sativa* (*japonica* cultivar-group)	8.00E-65
10	SofAffx.1146.1.S1_at	4.589	0.000	CF573503	beta-alanine-pyruvate aminotransferase	*Arabidopsis thaliana*	----
11	Sof.1173.1.S1_at	3.274	0.002	CA137663	beta-alanine-pyruvate aminotransferase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-48
12	AFFX-Sof-r2-Bs-lys-M_at	0.48	0.000	AFFX-Sof-r2-Bs-lys-M	diaminopimelate decarboxylase	*Bacillus subtillis*	----
13	AFFX-Sof-r2-Bs-lys-5_at	0.464	0.001	AFFX-Sof-r2-Bs-lys-5	diaminopimelate decarboxylase	*Bacillus subtillis*	----
14	AFFX-Sof-r2-Bs-lys-3_at	0.495	0.006	AFFX-Sof-r2-Bs-lys-3	diaminopimelate decarboxylase	*Bacillus subtillis*	----
15	AFFX-r2-Bs-lys-M_at	0.47	0.007	AFFX-r2-Bs-lys-M	diaminopimelate decarboxylase	*Bacillus subtillis*	----
16	AFFX-r2-Bs-lys-5_at	0.439	0.001	AFFX-r2-Bs-lys-5	diaminopimelate decarboxylase	*Bacillus subtillis*	----
17	AFFX-r2-Bs-lys-3_at	0.444	0.020	AFFX-r2-Bs-lys-3	diaminopimelate decarboxylase	*Bacillus subtillis*	----
18	AFFX-LysX-M_at	0.488	0.001	AFFX-LysX-M	diaminopimelate decarboxylase	*Bacillus subtillis*	----
19	AFFX-LysX-5_at	0.465	0.002	AFFX-LysX-5	diaminopimelate decarboxylase	*Bacillus subtillis*	----
20	AFFX-LysX-3_at	0.471	0.004	AFFX-LysX-3	diaminopimelate decarboxylase	*Bacillus subtillis*	----
21	SofAffx.1369.1.S1_s_at	2.806	0.000	CF572085	glutamate synthase (NADH dependent)	*Oryza sativa* (*japonica* cultivar-group)	4.00E-34
22	SofAffx.1369.1.S1_at	2.832	0.000	CF572085	glutamate synthase (NADH dependent)	*Oryza sativa* (*japonica* cultivar-group)	4.00E-34

23	Sof.78.1.S1_at	2.799	0.006	CA145696	glutamate synthase (NADH dependent)	*Oryza sativa* (*japonica* cultivar-group)	3.00E-77
24	SofAffx.2060.1.S1_s_at	0.459	0.002	CO373145	glutamine synthetase	*Saccharum officinarum*	5.00E-09
25	SofAffx.2060.1.S1_at	0.42	0.003	CO373145	glutamine synthetase	*Saccharum officinarum*	5.00E-09
26	Sof.2400.1.S1_at	0.489	0.002	CA279746	glutamine synthetase	*Saccharum officinarum*	0.001
27	AFFX-ThrX-M_at	0.452	0.003	AFFX-ThrX-M	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
28	AFFX-ThrX-5_at	0.429	0.015	AFFX-ThrX-5	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
29	AFFX-Sof-r2-Bs-thr-M_s_at	0.454	0.006	AFFX-Sof-r2-Bs-thr-M	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
30	AFFX-Sof-r2-Bs-thr-5_s_at	0.439	0.001	AFFX-Sof-r2-Bs-thr-5	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
31	AFFX-r2-Bs-thr-M_s_at	0.466	0.006	AFFX-r2-Bs-thr-M	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
32	AFFX-r2-Bs-thr-5_s_at	0.434	0.001	AFFX-r2-Bs-thr-5	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
33	AFFX-r2-Bs-phe-M_at	0.452	0.000	AFFX-r2-Bs-phe-M	homoserine kinase and threonine synthase	*Bacillus subtillis*	----
34	Sof.2004.2.S1_a_at	0.483	0.002	CA226154	lysine decarboxylase	*Arabidopsis thaliana*	5.00E-57
35	Sof.2004.1.S1_at	0.246	0.000	CA109074	lysine decarboxylase	*Arabidopsis thaliana*	----
36	Sof.2814.1.S1_at	2.045	0.000	BU102593	phospho-2-dehydro-3-deoxyheptonate aldolase	*Oryza sativa*	2.00E-52
37	AFFX-Sof-r2-Bs-phe-3_at	0.492	0.004	AFFX-Sof-r2-Bs-phe-3	phenylalanine biosynthesis associated protein	*Bacillus subtillis*	----
38	AFFX-r2-Bs-phe-3_at	0.483	0.002	AFFX-r2-Bs-phe-3	phenylalanine biosynthesis associated protein	*Bacillus subtillis*	----
39	AFFX-PheX-M_at	0.476	0.002	AFFX-PheX-M	phenylalanine biosynthesis associated protein	*Bacillus subtillis*	----
40	Sof.2974.2.S1_at	2.017	0.000	CA093542	phosphotransferase (amino)	*Oryza sativa* (*japonica* cultivar-group)	2.00E-58
41	Sof.2974.2.S1_a_at	2.507	0.000	CA093542	phosphotransferase (amino)	*Oryza sativa* (*japonica* cultivar-group)	2.00E-58
42	Sof.2974.1.A1_at	2.27	0.001	CA093606	phosphotransferase (amino)	*Oryza sativa* (*japonica* cultivar-group)	1.00E-39
43	Sof.301.1.S1_at	2.04	0.000	CA122377	prephenate dehydratase/chorismate mutase	*Arabidopsis thaliana*	0.054
44	Sof.3337.1.S1_at	0.464	0.001	CA296988	protein kinase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-04
45	Sof.2081.1.A1_at	0.438	0.000	CA068380	protein kinase	*Medicago truncatula*	5.00E-13
46	Sof.687.1.S1_at	2.312	0.010	CA230038	protein kinase (ATP binding)	*Oryza sativa* (*japonica* cultivar-group)	9.00E-12
47	Sof.4911.1.S1_at	4.487	0.000	CA123070	protein kinase (ATP binding)	*Arabidopsis thaliana*	2.00E-21

48	Sof.4465.1.S1_a_at	2.727	0.001	CA066873	protein kinase (ATP binding)	*Oryza sativa* (*japonica* cultivar-group)	2.00E-94
49	Sof.3922.3.S1_a_at	0.342	0.000	CA162299	protein kinase (calcium-dependent)	*Zea mays*	4.00E-44
50	Sof.2799.2.S1_at	2.735	0.000	CA296007	protein phosphatase 2C	*Hordeum vulgare*	1.00E-24
51	Sof.1143.2.S1_at	2.987	0.000	CA228512	protein phosphatase 2C	*Oryza sativa* (*japonica* cultivar-group)	3.00E-41
52	Sof.2799.3.S1_at	2.036	0.001	CA131632	protein phosphatase 2C	*Arabidopsis thaliana*	2.00E-33
53	Sof.934.1.S1_at	0.419	0.004	CA159968	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	5.00E-54
54	Sof.2538.3.S1_at	0.388	0.007	CA157758	serine / threonine kinase	*Sorghum bicolor*	8.00E-86
55	Sof.2538.2.S1_at	0.424	0.014	CA156919	serine / threonine kinase	*Sorghum bicolor*	4.00E-54
56	Sof.2538.1.A1_at	0.43	0.000	CA249568	serine / threonine kinase	*Sorghum bicolor*	2.00E-20
57	SofAffx.1839.1.S1_at	0.336	0.011	CF570918	serine / threonine kinase	*Saccharum* hybrid cultivar	---
58	Sof.2621.3.A1_a_at	5.246	0.000	CA267619	serine / threonine kinase	*Sorghum bicolor*	0.001
59	Sof.1250.2.S1_a_at	2.357	0.000	CA170162	serine / threonine kinase	*Sorghum bicolor*	2.00E-18
60	SofAffx.72.1.S1_at	2.763	0.001	CF576672	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	8.00E-22
61	SofAffx.45.1.S1_at	2.295	0.003	CF577437	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-06
62	Sof.680.1.S1_at	2.047	0.001	CA080210	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-49
63	Sof.4465.1.S1_at	2.243	0.000	CA066873	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-94
64	Sof.2120.1.A1_at	2.674	0.010	CA141566	serine / threonine kinase	*Oryza sativa* (*japonica* cultivar-group)	7.00E-18
65	Sof.1568.1.A1_at	0.404	0.000	CA070399	serine / threonine kinase receptor precursor	*Oryza sativa* (*japonica* cultivar-group)	3.00E-54
66	Sof.2799.1.S1_at	2.742	0.006	CA196644	serine / threonine phosphatase	*Arabidopsis thaliana*	1.00E-34
67	Sof.1143.1.S1_at	2.694	0.000	CA090531	serine / threonine phosphatase	*Oryza sativa* (*japonica* cultivar-group)	9.00E-24
68	Sof.2054.1.S1_at	2.143	0.000	CA087269	serine carboxypeptidase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-33
69	Sof.3249.1.S1_at	3.102	0.000	CA230383	serine hydrolase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-86
70	Sof.3361.2.S1_a_at	6.839	0.004	CA174854	serine protease	*Zea mays*	2.00E-27
71	Sof.4877.2.S1_at	0.491	0.001	CA152550	shikimate kinase (ATP binding)	*Oryza sativa* (*japonica* cultivar-group)	9.00E-93
72	Sof.4877.2.S1_a_at	0.494	0.003	CA152550	shikimate kinase (ATP binding)	*Oryza sativa* (*japonica* cultivar-group)	9.00E-93
73	Sof.2632.1.S1_at	3.143	0.002	CA148334	threonine aldolase (amino acid transport)	*Oryza sativa* (*japonica* cultivar-group)	4.00E-56
74	SofAffx.40.2.S1_at	2.483	0.000	CF577373	tyrosine phosphatase	*Oryza sativa* (*japonica* cultivar-	7.00E-40

						group)	
Heavy metal metabolism							
1	Sof.3446.3.S1_x_at	3.082	0.000	CA289431	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	2.00E-78
2	Sof.3446.3.S1_a_at	3.353	0.006	CA289431	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	2.00E-78
3	Sof.3446.2.S1_x_at	4.005	0.001	CA069720	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	1.00E-06
4	Sof.3446.2.S1_s_at	3.941	0.001	CA069720	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	1.00E-06
5	Sof.3446.2.S1_at	4.015	0.000	CA069720	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	1.00E-06
6	Sof.3446.1.S1_at	2.704	0.000	CA067108	ferritin (iron storage protein), chloroplast precursor	*Zea mays*	8.00E-05
7	Sof.1890.1.S1_at	0.371	0.003	CA163877	ferrochelatase	*Nicotiana tabacum*	5.00E-43
8	Sof.5293.1.S1_s_at	2.802	0.000	CA092887	metallothionein-like protein	*Saccharum* hybrid cultivar	2.00E-23
9	Sof.1428.1.S1_at	3.23	0.000	CA153302	molybdenum cofactor sulfurase-like protein	*Oryza sativa* (*japonica* cultivar-group)	3.00E-22
10	Sof.1057.1.S1_at	5.557	0.000	CA070876	phytochelatin synthase	*Zea mays*	3.00E-18
11	Sof.1057.2.S1_at	2.789	0.001	CA163307	phytochelatin synthase	*Sorghum bicolor*	2.00E-98
Cytoskeletal metabolism							
1	Sof.4683.1.S1_at	2.687	0.000	CA268837	Actin	*Oryza sativa* (*japonica* cultivar-group)	7.00E-28
2	Sof.926.1.S1_at	2.455	0.000	CA150227	Actin	*Oryza sativa* (*japonica* cultivar-group)	2.00E-14
3	Sof.4708.2.S1_at	2.835	0.002	CA196968	Actin	*Oryza sativa* (*japonica* cultivar-group)	3.00E-12
4	Sof.3247.1.S1_s_at	2.043	0.001	CA117568	actin depolymerizing factor	*Oryza sativa* (*japonica* cultivar-group)	2.00E-29
5	SofAffx.555.1.S1_at	2.208	0.000	CF575199	actin-binding FH2	*Arabidopsis thaliana*	1.00E-31
6	Sof.4196.1.S1_at	0.443	0.001	CA075025	ankyrin-repeat protein	*Oryza sativa* (*japonica* cultivar-group)	1.00E-32
7	SofAffx.194.1.S1_at	2.23	0.000	CF570826	aquaporin	*Hordeum vulgare*	2.00E-09
8	Sof.3973.1.S1_s_at	2.337	0.001	CA294123	aquaporin	*Hordeum vulgare*	4.00E-70
9	SofAffx.1774.1.S1_at	7.269	0.001	CF570745	beta tubulin	*Oryza sativa* (*japonica* cultivar-group)	6.00E-36
10	SofAffx.16.1.S1_s_at	2.217	0.001	CF577249	beta-tubulin	*Zea mays*	4.00E-32
11	Sof.4868.2.S1_at	3.182	0.007	CA172903	kinesin heavy chain	*Zea mays*	8.00E-86
12	Sof.4868.1.S1_a_at	2.555	0.000	CA089553	kinesin heavy chain	*Zea mays*	7.00E-86
13	Sof.4093.2.S1_s_at	2.158	0.010	CA255815	tubulin A	*Hyriopsis cumingii*	6.00E-88

Other metabolic events

1	SofAffx.1850.1.S1_at	0.486	0.000	CF570594	1-deoxy-D-xylulose 5-phosphate reductoisomerase	*Zea mays*	9.00E-21
2	AFFX-Sof-r2-Bs-dap-5_at	0.488	0.000	AFFX-Sof-r2-Bs-dap-5	acetyl-CoA-carboxylase ligase (birA) gene	*Bacillus subtillis*	----
3	Sof.3744.1.S1_at	2.723	0.000	CA234593	ankyrin	*Arabidopsis thaliana*	----
4	Sof.569.2.S1_a_at	0.295	0.010	CA293295	aspartic proteinase	*Arabidopsis thaliana*	----
5	Sof.973.1.A1_at	2.532	0.000	CA161592	aspartyl protease	*Oryza sativa* (*japonica* cultivar-group)	0.001
6	Sof.3025.1.A1_at	2.933	0.000	CA069871	AT-hook DNA-binding protein	*Oryza sativa* (*japonica* cultivar-group)	2.00E-13
7	SofAffx.2145.1.S1_s_at	0.472	0.000	50198865-85	ATP-dependent chloroplast protease	*Saccharum* hybrid cultivar	----
8	Sof.602.1.A1_at	0.089	0.001	CA140765	beta glucanase	*Triticum aestivum* (bread wheat)	3.00E-06
9	Sof.4235.1.S1_a_at	5.409	0.000	CA231339	beta-galactosidase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-41
10	Sof.962.2.S1_at	0.383	0.005	CA155393	beta-lactamase	*Arabidopsis thaliana*	2.00E-103
11	Sof.2798.1.S1_at	2.211	0.000	CA181553	Ca2+/H+-exchanging protein	*Oryza sativa* (*japonica* cultivar-group)	1.00E-08
12	Sof.5318.1.S1_at	0.383	0.000	BQ531226	Ca2+-binding protein	*Triticum aestivum*	7.00E-28
13	SofAffx.762.1.S1_at	2.088	0.005	CF574946	calmodulin binding protein	*Arabidopsis thaliana*	1.00E-04
14	Sof.2146.1.S1_at	2.228	0.033	CA065753	calmodulin binding protein	*Oryza sativa* (*japonica* cultivar-group)	4.00E-27
15	Sof.3347.2.S1_at	4.091	0.001	CA122155	CCT domain containing protein	*Oryza sativa* (*japonica* cultivar-group)	3.00E-57
16	Sof.4448.2.S1_at	0.499	0.002	CA269147	chloroplast inner envelope protein	*Oryza sativa* (*japonica* cultivar-group)	1.00E-97
17	SofAffx.2124.1.S1_at	0.381	0.001	50198865-74	chloroplast membrane protein A	*Saccharum* hybrid cultivar	----
18	Sof.2816.1.S1_at	3.6	0.002	CA240158	delta-1-pyrroline-5-carboxylate synthetase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-08
19	SofAffx.800.1.S1_s_at	0.388	0.002	CF573822	disease resistance protein	*Hordeum vulgare*	4.00E-59
20	Sof.970.1.S1_a_at	2.321	0.010	BQ533251	dTDP-glucose 4,6-dehydratase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-19
21	Sof.4150.1.S1_at	2.755	0.001	CA290341	early light-inducible protein	*Triticum aestivum*	9.00E-09
22	Sof.3713.2.S1_x_at	2.028	0.003	CA229648	exostosin	*Oryza sativa* (*japonica* cultivar-group)	3.00E-50
23	Sof.1606.1.A1_at	5.323	0.001	CA233374	exostosin	*Oryza sativa* (*japonica* cultivar-group)	2.00E-58
24	Sof.3590.1.S1_at	4.405	0.000	CA148883	fasciclin-like protein	*Triticum aestivum*	3.00E-57
25	SofAffx.313.1.S1_at	3.176	0.002	CF572801	flavonoid 3'-hydroxylase	*Triticum aestivum*	8.00E-08
26	Sof.4795.1.S1_at	0.409	0.003	CA288421	glutamate-1-semialdehyde aminotransferase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-09

27	Sof.4848.1.S1_a_at	0.363	0.000	CA076526	glutathione S-transferase	*Zea mays*	1.00E-63
28	Sof.5183.1.A1_at	0.454	0.000	CA069866	glutathione S-transferase	*Zea mays*	2.00E-54
29	Sof.1666.2.S1_x_at	0.314	0.000	CA218291	glycerophosphodiester phosphodiesterase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-55
30	Sof.1666.2.S1_at	0.407	0.006	CA218291	glycerophosphodiester phosphodiesterase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-55
31	Sof.1243.1.S1_at	0.453	0.032	CA260453	glycine dehydrogenase	*Pisum sativum*	3.00E-20
32	Sof.4382.1.S1_at	2.564	0.018	CA076530	glycine dehydrogenase	*Oryza sativa* (*japonica* cultivar-group)	6.00E-66
33	SofAffx.590.1.S1_at	2.277	0.001	CF575358	glycolate oxidase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-17
34	SofAffx.1366.1.S1_at	2.285	0.001	CF572238	glycoprotein 3-alpha-L-fucosyltransferase	*Saccharum* hybrid cultivar	----
35	Sof.2023.2.S1_at	0.479	0.000	BQ534217	GTP cyclohydrolase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-72
36	Sof.2023.2.S1_a_at	0.465	0.001	BQ534217	GTP cyclohydrolase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-72
37	Sof.4424.1.S1_at	3.174	0.001	CA293999	haloacid dehalogenase-like hydrolase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-40
38	Sof.434.2.S1_at	3.002	0.039	CA260585	haloacid dehalogenase-like hydrolase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-53
39	Sof.434.1.A1_at	2.098	0.000	CA133757	haloacid dehalogenase-like hydrolase	*Oryza sativa* (*japonica* cultivar-group)	4.00E-05
40	Sof.833.1.A1_a_at	0.412	0.022	CA270500	haloacid dehalogenase-like hydrolase	*Oryza sativa* (*japonica* cultivar-group)	7.00E-41
41	Sof.4394.1.A1_at	2.203	0.000	CA104142	harpin-induced protein	*Oryza sativa* (*japonica* cultivar-group)	3.00E-38
42	SofAffx.495.1.S1_at	6.832	0.002	CF575662	harpin-induced protein	*Oryza sativa* (*japonica* cultivar-group)	4.00E-05
43	Sof.3967.2.S1_a_at	2.524	0.001	CA265436	HR-like lesion-inducing	*Oryza sativa* (*japonica* cultivar-group)	8.00E-50
44	Sof.5330.1.A1_at	3.532	0.000	CA090956	Hsp70 protein	*Oryza sativa* (*japonica* cultivar-group)	2.00E-18
45	SofAffx.409.1.S1_at	2.264	0.005	CF575858	hydroxyanthranilate hydroxycinnamoyltransferase	*Oryza sativa* (*japonica* cultivar-group)	5.00E-41
46	Sof.2296.1.A1_at	2.53	0.000	CA210265	Hydroxycinnamoyl coenzyme A-quinate transferase	*Prunus mume*	7.00E-16
47	Sof.1497.2.S1_a_at	0.392	0.001	CA112568	hypersensitive-induced response protein	*Zea mays*	2.00E-91
48	Sof.1497.1.S1_at	0.449	0.006	CA164474	hypersensitive-induced response protein	*Zea mays*	7.00E-31
49	Sof.5265.1.A1_s_at	2.091	0.042	CA201098	hypoxia-responsive family protein	*Citrus sinensis*	7.00E-13
50	Sof.2341.1.S1_at	2.093	0.000	CA141276	hypoxia-responsive family protein	*Citrus sinensis*	6.00E-18
51	Sof.5015.1.S1_a_at	0.385	0.007	CA065869	KED-like protein	*Zea mays*	2.00E-20
52	Sof.1537.2.S1_at	0.361	0.003	CA186293	leucine zipper transcription factor	*Oryza sativa* (*japonica* cultivar-group)	7.00E-88

53	Sof.3557.1.S1_a_at	0.353	0.000	CA153687	light-induced protein	*Lolium perenne*	1.00E-16
54	SofAffx.10.1.S1_at	2.185	0.007	L13655	membrane protein	*Saccharum* hybrid cultivar	2.00E-147
55	Sof.5020.1.S1_at	2.064	0.000	CA253086	methionine synthase	*Sorghum bicolor*	2.00E-76
56	Sof.470.1.A1_at	0.431	0.000	CA070188	methylase	*Oryza sativa* (*japonica* cultivar-group)	8.00E-37
57	SofAffx.1998.1.S1_at	2.139	0.012	CO373068	multi antimicrobial extrusion protein	*Oryza sativa* (*japonica* cultivar-group)	4.00E-48
58	Sof.4918.1.A1_at	2.425	0.001	CA201075	multi antimicrobial extrusion protein	*Oryza sativa (japonica cultivar-* *Oryza sativa* (*japonica* cultivar-group)*group)*	2.00E-11
59	Sof.2262.1.S1_at	0.474	0.008	CA245123	N-acetylglucosaminyltransferase	*Oryza sativa (japonica cultivar-* *Oryza sativa* (*japonica* cultivar-group)*group)*	4.00E-29
60	Sof.2262.2.S1_a_at	0.412	0.000	CA266083	N-acetylglucosaminyltransferase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-57
61	SofAffx.304.1.S1_at	4.306	0.000	CF576287	NADPH oxidase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-34
62	Sof.4627.1.S1_at	4.1	0.000	CA210996	NADPH oxidase	*Oryza sativa* (*japonica* cultivar-group)	3.00E-33
63	Sof.5000.2.S1_a_at	2.048	0.000	CA105019	nonclathrin coat protein	*Zea mays*	1.00E-73
64	Sof.1213.1.S1_at	2.193	0.021	CA255843	O-acetyltransferase	*Arabidopsis thaliana*	6.00E-34
65	Sof.1611.1.A1_at	2.37	0.001	CA068161	oxidoreductase	*Arabidopsis thaliana*	1.00E-37
66	Sof.751.2.S1_at	0.278	0.000	CA144595	oxidoreductase	*Arabidopsis thaliana*	**---**
67	Sof.4713.1.A1_at	0.491	0.000	CA231851	oxidoreductase	*Medicago truncatula*	3.00E-35
68	SofAffx.733.1.S1_at	0.236	0.003	CF574561	pentatricopeptide (PPR) repeat-containing protein	*Saccharum* hybrid cultivar	----
69	SofAffx.533.1.S1_at	0.393	0.000	CF575318	peptidyl-prolyl cis-trans isomerase	*Arabidopsis thaliana*	2.00E-47
70	Sof.5015.1.S1_at	0.434	0.001	CA065869	peroxisomal membrane protein	*Oryza sativa* (*japonica* cultivar-group)	5.00E-61
71	Sof.2292.1.A1_at	0.384	0.001	CA201095	peroxisomal membrane protein	*Oryza sativa* (*japonica* cultivar-group)	1.00E-55
72	Sof.5015.2.S1_at	0.485	0.002	CA274216	peroxisomal membrane protein	*Oryza sativa* (*japonica* cultivar-group)	4.00E-13
73	Sof.3603.1.S1_s_at	3.337	0.029	CA067811	phenylalanine ammonia-lyase	*Oryza sativa* (*japonica* cultivar-group)	1.00E-46
74	Sof.3100.1.S1_at	2.419	0.002	CA234511	phenylalanine ammonia-lyase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-30
75	Sof.3419.1.A1_at	3.187	0.026	CA230737	phytosulfokine-alpha 1 precursor	*Triticum aestivum*	2.00E-04
76	Sof.4293.2.S1_s_at	3.085	0.000	BQ537007	plasma membrane integral protein	*Arabidopsis thaliana*	----
77	SofAffx.893.1.S1_at	0.482	0.000	CF574158	pore-forming toxin-like protein Hfr-2	*Triticum aestivum*	7.00E-10
78	Sof.4058.1.S1_at	5.142	0.000	CA128360	proline rich protein	*Arabidopsis thaliana*	----

79	Sof.3524.2.S1_at	0.439	0.001	CA184235	pseudo-response regulator 95 homologue	*Oryza sativa* (*japonica* cultivar-group)	1.00E-95
80	Sof.4601.1.S1_at	2.421	0.000	CA169316	purple acid phosphatase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-32
81	Sof.3476.1.S1_a_at	3.723	0.000	CA265648	rapid alkalinization factor	*Oryza sativa* (*japonica* cultivar-group)	8.00E-11
82	Sof.3476.2.S1_x_at	2.434	0.000	CA181867	rapid alkalinization factor	*Oryza sativa* (*japonica* cultivar-group)	5.00E-13
83	Sof.5197.2.S1_a_at	0.382	0.014	CA111700	RING-finger (Really Interesting New Gene) protein	*Capsicum annuum*	6.00E-11
84	Sof.2517.1.S1_at	2.407	0.001	CA174457	Rop1 small GTP binding protein	*Zea mays*	1.00E-51
85	Sof.4769.1.S1_at	3.248	0.023	CA135439	secretory carrier membrane protein	*Oryza sativa* (*japonica* cultivar-group)	5.00E-53
86	Sof.2889.1.S1_at	3.744	0.001	CA244470	secretory carrier membrane protein	*Oryza sativa* (*japonica* cultivar-group)	3.00E-26
87	Sof.3971.2.S1_a_at	2.103	0.000	CA265321	secretory carrier-associated membrane protein	*Arabidopsis thaliana*	8.00E-26
88	Sof.2383.1.A1_at	0.426	0.000	CA213987	signal recognition particle protein	*Oryza sativa* (*japonica* cultivar-group)	8.00E-47
89	SofAffx.738.1.S1_at	2.023	0.000	CF575031	strictosidine synthase	*Oryza sativa* (*japonica* cultivar-group)	2.00E-16
90	Sof.4841.2.S1_a_at	3.113	0.001	CA111464	succinate-semialdehyde dehydrogenase	*Arabidopsis thaliana*	1.00E-79
91	Sof.4841.1.S1_at	2.655	0.000	CA093279	succinate-semialdehyde dehydrogenase	*Magnetospirillum magneticum*	6.00E-59
92	Sof.1786.1.A1_at	0.355	0.000	CA218473	TPR domain containing protein	*Oryza sativa* (*japonica* cultivar-group)	6.00E-20
93	Sof.3285.1.S1_at	0.458	0.000	CA068473	translation elongation factor	*Oryza sativa* (*japonica* cultivar-group)	8.00E-49
94	Sof.4530.1.S1_at	2.23	0.010	CA219604	translation initiation factor	*Salinispora tropica*	0.56
95	Sof.267.1.A1_at	0.463	0.001	CA194629	TUB protein	*Arabidopsis thaliana*	----
96	SofAffx.91.1.S1_at	3.487	0.001	CF577273	vesicle soluble NSF attachment protein acceptor	*Saccharum* hybrid cultivar	----

Chapter 7:
Culm sucrose accumulation promotes physiological decline of mature leaves in sugarcane

7.1 Abstract

Photosynthetic activity in C_4 sugarcane has been suggested to be regulated by the demand for photoassimilate from sink tissues, including culm storage of sucrose and other components of the plant (e.g. roots). This study examined the extent to which sink demand effects source activity and regulates leaf turnover in field-grown sugarcane. To increase sink demand on selected leaves, plants were defoliated apart from the immature leaf before the first fully expanded leaf (2^{nd} leaf) and the mature 8^{th} leaf. Changes in leaf gas exchange and fluorescence characteristics were recorded for both leaves over a 28 d period. Furthermore, variations in sucrose and hexose concentrations in leaf and culm tissues were measured. The affects of partial defoliation were examined based on the fixation of $^{14}CO_2$ and translocation of radio-labelled photosynthate. A decrease in internodal sucrose concentrations in partially defoliated plants was associated with significant increases over time in assimilation (*A*) and electron transport rates (ETR) for both leaf 2 and 8. Conversely, culm sucrose accumulation in control plants was related to a decline in photosynthetic rates in leaf 8 during the treatment period. It was concluded that leaf physiological ageing in sugarcane is promoted by sucrose accumulation during culm maturation, and that the feedback effect on leaf physiology which leads to leaf senescence is a consequence of decreased sink demand. Results indicated that the signaling mechanisms regulating the decline in leaf photosynthetic activity are likely hexose-mediated.

Keywords: hexose, leaf, photosynthesis, senescence, sink, source, sucrose, sugarcane

7.2 Introduction

Sugarcane (*Saccharum* L. spp. hybrids) is a tropical C_4 crop characterised by its ability to store sucrose at levels up to 650 mM (Welbaum & Meinzer, 1990). As sucrose is stored in the stalk (culm) parenchyma tissue and not in specialized storage organs, sugarcane represents a complex source-sink system. Close co-ordination of source

photosynthetic activity with carbon demand of sinks is apparent in many plant species, including sugarcane, where a decrease in source photosynthetic assimilation rates is observed when sink demand for carbohydrate is limited (Amaya *et al*., 1995; Gucci *et al.,* 1994; Basu *et al*., 1999; Iglesias *et al*., 2002; De Groot *et al*., 2003; Quilot *et al*., 2004; Franck *et al*., 2006). Conversely, photosynthetic rates have been shown to increase when sink carbon requirements are higher (Dosskey *et al*., 1990; Jeschke & Hilpert, 1997; McCormick *et al*., 2006 [Chapter 3]). Together, these studies strongly suggest the source-sink relationship in plants is sink-regulated (Paul & Foyer, 2001, Watt *et al*., 2005). Studies from C_3 crops indicate that the metabolic control of source activity by sink demand is the result of a sugar-mediated feedback signal, which responds to the status of the primary transport sugar, sucrose, and its catalytic hexose products (Abdin *et al*., 1998; Rolland *et al*., 2002; Gibson, 2005, Franck *et al*., 2006). The involvement of several sugar-sensing systems has since been revealed in a number of species (Paul & Pellny, 2003; Rolland *et al*., 2006; Paul, 2007).

Sucrose accumulation within the maturing sugarcane stem is likely the result of a strong sink-demand for photoassimilate (Marcelis, 1996), however, the activity of the source leaves is closely linked to culm maturity, with young plants typically assimilating at significantly higher rates than older plants (Hartt & Burr, 1967; Bull & Tovey, 1974). Three-month-old sugarcane leaves have been shown to assimilate at rates of 45 µmol m^{-2} s^{-1} under intense illumination, while young leaves on ten-month-old plants only fixed CO_2 at only 25 µmol m^{-2} s^{-1} (Amaya *et al*., 1995). Gross photosynthesis is reportedly lower in eight-month-old sugarcane plants compared to four-month-old plants, regardless of leaf age and light intensity (Allison *et al*., 1997). However, in a recent report where all but one leaf of sugarcane plants was shaded, a significant increase in both the maximum photosynthetic rate (J_{max}) and carboxylation efficiency (*CE*) in the unshaded source leaf was observed over time (McCormick *et al*., 2006 [Chapter 3]). A negative correlation between leaf hexose concentration and photosynthetic rates was found, indicating that hexose may play a key role in regulating the perceived demand for carbon from the sink (McCormick *et al*., 2006 [Chapter 3]). Furthermore, Gutiérrez-Miceli *et al*. (2004) have recently demonstrated that partial defoliation does not significantly influence culm sucrose concentrations, which indicates a substantial flexibility in the assimilation rates of the remaining intact leaves towards meeting the carbon demands of the sink. Together, these studies suggest that photosynthetic

capacity in sugarcane leaves is determined by the carbon requirements of the culm (McCormick *et al*., 2006 [Chapter 3]).

The processes of leaf appearance, expansion and eventual senescence are closely correlated in sugarcane (Robertson *et al*., 1998; Inman-Bamber, 2004), and the dynamics of this relationship are affected by both environmental and metabolic factors. Agronomic studies have extensively documented both environmental and climatic factors effecting leaf turnover, including temperature (Inman-Bamber, 1994; Robertson *et al*., 1998), soil pH (Plaut *et al*., 2000) and water stress (Inman-Bamber, 2004; Smit & Singels, 2006). Restriction of water availability (a practice known as 'drying off') or application of ripening agents, are often implemented in agricultural practices to increase overall culm sucrose levels prior to harvest (Donaldson, 1999; Inman-Bamber, 2004). Although a metabolic understanding of the physiological aspects of this practice is limited, it has been suggested that sugarcane culm under conditions of stress favours the partitioning of source assimilate to sucrose storage over expansive growth (Pammenter & Allison, 2002; Inman-Bamber, 2004). Manipulation of sink carbon partitioning by water deficit has been shown to result in significantly increased culm sucrose content (Inman-Bamber & Smith, 2005). During the 'artificial' water-deficit-induced maturation period prior to harvest, a decrease in leaf appearance and increased senescence of older leaves results in a decline in green leaves per stalk (Inman-Bamber, 2004; Inman-Bamber & Smith, 2005). The senescence of leaves in ripening sugarcane may be partly due to a perturbation of source-sink signaling resulting from an increase in sucrose accumulation in the culm. However, it is difficult to isolate this phenomenon from stress responses induced by water deficit or chemical ripeners. Although leaf turnover is well documented in sugarcane agriculture, the metabolic factors regulating leaf photosynthesis are not well characterized. Moreover, the possible impact of sink demand on leaf senescence processes, and consequent interaction with leaf sugar-signaling mechanisms (Wingler *et al*., 2006), has not been investigated in sugarcane.

The current study used a partial defoliation technique to examine the relationship between source leaf development and culm sink tissues in twelve-month-old, field-grown sugarcane plants over a period of approximately one month (28 d). Changes in photosynthetic rates and sugar concentrations in leaves and culm of plants completely defoliated except for two leaves, an immature leaf (leaf 2) and an older leaf nearing senescence (leaf 8) (McCormick *et al*., 2006 [Chapter 3]), were compared to non-

defoliated plants (controls) over time. Furthermore, the carbon allocation patterns in the culm of a ^{14}C label supplied to these leaves following 27 d of partial defoliation were examined. This work demonstrated the ability of sink demand to determine source leaf photosynthetic capacity, leaf carbon partitioning and the onset of leaf senescence in sugarcane. Decreased hexose levels observed in leaves of defoliated plants indicated that a hexose-mediated signal may play a role in triggering leaf senescence in sugarcane.

7.3 Materials and methods

7.3.1 Plant material

A 5 x 15 m plot of twelve-month-old field-grown *Saccharum* spp. (L.) hybrid *cv.* N19 (N19), cultivated at Mount Edgecombe, KwaZulu-Natal (SASRI), South Africa (29° 42'S, 31° 2' E, 96 m), was used in this study during March, 2007. The plot was located on a north-east facing slope with a gradient of *ca.* 10°.

7.3.2 Plant treatment

Sugarcane plants were randomly selected for defoliation or as controls within two rows (15 m) of the selected plot. To minimise mutual shading effects, every second row of cane was cut-back, as well as every second stool within the remaining rows. The plot was irrigated (26 mm) a week prior to the commencement of the experiment and every week at the same rate for the duration of the investigation. The plot was not irrigated during the second week due to a substantial rainfall event (57 mm). Plants (n=20) were completely defoliated except for the leaf prior to the first fully formed dewlap (leaf 2) and leaf 8, as described by McCormick *et al*. (2006) (Chapter 3). During the experiment, leaf growth in defoliated plants was restricted by trimming every week to limit the available photosynthetic source material to these two leaves, however the central leaf bundle was kept intact, so as not to disturb the sink represented by this region. Leaf 2 and leaf 8 were marked on a set of intact control plants (n=20). Light conditions were measured regularly throughout the experiment using a LI-6400 portable photosystem unit (LI-COR Biosciences Inc., Nebraska, USA) to ensure that leaf 2 and 8 from control and treated plants received similar levels of light exposure. Plants were harvested (n=5) for sugar

analysis every 8 d at 14h00. When harvested, leaf and separated internode samples were immediately frozen in liquid nitrogen (–196° C) and then milled in an A11 Basic Analysis Mill (IKA®, Staufen, Germany) and stored at –80°C until analysis.

7.3.3 Sugar determination

Approximately 100 mg powdered sample tissue was incubated overnight in 10 volumes of sugar extraction buffer containing 30 mM HEPES (pH 7. 8), 6 mM $MgCl_2$ and ethanol 70% (v/v) at 70°C. Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic coupling assay as described previously (McCormick *et al*., 2006 [Chapter 3]). The phosphorylation of glucose by hexokinase/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Roche, Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5.3.1.9) (Roche) was quantified by following the reduction of $NADP^+$ to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader (Biotek Instrument, Inc., Vermont, USA) using KC4 software (Biotek Instrument, Inc), respectively.

7.3.4 $^{14}CO_2$ labelling

The influence of defoliation on carbon allocation after 27 d was measured by supplying leaf 2 or 8 (n=6) with $^{14}CO_2$ using a protocol modified from Hartt *et al.* (1963). A portion of leaf (5 x 20 cm) weighing approximately 5 g was sealed in a cylindrical glass tube (10 dm^3) containing 50 µl $NaH^{14}CO_3$ (specific activity, 60 mCi $mmol^{-1}$, Amersham Biosciences, UK) to which 1 ml 10% (v/v) lactic acid was added to release $^{14}CO_2$. The glass tubes were initially covered in a black bag (5 min) to ensure equilibration of released $^{14}CO_2$ and even distribution of uptake over the leaf surface. After 45 min, tubes were removed and a leaf disc (*ca.* 100 mg) of the labelled region of leaf 2 or 8 was excised and stored in liquid nitrogen. The plants were harvested 24 h after $^{14}CO_2$ supply and tissue samples milled in an A11 Basic Analysis Mill and incubated overnight in twenty volumes of sugar extraction buffer made from 30 mM HEPES (pH 7. 8), 6 mM $MgCl_2$ and ethanol 70% (v/v) at 70°C. The radioactivity in the 70% (v/v) alcohol-soluble component was measured with a Tri-Carb Liquid Scintillation Analyzer (Packard, Milford, MA, USA) using Ultima Gold™ XR (Packard).

7.3.5 Gas exchange and fluorescence determinations

Every 5 d, for a period of 25 d, gas exchange measurements were made on 2 cm^2 portions of leaf tissue using a LI-6400 portable photosystem. Light was provided by a red/blue LED light source (LI-COR Biosciences Inc.) at photon irradiance of 2 000 µmol m^{-2} s^{-1}. All leaf measurements were taken under ambient CO_2 conditions (370 µmol mol^{-1}) at a constant leaf temperature of 28°C. Gas exchange variables measured included photosynthetic assimilation (*A*), transpiration rate (*E*), stomatal conductance (G_s), intercellular CO_2 (C_i) and leaf temperature at ambient CO_2 (370 µmol mol^{-1}).

After 28 d, the response of *A* to C_i (*A*:C_i) was measured for leaf 2 and leaf 8 of defoliated and control plants (n=4) by varying the external CO_2 concentration from 0 to 1 000 µmol mol^{-1} under a constant photosynthetically active radiation (PAR) of 2 000 µmol m^{-2} s^{-1}. An equation $A = a\left(1 - e^{(-bC_i)}\right) - c$ was fitted to the *A*:C_i data using least squares. The portion of the curve where the slope approaches zero due to limitation in the supply of substrate (ribulose-1,5-bisphosphate), which is equivalent to the CO_2- and light-saturated photosynthetic rate (J_{max}) (Lawlor, 1987), was calculated from this equation (a, J_{max}; b, curvature parameter; c, dark respiration (R_d)). Linear regression was performed on the data between a C_i of 0 and 200 µmol mol^{-1} to determine the efficiency of carboxylation (*CE*; Lawlor, 1987). The assimilation rate in the absence of stomatal limitations (A_a) was as calculated as *A* interpolated from the response curve at C_i = 380 µmol mol^{-1}.

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0.8 s, 6 000 µmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm') at varying external CO_2 concentrations (0 - 1 000 µmol mol^{-1}). The electron transport rate (ETR), defined as the actual flux of photons driving photosystem II (PSII) was calculated from $ETR = \left(\frac{Fm' - Fs}{Fm'}\right) f I \alpha_{leaf}$, where *Fs* is "steady-state" fluorescence (at 2 000 µmol m^{-2} s^{-1}), *Fm'* is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by PSII, typically assumed to be 0.4 for C_4 plant species (Edwards & Baker, 1993), *I* is incident photon flux density and α_{leaf} is leaf absorptance (0.85, LI-COR manual). The component fluorescence variables were derived as described by Maxwell & Johnson (2000).

7.3.6 Statistical analysis

Results were subjected to analysis of variants (ANOVA) to determine the significance of difference between responses to treatments. When ANOVA was performed, Tukey's honest significant difference (HSD) *post-hoc* tests were conducted to determine the differences between the individual treatments (SPSS Ver. 11.5, SPSS Inc., Illinois, USA). SPSS was also used to calculate the Pearson's correlation coefficients for correlation analyses.

7.4 Results

7.4.1 Changes in sugar levels of defoliated and untreated plants

Hexose levels in leaves 2 and 8 of partially defoliated plants decreased significantly (36% and 48% decrease in leaves 2 and 8, respectively) compared to corresponding leaves of untreated controls over the duration of the treatment (Fig. 7.1). Leaf sucrose concentrations were, on average, slightly higher in leaves of partially defoliated plants after 12 d, but only significantly so at 20 d and 28 d for leaf 2 and 8, respectively.

In both partially defoliated and controls plants, culm sugar concentrations followed similar patterns, with older internodes characterised by increased sucrose concentrations (Fig. 7.2). During the treatment period, changes in culm sugar concentrations differed depending on internode maturity. Immature internodes (internodes 3 – 6) exhibited a small, but significant decline in sucrose (Fig. 7.3). Conversely, sucrose concentrations in mature internodes (internodes 7 – 10) increased over time, but only significantly so in control plants. This resulted in defoliated plants accumulating significantly lower culm sucrose concentrations during the treatment period (Fig. 7.2). Mature internodes of control plants were characterised by an overall decline in hexose concentrations (Fig. 7.3). However, this trend was not as clearly apparent in the partially defoliated plants, in which no significant decrease in hexose levels was observed over time.

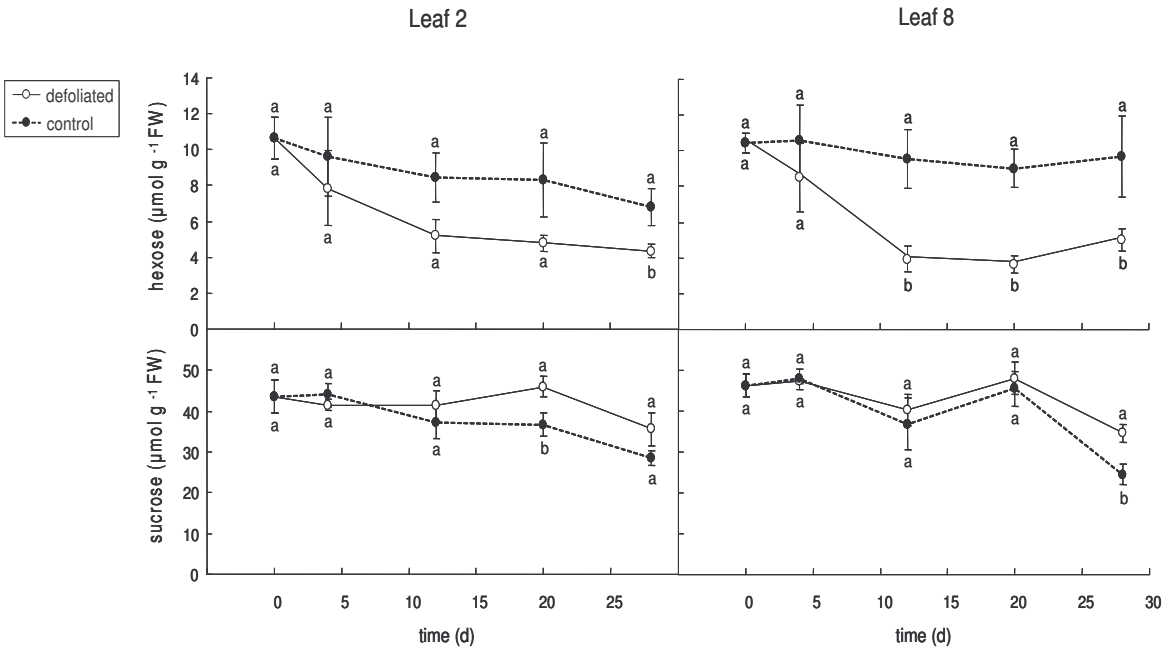

Fig. 7.1. Hexose and sucrose (µmol g^{-1} FW) concentrations for leaf 2 and 8 from field-grown sugarcane plants kept partially defoliated or untreated (controls) for a 28 d period (n=5). Letters above the SE bars indicate whether defoliation had a significant influence compared to controls ($P<0.05$) as determined by ANOVA.

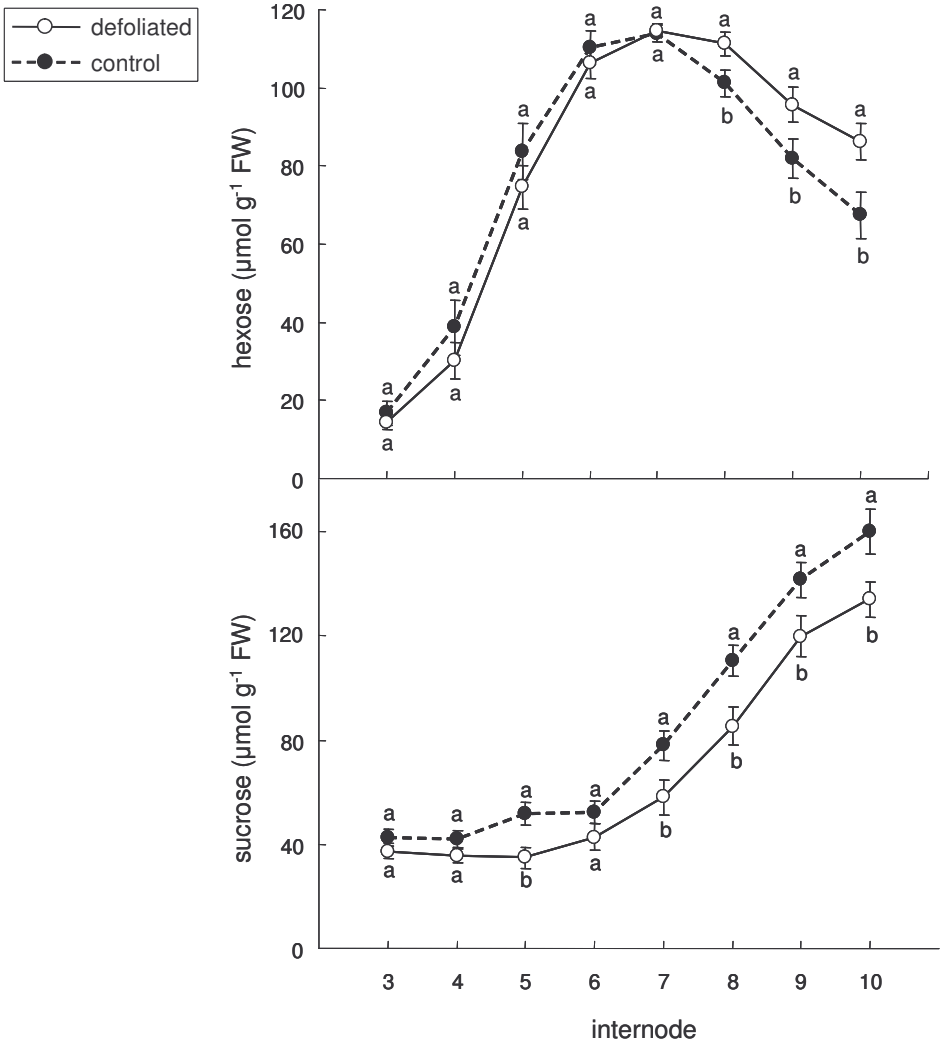

Fig. 7.2. Sugar distribution patterns for hexose and sucrose (µmol g^{-1} FW) in internodes 3 – 10 of field-grown sugarcane plants over a 28 d period. Plants were either defoliated except for leaf 2 and 8 or untreated (controls) (n=5). Values represent the average concentration (n=5) within each internode over four time points (4, 12, 20 and 28 d). Letters above the SE bars indicate whether defoliation had a significant influence compared to controls ($P<0.05$) as determined by ANOVA.

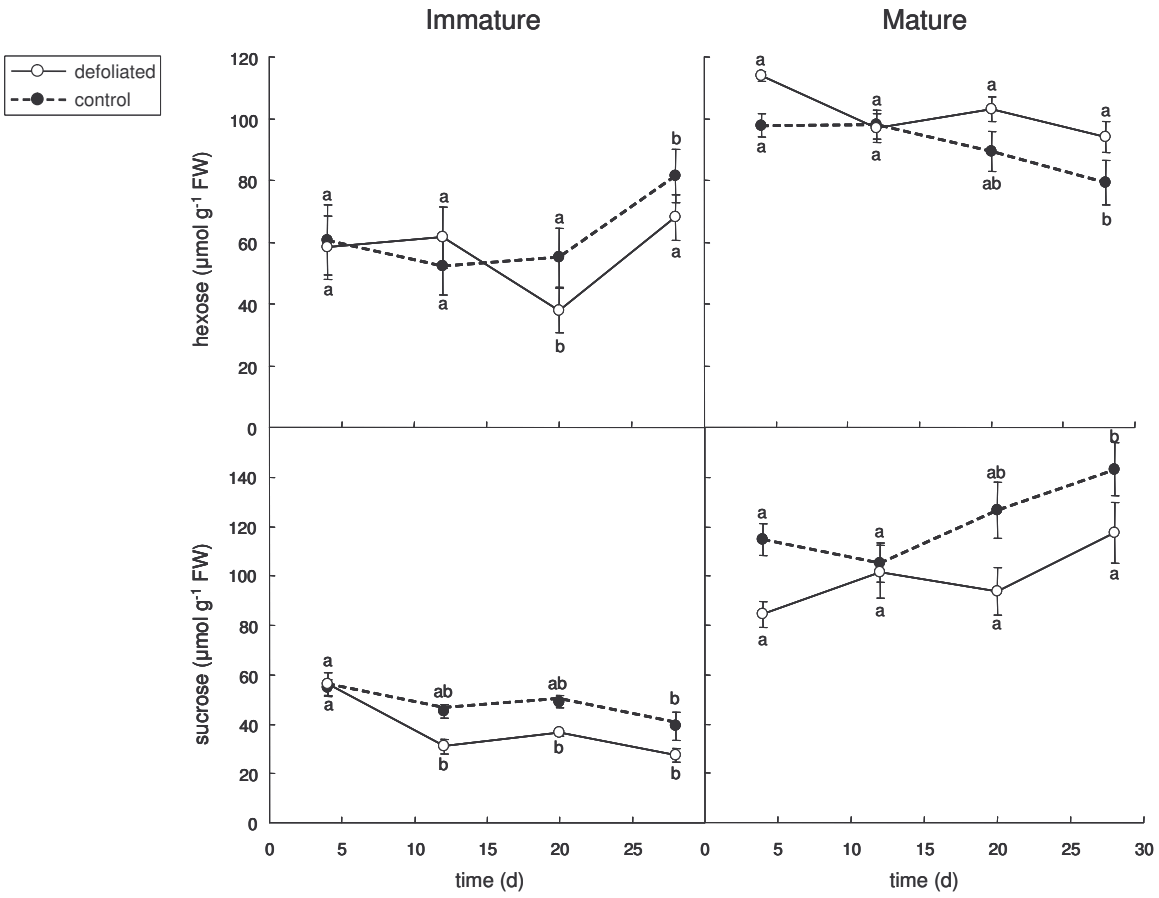

Fig. 7.3. Average hexose and sucrose (µmol g^{-1} FW) concentrations of immature (3 – 6) or mature (7 – 10) internodes for partially defoliated or untreated (control) field-grown sugarcane plants over time (28 d). Treatment values are the mean ± SE of four internodes (n=20) and are followed by letters indicating whether a significant change ($P<0.05$) was observed over time for partially defoliated or control plants, as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests.

7.4.2 Effects of partial defoliation on ^{14}C partitioning

The levels of fixed ^{14}C detected in samples of leaves 2 and 8 immediately after the 45 min feeding period were statistically indistinguishable between the control and partially defoliated sample groups (leaf 2 and 8) (Table 7.1). However, following the 24 h chase period the amount of ^{14}C in labelled leaf 2 and 8 of partially defoliated plants was significantly less than in similar labelled leaves of untreated plants.

In all plants, carbon allocation from leaf 2 and 8 to mature internodes was higher than to immature internodes, indicating that larger sinks acquire more assimilate per unit mass (Table 7.1). Carbon partitioning to immature internodes was higher in both labelled leaf 2 sample groups (control and defoliated) compared to the labelled leaf 8 control group. However, a significant increase in allocation of ^{14}C from leaf 2 to older internodes (9 and 10) occurred in partially defoliated plants. In contrast, ^{14}C from leaf 8 of defoliated plants exhibited significant increases in acropetal translocation to immature internodes 3, 4, and 5 (Table 7.1).

Table 7.1. Incorporation and distribution of a ^{14}C label in field-grown sugarcane plants that were either untreated or partially defoliated (not leaf 2 and 8) for 28 d. Plants were supplied with 100 µCi $^{14}CO_2$ to leaf 2 (top) or 8 (bottom) followed by a 24 h chase period. The means ± standard errors (n=6) are followed by letters indicating for each tissue type whether the treatments had a significant influence (P<0.05) as determined by ANOVA.

	Control	Defoliated
Leaf 2		
Leaf 2* (KBq g^{-1} FW)	468 ± 71 a	531 ± 55 a
Leaf 2	24.2 ± 2.5 a	7.3 ± 0.7 b
Leaf 8	0.3 ± 0.01 a	0.02 ± 0.0 b
Internode 3 (KBq int^{-1} FW)	0.6 ± 0.1 a	0.5 ± 0.2 a
Internode 4	1.5 ± 0.1 a	1.9 ± 0.7 a
Internode 5	4.2 ± 0.3 a	3.1 ± 0.8 a
Internode 6	5.9 ± 0.5 a	6.4 ± 0.7 a
Internode 7	10.4 ± 2.1 a	12 ± 1.3 a
Internode 8	12.4 ± 1.7 a	15.9 ± 4.8 a
Internode 9	9.9 ± 0.6 a	14.2 ± 0.7 b
Internode 10	7.3 ± 1.5 a	17.8 ± 3.4 b
Leaf 8		
Leaf 8* (KBq g^{-1} FW)	599 ± 54 a	472 ± 98 a
Leaf 2	0.02 ± 0.0 a	0.07 ± 0.0 a
Leaf 8	24.1 ± 0.5 a	12.8 ± 1.6 b
Internode 3 (KBq int^{-1} FW)	0.2 ± 0.1 a	0.6 ± 0.1 b
Internode 4	0.4 ± 0.1 a	1.3 ± 0.3 b
Internode 5	1.6 ± 0.6 a	3.5 ± 0.2 b
Internode 6	6.9 ± 2 a	8.5 ± 1.7 a
Internode 7	17.5 ± 5.3 a	23.1 ± 6.6 a
Internode 8	20 ± 3.8 a	26 ± 0.3 a
Internode 9	16.8 ± 2.5 a	21.5 ± 3.9 a
Internode 10	21.6 ± 1.6 a	20.1 ± 2.5 a

Leaf 2* and 8* samples taken directly after labelling.

FW, fresh weight.

7.4.3 Changes in source leaf photosynthesis

Photosynthetic gas exchange characteristics and leaf chlorophyll fluorescence activities were determined on leaves from partially defoliated plants and untreated controls plants (Fig. 7.4). Both leaf 2 and 8 from partially defoliated plants exhibited increased *A* (51% and 84% increase in leaf 2 and 8, respectively) and ETR (32% and 85% increase in leaf

2 and 8, respectively) over time compared to controls (Fig. 7.4). Conversely, *A* and ETR declined over time in leaf 8 of control plants, while leaf 2 did not show any significant change. Due to homogeneity of available ambient light to both treatments, the observed increases in photosynthetic rates in partially defoliated plants could not be attributed to a release from canopy shading. Of note is that C_i and G_s increased over the duration of the experiment in all leaves measured, however this increase was generally more prominent in leaves from defoliated plants. Furthermore, several of the gas exchange variables derived from A:C_i curves, including J_{max} and *CE*, measured after 28 d of defoliation were significantly higher in both leaf 2 and 8 compared to the same leaves on controls plants (Table 7.2). However, exceptions for this trend were C_i (measured at ambient CO_2) and R_d, which were not significantly different between leaf treatments (Table 7.2).

7.4.4 Correlations between sugars and photosynthesis

In both control and partially defoliated plants, hexose and sucrose concentrations in mature culm were negatively correlated, but not in immature culm (Table 7.3). Photosynthetic rates in leaf 2 and leaf 8 of control plants exhibited contrasting correlations with sucrose and hexose concentrations measured in immature and mature culm, respectively. Leaf sucrose concentrations were negatively correlated with *A* in control leaf 2, and positively correlated in control leaf 8. Notably, no links were observed between photosynthetic rates and leaf hexose concentrations in control plants.

The partial defoliation treatment resulted in a negative correlation between photosynthetic rates and leaf hexose concentrations in both leaf 2 and 8, while no correlation was found with leaf sucrose (Table 7.3). Although photosynthetic rates in leaf 8 were negatively correlated with hexose in immature culm, no other culm correlations were found for this leaf.

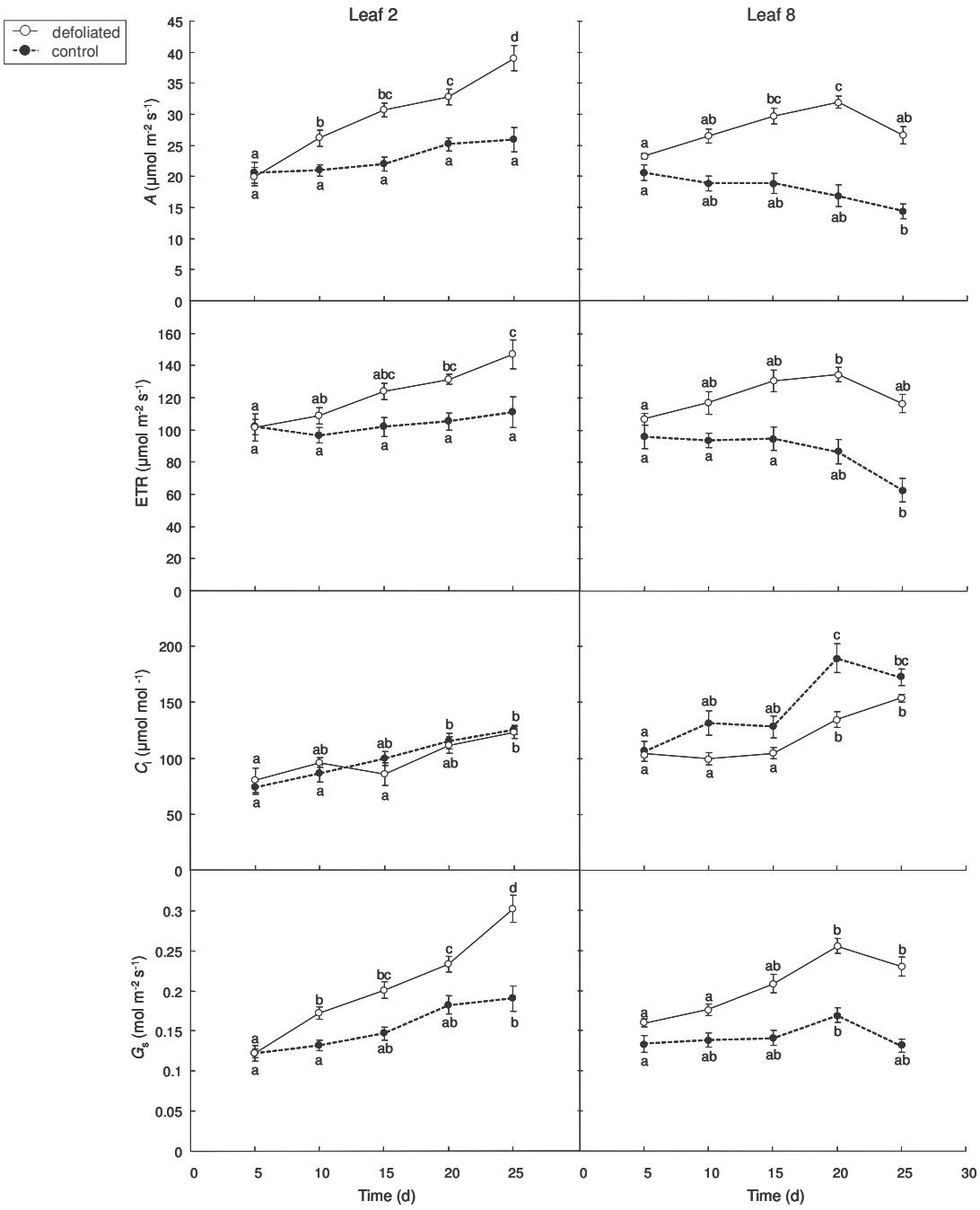

Fig. 7.4. Change in gas exchange variables of leaf 2 and 8 of partially defoliated and ntreated (control) field-grown sugarcane plants: assimilation rate (A), electron transport rate (ETR), intercellular CO_2 concentration (C_i) and stomatal conductance (G_s). Measurements were performed over a period of 28 d at an ambient RH of 42.6% ± 1.7 (mean ± SE) and an irradiance of 2000 µmol m^{-2} s^{-1}. Treatment values are the mean ± SE (n=5) and are followed by letters indicating, for leaf 2 and 8, whether a significant change ($P<0.05$) was observed over time in leaves of partially defoliated or control plants, as determined by ANOVA followed by Tukey's honest significant difference (HSD) tests.

Table 7.2. Variables from A:C_i curves based on gas exchange analysis and leaf fluorescence of leaf 2 and 8 from field-grown sugarcane plants partially defoliated for 28 d or untreated controls: substrate supply limited assimilation (J_{max}), dark respiration (R_d), carboxylation efficiency (CE), photosynthetic rate in the presence (A_i) and absence of stomatal limitation (A_a), intercellular CO_2 concentration at ambient CO_2 (C_i at C_a = 370) and electron transport rate (ETR) at C_a = 370. Measurements were performed at an ambient RH of 40.9% ± 2.2 (mean ± SE) and an irradiance of 2000 µmol m^{-2} s^{-1}. The treatment values are the mean ± SE (n=5) and are followed by letters indicating whether partial defoliation had a significant influence (P<0.05), as determined by ANOVA.

	Leaf 2		Leaf 8	
	Control	**Defoliated**	**Control**	**Defoliated**
J_{max} (µmol m^2 s^{-1})	35.9 ± 4.4 a	44.3 ± 2.7 b	24.8 ± 2.6 a	35.1 ± 2.5 b
R_d (µmol m^2 s^{-1})	4 ± 0.6 a	3.4 ± 0.3 a	2.8 ± 0.2 a	3.4 ± 1.2 a
CE (mmol m^2 s^{-1})	220.3 ± 47 a	263 ± 10 a	100.1 ± 7 a	185.5 ± 16 b
A_a (µmol m^2 s^{-1})	29.3 ± 4.2 a	40.5 ± 2.3 b	20.9 ± 2 a	31.1 ± 3.3 b
A_i (µmol m^2 s^{-1})	25.7 ± 3.4 a	32 ± 1.3 b	16.6 ± 0.6 a	25.3 ± 3.1 b
C_i at C_a=370 (µmol mol^{-1})	123 ± 15.8 a	133 ± 10.9 a	176 ± 6.9 a	144 ± 22 a
ETR at C_a=370 (µmol mol^{-1})	90.8 ± 12.6 a	140 ± 8.2 b	69.6 ± 10.8 a	104 ± 7.3 b

Table 7.3. Bivariate Pearson's correlation coefficients between sugar concentrations (sucrose, hexose), assimilation rate (A) of leaf 2 and 8 from field-grown sugarcane either untreated controls (left) or partially defoliated except for leaf 2 and leaf 8 (right) over a 28 d period. Immature and mature culm values represent the average sugar concentrations of internodes 3 - 6 and 7-10, respectively, for each treatment. Significance levels (P) are reported for the Pearson's correlation coefficients (in brackets).

	Control			**Defoliated**		
	Leaf 2	**Immature Culm**	**Mature Culm**	**Leaf 2**	**Immature Culm**	**Mature Culm**
Sucrose:Hexose			-.89 (0.00)			-.93 (0.00)
Sucrose:*A*	-.55 (0.01)	-.52 (0.01)	.55 (0.01)		-.73 (0.00)	.48 (0.02)
Hexose:*A*		.47 (0.04)	-.66 (0.01)	-.48 (0.03)		-.47 (0.03)
	Leaf 8			**Leaf 8**		
Sucrose:A	.57 (0.01)	.59 (0.01)	-.66 (0.00)			
Hexose:*A*		-.56 (0.01)	.69 (0.01)	-.51 (0.02)	-.58 (0.01)	

7.5 Discussion

7.5.1 Partial defoliation effected culm sucrose status and carbon partitioning

Manipulations of the source-sink balance though a partial defoliation treatment resulted in reduced rates of culm sucrose accumulation during the 28 d ripening period (Fig. 7.2, 7.3). Changes in culm sucrose content were comparable to those observed in a previous study where the source-sink balance was manipulated by shading all leaves but one, resulting in only a single leaf for source supply (McCormick *et al*., 2006 [Chapter 3]). Previously, partial defoliation in sugarcane (i.e. removal of half the leaves) has been shown to not have a long-term negative effect on the sucrose content (Pammenter & Allison, 2002; Gutiérrez-Miceli *et al*., 2004). The significant changes in culm sucrose levels observed in this study may be a result of the intensity of the defoliation treatment.

The defoliation treatment resulted in significant changes in leaf assimilate partitioning. In control plants, ^{14}C analyses confirmed that sugarcane leaves export assimilate primarily in a basipetal direction (MacDonald, 2000), with leaf 2 allocating more carbon to immature internodes when compared to leaf 8 (Table 7.1). Leaf 8 was characterised by significantly increased partitioning of carbon to young culm tissues, likely as a response to increased demand from immature sink tissues due to reduced supply from other sources. However, neither leaf 2 nor 8 exhibited a decrease in the supply of carbon to mature internodes, with leaf 2 showing significantly increased basipetal partitioning compared to control (Table 7.1). These data suggest that carbon supply to the active sinks (i.e. leaf bundle and roots) was maintained during partial defoliation treatment, possibly at the expense of allocation to culm sucrose accumulation.

In the partially defoliated plants, both leaf 2 and 8 had significantly reduced levels of ^{14}C label after 24 hr compared to controls (Table 7.1), indicating an increase in sucrose export for source leaves of plants with increased sink demand. Thus, although the destination of assimilate from source leaves is typically linked to the location of the leaf (MacDonald, 2000), the current study demonstrates that sugarcane phloem loading and transport system is capable of rapidly adapting to changing sink demand and priority, similar to C_3 species (Minchin *et al*., 2002; Vaughn *et al*., 2002). In sugarcane, the export of carbon at the source and import at the sink is a current and active area of research (Walsh *et al*., 2005; Rae *et al*., 2005a). Unlike C_3 species, C_4 plants primarily

produce sucrose in the mesophyll (Lunn & Furbank, 1997; 1999), thus exported assimilate must additionally pass through the bundle sheath cells to be loaded into the phloem. Phloem loading and unloading appear to involve an apoplastic step in sugarcane (Walsh et al. 2005; Rae *et al*., 2005a), suggesting that the activity of sugar transporters may be crucial in regulating the sugarcane source-sink relationship (Rae *et al*., 2005b).

7.5.2 Increased sink demand resulted in significantly increased photosynthetic activity

Partial defoliation resulted in significant changes in gas exchange characteristics and fluorescence in the remaining source leaves (Fig. 7.4, Table 7.2). Based on the observed reductions in culm sugar content (Fig. 7.2) and changes in carbon partitioning (Table 7.1), the increases in A, ETR, J_{max} and CE in leaves from partially defoliated plants are likely a result of increased assimilate demand from sink tissues (Ho, 1992; Wardlaw, 1990; McCormick *et al*., 2006 [Chapter 3]). Notably, changes in C_i and G_s were not as pronounced as those observed for A and ETR (Fig. 7.4), suggesting that photosynthesis in defoliated plants was up-regulated through biochemical modulation, rather than simply through control of stomata. Culm sucrose accumulation in partially defoliated plants was reduced compared to controls (Fig. 7.2, 7.3), which indicates that the observed increase in photosynthetic activity in leaf 2 and 8 was likely to facilitate, not only increased culm demand, but also demand from additional sinks previously supplied by excised leaves (e.g. roots, shoot meristematic region).

In the N19 sugarcane hybrid cultivar used in the current study, leaf senescence commenced between leaf 8 and 10, when the adjacent internode sink exceeded 120 – 150 µmol g^{-1} FW sucrose (Fig. 7.2). The decline of photosynthetic activity in leaf 8 of control plants was negatively correlated with mature culm sucrose concentrations (Table 7.3), indicating that the culm ripening process, and consequent accumulation of sucrose, resulted in a negative feedback down-regulation of leaf photosynthetic rates. In a recent study, where transgenic sugarcane plants were made to produce an additional sucrose isomer, source leaves were characterised by increased photosynthetic rates and enhanced sucrose loading rates, while leaf senescence was delayed by 15 – 20 days (Wu & Birch, 2007). This suggests that sink demand, rather than sugar status per se, mediates the source-sink relationship in sugarcane, and furthermore, receives some priority during the process of leaf senescence in sugarcane.

Although the physiological decline in leaf 8 activity observed in the current study was associated with accumulation of culm sucrose, significant changes in culm hexose status were also measured in control plants. In sugarcane culm tissues, hexose levels have been shown to peak in young maturing internodes, but then subside, while sucrose concentrations increase as the internodes get older (Whittaker & Botha, 1997; McCormick *et al*., 2006 [Chapter 3]). This pattern was observed in the current study (Fig. 7.2, 7.3), however further correlations were observed between leaf age, photosynthetic rates and culm sugar concentrations (Table 7.3). In mature internodal tissues, the negative correlation between culm hexose and sucrose concentrations indicates that the balance between these sugars may be tightly controlled (Table 7.3), a phenomenon that has been observed previously (McCormick *et al*., 2006 [Chapter 3]). The sucrose: hexose ratio is an important metabolic signal which affects almost every aspect of plant development, including programmed cell death (Wobus & Weber, 1999; Koch, 2004). Notably, in the current study culm hexose concentrations did not significantly decrease in mature internodes of partially defoliated plants during the culm ripening period (Fig. 7.3). These results suggest that the sensing mechanisms signaling the decline of physiological activity in older leaves may result not only from the accumulation of culm sucrose, but also the changing sucrose: hexose ratio (Paul & Foyer, 2001; Iglesias *et al*., 2002; Koch, 2004).

7.5.3 The role of sugars in mediating sink-dependent changes in leaf physiological status

Aside from a slight decline in leaf 8 sucrose in control plants after 28 d, leaf sucrose levels were similar between treatments during the experiment (Fig. 7.1). However, both leaf 2 and 8 from partially defoliated plants were characterised by a decrease in hexose concentrations compared to control plants over time (Fig. 7.1). A significant negative correlation between leaf hexose concentrations and *A* in partially defoliated plants provided evidence for the involvement of hexose in facilitating the regulatory signal between sink demand and photosynthetic activity (Table 7.3) (Pego *et al*., 2000; McCormick *et al*., 2006 [Chapter 3]). This relationship was not observed in control plants, suggesting that, in sugarcane, leaf hexose concentrations are carefully maintained under normal conditions. In plants, hexose appears to have extensive interactions with several key source regulatory mechanisms. Increases in leaf hexose

has been shown to directly affect the expression of enzymes related to sucrose and starch synthesis (Koch, 1996), sugar transporters (Chiou & Bush, 1998) and photosynthesis (Sheen, 1990; Krapp *et al*., 1993; Krapp & Stitt, 1995).

More recently, the role of sugars in integrating environmental signals during the regulation of leaf senescence has been highlighted (Wingler *et al*., 2006). The accumulation of sugars in non-senescent leaves, through sugar feeding or cold-girdling treatments, has been shown to lead to decreased photosynthetic rates and chlorophyll content, and down-regulation of transcript expression and activities of enzymes related to photosynthesis (von Schaewen *et al*., 1990; Krapp *et al*., 1991; 1993; Krapp & Stitt; 1995). However, the precise involvement of sugar-mediated repression of genes and enzymes in the regulation of natural senescence is less clear (Feller & Fischer, 1994), as senescence is also co-regulated by several additional factors, including the leaf carbon: nitrogen ratio (Masclaux *et al*., 2000; Wingler *et al*., 2004), light (Dijkwel *et al*., 1997) and plant growth regulators (Jang *et al*., 1997; Wingler *et al*., 1998; Pourtau *et al*., 2004). In evergreen broad leaf species, leaf age has further been implicated as a possible factor affecting physiological decline as older leaves are characterised by increased cell wall thickening and intracellar CO_2 diffusion limitations (Damesin *et al*., 1998; Miyazawa *et al*., 2003). The interactions between leaf sugar signaling, sink demand, and these additional factors, remain to be explored in sugarcane. However, results from the current study suggest that hexose play an important role in co-regulating several leaf systems in sugarcane, including photosynthesis, phloem loading, sugar signaling and senescence.

7.5.4 Potential for influencing source-sink relations in sugarcane

Improved sucrose yields through artificial ripening have been attributed to a better understanding of the source-sink mechanisms (Inman-Bamber & Smith, 2005). However, results following water stress or application of chemical ripeners have indicated that ripening treatments manipulate the partitioning of sink assimilate, and not source photosynthetic activity (Inman-Bamber, 2004; Inman-Bamber & Smith, 2005). Similarly, increased culm sucrose content following the practice of ‘topping’, an artificial ripening technique where the entire leaf bundle is removed, is likely due to the lack of carbon demand from the absent meristematic sink. Thus, current techniques to increase sink sucrose only focus on one half of the system, and do not engage the supply pathway of the primary carbon source leaf.

Although the current study demonstrates that culm maturation is linked the physiological decline and eventual senescence in mature leaves, results further indicated that mature leaves readily increase the supply of assimilate under conditions of increased sink demand (Fig. 7.4). Renewed focus on the manipulation of regulatory leaf signaling components to increase photosynthetic rates and sustain mature leaf photosynthetic activity may result in further increases in culm sucrose yields. Based on current data and previous work (McCormick *et al*., 2006 [Chapter 3]), a useful starting point should be hexose sensors, such as hexokinase or the trehalose metabolic pathway, which are well documented in C_3 plants (Dai *et al*., 1999; Rolland *et al*., 2002; Rolland *et al*., 2006; Paul, 2007; Ramon & Rolland, 2007).

7.6 Concluding remarks

Photosynthetic rates in sugarcane leaves are determined by the demand for carbon from sink tissues. As leaves and culm mature, the accumulation of culm sucrose typically results in a feedback signal leading to a decline in leaf carbon assimilation and the onset of leaf senescence. However, mature sugarcane leaves retain the resources to significantly increase photoassimilate availability under conditions of increased sink demand. The sink-dependent feedback mechanisms which regulate source photosynthetic activity appear to be linked to leaf hexose status, indicating that the targeting of hexose-mediating sugar signaling systems should inform biotechnological efforts to modify metabolism for the improvement of culm sucrose accumulation.

7.7 References

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Chapter 8:
General Discussion

8.1 Supply and demand: a novel paradigm for the source-sink regulation of carbon accumulation in sugarcane

Almost all higher plant systems can be defined as an integrated organization of photosynthetic carbon sources (i.e. leaves) and non-photosynthetic carbon consuming sink tissues. Plant growth can thus be viewed as a function of the balance between the supply of carbon from the source and carbon demand from sinks. Sugarcane represents a somewhat unique source-sink system, for two reasons: 1) storage of assimilate at exceptionally high concentrations is in the form of sucrose which is an osmotically active solute whereas most plants store insoluble polysaccharides such as starch; and 2) storage occurs in non-specialised parenchyma culm tissue. In order to increase sucrose yields in this crop, sugar industries around the globe have encouraged the development and utilisation of new molecular techniques to act in concert with conventional sugarcane breeding programs (e.g. Carson & Botha 2002; Casu *et al*., 2007; Wu & Birch, 2007). Although much progress has been made in understanding the fate of sucrose in the culm and the mechanisms that regulate sucrose accumulation, little or no progress has been made in improving sucrose accumulation in the culm.

To-date, most efforts to manipulate sucrose concentrations by transgenesis have targeted single genes encoding putative rate-limiting sucrolytic enzymes in the culm (Ma *et al*. 2000; Botha *et al*., 2001; Watt *et al*., 2005; Groenewald & Botha, 2007). However, these attempts have, thus far, met with little success. This may be attributed to increasing the potential for sucrose accumulation in culm tissue to the maximum by conventional breeding (Grof & Campbell, 2001); however, several reports indicate while current sugarcane cultivars appear to accumulate sucrose to maximum levels, the potential exists for substantial further gains (Bull & Glasziou 1963; Moore *et al*., 1997; Wu & Birch, 2007). This suggests that future strategies to increase sugarcane via transgenic manipulation may require a broader understanding of the processes involved in sucrose accumulation (Watt *et al*., 2005).

There are several physiological factors that may limit the accumulation of sucrose in sugarcane: i) leaf photosynthetic rates and carbon partitioning into different pools; ii)

phloem loading in the leaf and unloading in the culm; iii) culm metabolism, including membrane transport, and sucrose partitioning, turnover and re-mobilisation; and iv) developmental constraints, such as duration and timing of maturation (Moore *et al*., 1997). Notably, the active loading and unloading of sucrose from the phloem by sugar transporters are suggested as pivotal rate-limiting processes for sucrose accumulation in sugarcane (Lalonde *et al*., 2003; Walsh *et al*., 2005). However, an additional constraint to crop yields may result from the 'sensitivity' of source photosynthesis to sink demand.

Co-ordination between source leaf photosynthetic rates and culm sink sucrose accumulation has been known for sometime. For example, *Saccharum spontaneum* (L.), a low sucrose accumulator, has a 30% higher photosynthetic rate compared to higher sucrose accumulating *Saccharum* spp. (L.) hybrids (Irvine, 1975). More recently, sugarcane leaf shading studies have shown that the demand for carbon from culm sinks resulted in increased rates of photosynthesis and sucrose export in source tissues (McCormick *et al*., 2006 [Chapter 3]), indicating that photosynthetic rates are typically limited by culm requirements. It has been suggested that sucrose accumulation in sugarcane may be regulated by the demand of sink tissues (Watt *et al*., 2005). Nevertheless, the relationship between source photosynthetic rates and sink demand in sugarcane is not well documented, and several important questions remain to be answered. What are the precise signaling mechanisms whereby the requirements of the sink may be transmitted to the source? How does the source sense these signals? How is this relationship effected between dissimilar cultivars or under different environmental conditions? To address these questions, a paradigm of an integrated supply and demand system should be employed, where an awareness of both source and sink activities is used to understand carbon flux and accumulation processes (Minchin & Lacointe, 2004).

In C_3 plants, there is ample evidence for source response to sink requirement (Basu *et al*., 1999; Iglesias *et al*., 2004; Franck *et al*., 2006), a relationship which has been shown to be regulated by several sugar-sensitive feedback systems (Rolland *et al*., 2006; Paul, 2007). Sugar-induced feedback inhibition of photosynthesis overrides regulation of photosynthesis by light, tissue type, and developmental stage (von Schaewen *et al*., 1990; Krapp *et al*., 1993; Sheen, 1994). Previously, the accumulation of photo-assimilate at the source, through application of sugar feeding or cold-girdling techniques, has been shown to lead to a feedback effect on leaf sucrose metabolism and

photosynthetic activity (Krapp *et al*., 1993; Krapp & Stitt, 1995). Furthermore, a disruption in the 'product export pathway', through the over-expression of a yeast-derived invertase in the cell walls of tobacco leaves, resulted in a negative affect on the phloem loading, which led to an increase in leaf sugar concentrations, photosynthetic inhibition, and a limitation of overall growth (Stitt *et al*., 1991). In addition, removal of sink demand by excising the growing tuber has been shown to lead to decrease photosynthetic rates in potato (Basu *et al*., 1999). A subsequent decline in photosynthetic rates following sugar accumulation in excised and intact sugarcane leaves (Chapter 5 and 6), has further corroborated the existence of sugar-sensing systems in C_4 crops. Together, these studies indicate that, although local sugar-sensing mechanisms are active at the source, the principal intermediate linking the supply and demand pathway is the phloematic transport product, sucrose.

The source-sink relationship between leaf and culm can be thought of as being similar to a factory production line (Fig. 8.1A). Production at the source must be carefully regulated by consumption demand, or else the product will either deplete or accumulate (Hofmeyr & Cornish-Bowden, 2000). Thus, in this model the control of flux through the system is not only regulated by demand, but also by the sensitivity of supply to demand. Studies of glycolysis in yeast have indicated that rate-limiting enzymatic steps do not exist, but rather that the control of flux is shared among all enzymes in the metabolic system (Schaaf *et al*., 1989; Fell, 1996). Furthermore, the control of flux has been shown to be regulated by uptake of glucose into the cell (Galazzo & Bailey, 1990; Bisson *et al*., 1993), suggesting that the ability to sense and receive carbon limits system capacity, and not the individual components within (Hofmeyr & Cornish-Bowden, 2000). It is possible that the import and immobilisation of sucrose to vacuolar storage in culm parenchyma cells may act to as an additional demand component, and furthermore maintain a high sink demand for sucrose, resulting in the extraordinary sucrose yields observed in sugarcane (Fig. 8.1B). In this model, changes in sink activity (i.e. storage and demand) would result in adjustments in the rates of symplastic and apoplastic phloem loading and flow processes (Lalonde *et al*., 2003). This would, in turn, feedback on the source sucrose pool, thus regulating supply.

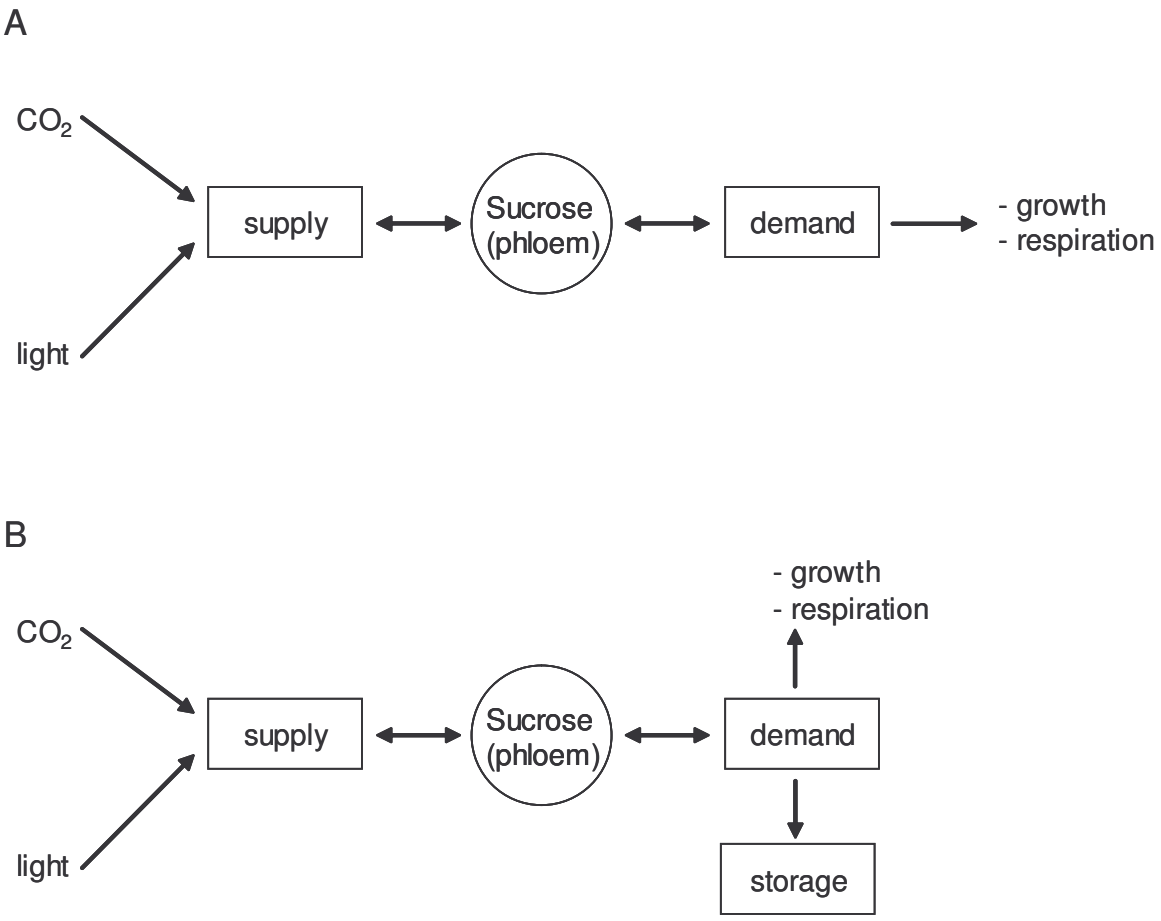

Fig. 8.1. A metabolic supply-demand system in conventional plants (a), and in plants with high carbon accumulation, such as sugarcane (b). Modified from Hofmeyr and Cornish Bowden (2000) (Elsevier, with permission).

If sucrose is considered as the intermediate linking supply and demand processes, a low demand will result in the demand pathway controlling the flux of sucrose through the overall system (Fig. 8.2) (Hofmeyr & Cornish-Bowden, 2000). In sugarcane, it is likely that the demand pathway is typically saturated and exerts little control over the concentration of sucrose. However, feedback control from the demand pathway will limit sucrose production, even though the concentration of sucrose is largely controlled by the supply. In order to increase sucrose concentration in the system a reduction in the elasticity of the supply is required, such that supply is less sensitive to inhibition by its sucrose product (Fig. 8.2).

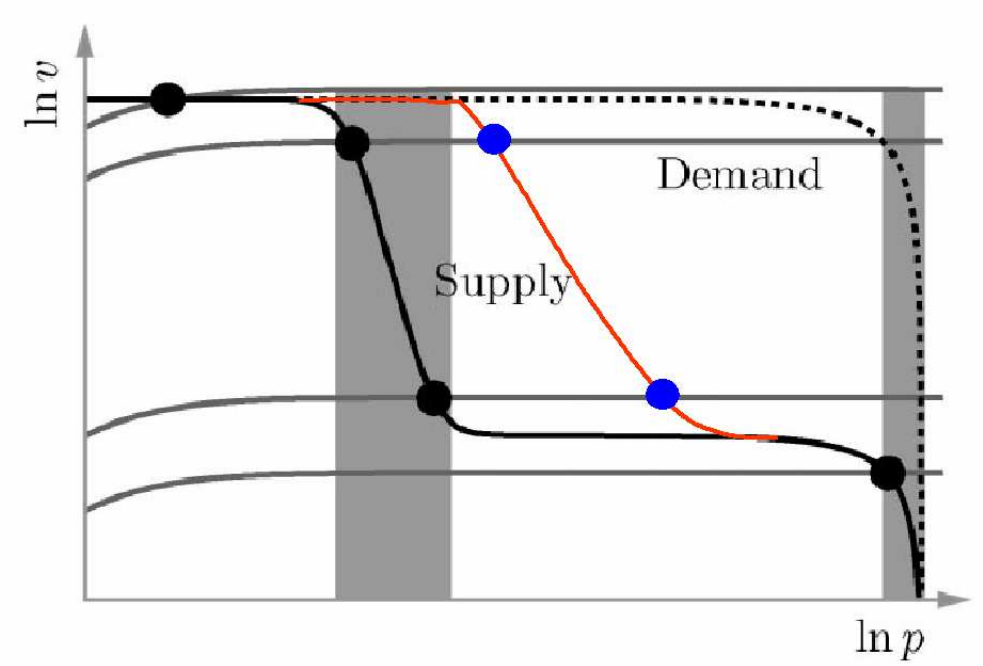

Fig. 8.2. The steady state behaviour of a supply-demand system (modified from Hofmeyr & Cornish Bowden, 2000) (Elsevier, with permission). Black lines indicate inhibition (solid) and no inhibition (dashed) of supply by its product *p*. To decrease inhibition of supply by demand, the elasticity, or insensitivity, of the supply pathway must be increased (red).

In some species, a lowered 'sensitivity' to source sugar accumulation has been demonstrated. Photosynthetic rates in C_4 *Flaveria bidentis* (L.) and C_3 tobacco (*Nicotiana tabacum* L.) remained unaffected when sucrose concentrations were increased 2 – 3 fold through sugar feeding (Furbank *et al*., 1997). Furthermore, spinach and rye grass, can accumulate a greater than 50-fold increase in normal source leaf sucrose concentrations before limiting photosynthesis or growth (Housley & Pollock, 1985; Goldschmidt & Huber, 1992; Krapp & Stitt, 1995). Conversely, tomato and *Arabidopsis* are highly sensitive to leaf sucrose accumulation and are characterized by severe photoinhibition and growth retardation (Goldschmidt & Huber, 1992; Furbank *et al*., 1997). However, several *Arabidopsis* mutants have been identified as insensitive to high glucose (Zhou *et al*., 1998) or sucrose (Pego *et al*., 2000), or both (Laby *et al*., 2000), indicating that inhibition by sugars can be uncoupled from feedback mechanisms regulating growth and development.

Sugarcane source leaves are characterized by the accumulation of sucrose during the diurnal period (Du *et al*., 2000; McCormick *et al*., 2006 [Chapter 3]) and high sucrose concentrations in the culm sink. Based on supply-demand analysis of relationship between source and sink tissues in sugarcane (Hofmeyr & Cornish-Bowden, 2000), it is possible that sugarcane does not accumulate high sucrose to simply supply a high sink demand, but rather that that a decreased sensitivity to the sugar-feedback mechanisms at the source has been inadvertently selected for in sugarcane agriculture. This implies that the rates of carbon accumulation in sugarcane may be dependent, at least partially, on source leaf supply. Moreover, sugarcane leaves appear to have great capacity for adapting to increased demand from culm sinks. Under conditions of limited source supply, through partial shading or defoliation treatments, significant increases in photosynthetic rates have been observed in the remaining source leaves, while mature culm sucrose status was not significantly affected (Pammenter & Allison, 2002, Gutiérrez-Miceli *et al*., 2004, McCormick *et al*., 2006 [Chapter 3]). Furthermore, culm sucrose storage appears to receive priority in the partitioning of assimilate above respiratory and growth requirements (Pammenter & Allison, 2002; Inman-Bamber & Smith, 2005). In immature culm tissues, this phenomenon may be limited by the futile cycling of sugars between the vacuole and cytosol (Whittaker & Botha, 1997). However, as cytosolic and vacuolar invertase activities declines as the culm matures (Zhu *et al*., 1997; Rose & Botha, 2000), the effect of futile cycling on sucrose demand is likely less prominent in older internodes. The primary role of invertases in both source and sink tissues (Goldschmidt & Huber, 1992, Zhu *et al*., 1997; Rose & Botha, 2000), indicates that the hexose products of sucrose may be important in sugar-signaling.

Recently, transgenic sugarcane producing an additional sucrose isomer has demonstrated a doubling of sugar content (Wu & Birch, 2007). Furthermore, source leaf photosynthetic rates were significantly increased and leaf senescence was delayed by 15 – 20 days (Wu & Birch, 2007). This study corroborates the previously observed innate capacity of source leaves to augment supply under conditions of increased sink demand (McCormick *et al*., 2006 [Chapter 3]), and furthermore, indicates that culm tissues in current cultivars possess the capacity to store more sucrose. Thus, the successful manipulation of sucrose yield in sugarcane may not necessitate an increase in culm demand, as has been previously attempted (Ma *et al*. 2000; Botha *et al*., 2001; Groenewald & Botha, 2007), but rather an uncoupling of the signal pathways that mediate negative feedback between source and sink tissues.

In conclusion, current knowledge suggests that up-regulating culm sucrose accumulation will require a down-regulation of signaling mechanisms that limit leaf photosynthesis. The source leaf is an appropriate target due to the considerable malleability of the carbon supply pathway (McCormick *et al*., 2006 [Chapter 3]; Wu & Birch, 2007). As the partitioning of carbon to sucrose or fiber (i.e growth) differs substantially between sugarcane cultivars and environmental conditions, it is likely that changes to signaling mechanisms will require appropriate adjustment. However, if supply is increased and not taken up by storage tissues due to physiological limitations, such as osmotic thresholds or constraints in the uptake process, it is likely that only bigger stalks will suffice to increase sugarcane sucrose yields. Several sugar-signaling mechanisms have been documented (Rolland *et al*., 2006; Paul, 2007) and, thus far, evidence indicates hexokinase (HXK; EC 2.7.1.1), sugar transporters, and the trehalose metabolic pathway as suitable targets for further investigation.

8.2 Concluding remarks

The current study has examined the nature of the relationship between source and sink tissues in sugarcane, with emphasis on the identification of mechanisms that contribute to the modulation of leaf photosynthetic activity. Initial analyses indicated that demand for carbon from culm sinks was closely linked to the rates of photosynthesis and sucrose export in source leaves (Chapters 3 and 7). The observed increases in photosynthetic rates were shown to correlate negatively with concentrations of leaf hexose, but not sucrose. Together, these results highlighted the integrated relationship amongst leaf photosynthetic rates, leaf sugar status, and culm demand. Reduced leaf hexose concentrations were further implicated in delaying the onset of senescence (Chapter 7), indicating that hexoses play a potentially important role in leaf turnover. Additional study, using a macroarray bearing a defined set of carbohydrate metabolism and photosynthesis related ESTs, revealed that the decrease in leaf hexoses correlated to an increase in expression of several genes related to C_4-photosynthesis and sugar transport, including, PEPC, Rubisco, a monosaccharide transporter and a triose phosphate translocator (Chapter 4). A further correlation amongst changes in photosynthesis, leaf hexose concentrations and HXK, provided evidence for the involvement of a putative HXK-dependent sugar-sensing mechanism (Harrington & Bush, 2003).

To explore the relationship between leaf sugar status and photosynthesis, excised sugarcane leaves were fed sucrose or hexoses, which resulted in an increase in leaf sugars and a decrease in leaf photosynthetic rates (Chapter 5). Conversely, when leaf sugar levels were suppressed through shading, photosynthetic rates were significantly increased upon return to light. Notably, a concurrent suppression of leaf hexose concentrations was observed, while no significant differences were found in stomatal conductance between treatments, indicating that photosynthesis was constrained by biochemical restrictions, rather than stomatal limitation. This work provided additional evidence of a direct role for sucrose and hexose in modulating leaf photosynthetic rates (Krapp *et al*., 1993; Chiou & Bush, 1998; Gibson, 2005), which suggests that these sugars are key signal molecules in regulating the carbon supply between source and sink tissues in sugarcane.

The relationship between sugars and photosynthesis in intact sugarcane leaves was further investigated using a leaf cold-girdling technique (Chapters 5 and 6). In these experiments, increased sugar accumulation resulted in a decline in leaf assimilation rates and fluorescent activity over time, in both greenhouse- and field-grown sugarcane. An analysis of gene expression in cold-girdled field-grown sugarcane, using a commercial microarray technique, identified significant changes within several diverse metabolic pathways. These included decreased expression of genes related to photosynthesis, and an increase in expression of genes involved in glycolytic carbon partitioning, cell wall metabolism, P_i cycling, stress response and sugar signaling (Chapter 6). These results were indicative of the integrated feedback role of sugars status on several pathways, possibly via a common phosphorylating signal transduction pathway (Ehness *et al*., 1997; Müller *et al*., 2007). Thus, photosynthesis may be modulated by several different routes in sugarcane. Changes in the expression of P_i-related genes, further suggested that leaf photosynthetic rates were limited by the depletion of cytosolic P_i availability, due to the accumulation of sugars and phosphorylated intermediates (Paul & Pellny, 2003). Although no significant change in HXK gene expression were observed, respective up- and down-regulation of TPP and TPS provided evidence for a T6P-mediated sugar-signaling mechanism in sugarcane leaves.

Notably, limited changes in expression of sucrolysis-related genes implicated in regulating carbon flux, such as SPS and SuSy, were observed from macroarray and

microarray expression data (Chapters 4 and 6). However, in both studies a potential transcriptional regulation of glycolysis mediated through FBPase and GPD was apparent. This suggests that, at the transcript level, the initial uptake and distribution of triose-P into the glycolytic cycle may be more closely modulated than downstream processes. However, based on the reported roles of SPS and SuSy in leaves of other species, subsequent post-translational and allosteric regulation of SPS and SuSy is highly likely. Future research into source-sink relations in sugarcane should not only focus on the identity of putative regulatory genes and gene networks, but also examine the relationship between metabolic events across hierarchical scales (transcript, enzyme and metabolites) and the consequent impact on crop performance (Edmeades *et al*., 2004; Sinclair & Purcell, 2005).

8.3 References

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