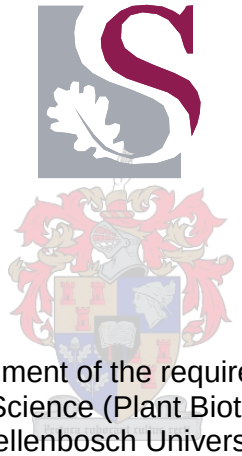
Comparative Analysis of Differential Gene Expression in the Culms of Sorghum

by

Gordon Sandile Ndimande



Thesis presented in fulfilment of the requirements for the degree of
Master of Science (Plant Biotechnology)
Stellenbosch University

December 2007

Supervisor:
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DECLARATION

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

Signature:

Date:

Summary

Despite numerous attempts involving a variety of target genes, the identity of the key regulatory genes of sucrose metabolism in sugarcane is still illusive. To date, genomic research into sucrose accumulation in sugarcane has focused on genes that are expressed in association with stalk development/maturation, with the aim of identifying key regulatory steps in sucrose metabolism. The identification of possible controlling points, however, is complicated by the polyploid nature of sugarcane. Although these studies have yielded extensive annotated gene lists and correlative data, the identity of key regulatory genes remains elusive. A close relative of sugarcane, *Sorghum bicolor,* is diploid, has a small genome size and accumulates sucrose in the stalk parenchyma. The main aim of the work presented in this thesis was to use *S. bicolor* as a model to identify genes that are differentially expressed during sucrose accumulation in the stalk of low and high sucrose genotypes.

In the first part of the study, a macroarray protocol for identification of differentially expressed genes during sorghum development was established. Firstly, the macroarray sensitivity of probe-target hybridisation was optimised with increasing amounts of target DNA i.e. 0.005-0.075 pmol. The hybridisation signal intensity increased as expected with increasing amounts of probe until the hybridisation signals reached maximum levels at 0.05 pmol. As a result, to ensure quantitative cDNA detection, probes were arrayed at 0.05 pmol when 1 µg target cDNA was used. Secondly, intra-array and inter-array membrane reproducibility was found to be high. In addition, the protocol was able to detect species of mRNA at the lowest detection limit tested (0.06%) and permits the detection of an eight-fold variation in transcript levels. The conclusion was therefore that the protocol was reproducible, robust and can reliably detect changes in mRNA levels.

In the second part of the study, sugar accumulation levels in the immature and maturing internodal tissues of sorghum GH1 and SH2 genotypes were compared during the boot and softdough stages. Sugars (i.e. fructose, glucose and sucrose) accumulated differently in the immature and maturing internodes in both sorghum genotypes during the boot and softdough stages, with sucrose being the dominant sugar in both stages. Based on these differences in sugar accumulation patterns, immature and maturing internodal tissues of sorghum genotypes were compared for

differentially expressed genes. A number of genes were found to be significantly differentially expressed during both stages.

In order to validate the reliability of the macroarray analysis, fourteen genes were arbitrarily selected for semi-quantitative RT-PCR. Seven genes (50%) revealed a similar pattern of transcript expression, confirming the macroarray results. The other seven genes, however, showed a different expression trend compared with the macroarrays. In this study, ESTs from rice and sugarcane were used for probing sorghum. The probability of cross-hybridisation between the probes and various isoforms of the homologous sorghum sequences is thus high, potentially leading to the identification of false positives. In addition, variation in expression patterns could have been introduced by technical and biological variation.

Lastly, to verify that changes in the levels of a transcript are also reflected in changes in enzyme activity, seven candidates were tested for enzyme activity. Only three i.e. soluble acid invertase (SAI), sucrose synthase (SuSy) and alcohol dehydrogenase (ADH), out of these seven genes showed enzyme activity levels reflective of the relative transcript expression. We concluded that changes in transcript levels may or may not immediately lead to similar changes in enzyme activity. In addition, enzyme activity may be controlled at transcriptional and at posttranscriptional levels.

In conclusion, sugar accumulation in low (GH1) and high (SH2) sucrose sorghum genotypes is influenced by differences in gene expression. In addition, the power of macroarrays and confirmation with semi-quantitative RT-PCR for identification of differentially expressed genes in sorghum genotypes was demonstrated. Moreover, the transcript and enzyme activity patterns of SAI, SuSy and ADH genes showed expression patterns similar to those of sugarcane during sucrose accumulation. Therefore, using sorghum as a model promises to enhance and refine our understanding of sucrose accumulation in sugarcane.

Opsomming

Ten spyte van veelvuldige pogings is die identiteit van die sleutel regulerende gene in die sukrosemetabolisme van suikerriet steeds onbekend. Tot dusver het genomiese navorsing, met die doel om die sleutel regulatoriese stappe in suikermetabolisme te identifiseer, op gene waarvan die uitdrukking met stingelontwikkeling geassosieer is, gefokus. Ongelukkig word die identifisering van moontlike beheerpunte bemoeilik deur die poliploïede-natuur van suikerriet. Alhoewel hierdie studies breë geenlyste en vergelykende data opgelewer het, is die identeit van sleutel regulatoriese gene steeds onbekend. ‘n Nabye verwant van sukerriet, *Sorghum bicolor*, is ‘n diploied, het ‘n klein genoom en akkumuleer ook sukrose in die stingel parenkiemselle. Die hoofdoel van die werk soos uiteengesit in hierdie tesis, was dus om *S. bicolor* te gebruik as ‘n modelplant om gene, wat differensieel uitgedruk word tydens suikerakkumulering, in die stingel van lae of hoë sukrose sukroseakkumulerende genotipes, te identifiseer.

In die eerste deel van die studie is ‘n makromatriks-protokol vir die identifisering van differensieel uitgedrukte gene ontwikkel. Eerstens is die sensitiwiteit van die makromatriks bevestig deur peiler-teiken hibridisering te optimiseer vir peiler DNA hoeveelhede tussen 0.005 en 0.075 pmol. Soos verwag, het die intensiteit van die hibridiseringsein verhoog soos die hoeveelheid peiler toegeneem het totdat die hibridiseringsein ‘n maksimum vlak by 0.05 pmol peiler bereik het. Peilers is hierna dus teen 0.05 pmol op die matrikse aangebring, wanneer 1 µg teiken cDNA gebruik is, om die kwantitatiewe deteksie van teikens te verseker. Tweedens, is die inter- en intra-membraan herproduseerbaarheid van spesifieke seinintensiteite bevestig. Die protokol was daartoe in staat om mRNA-spesies by die laagste konsentrasie wat getoets is (0.06%) te meet en kon ook ‘n tot agt voudige variasie in transkripsie vlakke akkuraat meet. Die gevolgtrekking was dus dat die protokol, herproduseerbaar en betroubaar, veranderinge in die mRNA vlakke kan meet.

In die tweede deel van die studie is die suikervlakke in die onvolwasse en volwasse internodale weefsels van die sorgum genotipes GH1 en SH2 tydens twee verskillende ontwikkelingstadiums vergelyk. Daar is gevind dat die suikers (fruktose, glukose en sukrose) verskillend in die volwasse en onvolwasse internodes, van beide sorgum genotypes, tydens beide stadiums akkumuleer en sukrose is in al die gevalle die dominerende suiker. Weens hierdie verskille in die suikerakkumuleringspatrone is

die uitdrukking van verskillende gene in hierdie verskillende weefsels ondersoek en ‘n aantal gene is geïdentifiseer, wat beduidend verskillend uitgedruk word.

In ‘n poging om die betroubaarheid van die makromatriksanalises te bevestig, is viertien gene willekeuring geselekteer vir semi-kwantitatiewe RT-PCR analises. Sewe van hierdie gene (50%) het ‘n soortgelyke uitdrukkingspatroon as in die makromatriksanalises getoon, terwyl vyf van hulle nie-beduidende verskille getoon het. Hierdie verskille tussen die makromatriks- en RT-PCR resultate kan moontlik verduidelik word aan die hand van verskille in die spesifisiteit tussen peelers en voorvoerders. Daarmee saam, kan variasie in die uitdrukkingspatrone ook veroorsaak word deur tegniese en/of biologiese variasie.

Laastens, om te bevestig of die waargenome veranderinge in die hoeveelheid boodskapper, aanleiding gee tot ‘n verandering in die hoeveelheid ensiemaktiwiteit, is sewe kandidaatgene se ensiem aktiwiteit bepaal. Slegs drie van die ensieme, oplosbare suur invertase (SAI), sukrose sintase (SuSY) en alkohol dehidrogenase (ADH), se aktiwiteitsvlakke het hul relatiewe boodskappervlakke weerspieël. Hierdie data ondersteun gegewens uit die literatuur wat wys dat die ensiem aktiwiteit ook op die translasionele en post-translasionele vlak beheer kan word.

In opsomming, suikerakkumulering in sorgum genotipes met lae (GH1) en hoë (SH2) sukrosevlakke, word geäsosieer met verskille in geenuitdrukking. Die doeltreffendheid van ‘n kombinasie van makromatriks en semi-kwantitatiewe RT-PCR analyses, vir die identifisering van differensiëel uitgedrukte gene in sorgum, is gedemostreer. Boodskapper-RNA-vlakke en ensiemaktiwiteitspatrone van SAI, SuSy en ADH is soortelyk aan die van suikerriet tydens sukroseakkumulering. Dus, die gebruik van sorgum as ‘n modelplant, om ons kennis van sukroseakkumulering in suikerriet te verbeter, lyk belowend.

FOR THOBILE and LWAZI

"I see education as playing a vital role in personal growth and in institutionalizing a way of life that a people choose as its highest ideal".

Prof. Ezekiel Mphahlele

ACKNOWLEGMENTS

I would like to thank Dr Hennie Groenewald for supervision of this study.
Hennie, your ubuntu in my life will never be forgotten.

Thanks to Prof Jens Kossmann and Dr James Lloyd, for the many discussions we had.

To Dr Paul Hills and Dr James Lloyd, thanks for proof reading, criticising and improving what I wrote.

To Dr Nokwanda Makunga, thanks for listening, helping in many ways and the “field work”.

To all my friends at the IPB who helped and supported me in so many ways.

SASRI, the NRF and Stellenbosch University for financial and other support.

Thanks to my parents and brother. Mama noBaba, nani boMalambule boKhozakhulu boMhlumaye banda baphangalala for your love and encouragement.

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ABBREVIATIONS

pmol	picomoles
µg	microgram
µl	microlitre
°C	degrees Celsius
3 PGA	phosphoglycerate
A_{340}	absorbance at 340 nanometers
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AGPase	adenosine 5`- diphosphate glucose pyrophosphorylase
AI	acid invertase
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary DNA
CWI	cell wall invertase
CV	co-efficient of variation
DEPC	diethyl pyrocarbonate
DTT	1,4-dithiolthreitol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
e.g.	for example
FBPase	fructose-1,6-bisphosphatase
fru-1,6-P	fructose-1,6-bisphosphate
fruc-6-P	fructose-6-phosphate
g	gram
g	relative centrifugal force
Gluc-1-P	glucose-1-phosphate
Gluc-6-P	glucose-6-phosphate
H^+	proton
HEPES	4-(2-hydroxyethyl)-1piperazineethanesulfonic acid

hexose-P	hexose phosphate
HX/G6-PDH	hexokinase/glucose-6-phosphate dehydrogenase
HXK	Hexokinase
HXT	Hexose transporter
kJ	KiloJoule
LB	Luria-Bertani
M	molar
MAFF	Ministry of Agriculture, Forestry and Fisheries
malate DH	malate dehydrogenase
ml	millilitre
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
ng	nanogram
NAD^+	oxidised nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
$NADP^+$	oxidised nicotinamide-adenine phosphate dinucleotide
NADPH	reduced nicotinamide-adenine phosphate dinucleotide
NCBI	National Centre for Biotechnology Information
NI	neutral invertase
OAA	oxaloacetate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
PEP	phenolenolpyruvate
PFK	6-phosphofructokinase
PFP	pyrophosphate-dependent phosphofructokinase
RNA	ribonucleic acid
Rubisco	ribulose bisphosphate carboxylase/ oxygenase
RuBP	ribulose bisphosphate
SAGE	serial analysis of gene expression
SAI	soluble acid invertase
SASRI	South African Sugar Research Institute

SDS	sodium dodecyl sulphate
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
suc-6-P	sucrose-6-phosphate
SuSy	sucrose synthase
SUT	sucrose transporter
TE	Tris EDTA
triose-P	triose phosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-HCl	Tris hydrochloric acid
UDP	uridine diphosphate
UDP-Gluc	uridine diphosphate glucose
UGPase	uridine diphosphate glucose pyrophosphorylase
UV	ultra-violet
V	volt

Chapter 1

GENERAL INTRODUCTION

Sugarcane (*Saccharum* L. spp. hybrids) is one of the most important agricultural crops in the world. This importance is due to its ability to produce cane sugar, i.e. sucrose, at high yields. South Africa is ranked as the thirteenth largest cane sugar producer out of 121 countries (www.sugarcanecrops.com). The refined sucrose is estimated to generate an annual income of approximately six billion Rands and contributes an estimated two billion Rands to the economy in foreign exchange earnings (www.sasa.org.za). To stay competitive in a global commodity market prone to overproduction, the South African industry has to focus on more cost effective production systems. Increasing the sucrose concentration in commercial sugarcane varieties will be one of the most important factors contributing towards improved cost effectiveness. The improvement of sucrose yield per unit cane through scientific research is therefore an essential objective of the local industry (Inman-Bamber *et al*. 2005).

Commercial sugarcane varieties are interspecific hybrids with the estimated biophysiological capability to store up to 62% of the dry weight or 25% of the fresh weight of their stems as sucrose (Bull and Glasziou, 1963; Grof and Campbell, 2001). This estimate is almost double the current commercial varieties' yields. Increases in sucrose yields and the incorporation of important agronomical traits have been achieved traditionally by using crossing and screening methods. However, it seems as if the natural genetic potential has been exhausted as sucrose yields have reached a plateau (Grof and Campbell, 2001; Moore, 2005). Novel approaches, e.g. biotechnological interventions, are therefore needed to break through this apparent yield ceiling (Moore, 1995; Grof and Campbell, 2001; Moore, 2005). Unfortunately, despite a fair amount of research on the primary carbohydrate metabolism of sugarcane, our understanding of sucrose accumulation in the plant is still incomplete (Moore, 1995; 2005). High amounts of sucrose accumulate in the stalk and its

concentration increases as the internodes mature (Rose and Botha, 2000). It has been suggested that sucrose accumulation in sugarcane is regulated at the sink level, where it is subjected to a continuous cycle of degradation and re-synthesis (Whittaker and Botha, 1997; Lingle, 1999). Consequently, studies on sugarcane enzymes are limited to a few that are directly linked to sucrose, i.e. sucrose phosphate synthase (SPS) (EC 2.4.1.14, Botha and Black, 2000); sucrose synthase (SuSy) (EC 2.4.1.13, Botha and Black, 2000; Schäfer *et al.,* 2004); the invertases (EC 3.2.1.26, Rose and Botha, 2000; Bosch *et al*., 2004) and two enzymes of glycolysis: fructokinase (EC 2.7.1.4, Hoepfner and Botha, 2003) and pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90, Whittaker and Botha, 1999; Groenewald and Botha, 2007a). In addition, several attempts to increase sucrose in sugarcane through transgenesis have focused on single genes encoding sucrolytic enzymes (Ma *et al.,* 2000; Botha *et al*., 2001; Groenewald and Botha, 2001; 2007b; Rossouw *et al.* 2007). The mixed success of these studies illustrates the need for continued research to identify key regulatory steps.

Global analyses of expressed sequence tags (ESTs) in immature and maturing sugarcane internodes paint an intricate picture of carbohydrate metabolism during sucrose accumulation (Carson *et al*., 2002; Casu *et al.,* 2003; Casu *et al.,* 2005). The great majority of transcripts identified in these tissues cannot be readily assigned functions associated with sucrose accumulation. Casu *et al.* (2003) found that only 2.1 to 2.4% of the transcripts in sucrose accumulating tissues are known genes in carbohydrate metabolism. These transcripts with putative functions in carbohydrate metabolism include enzymes of sucrose synthesis and cleavage, and enzymes involved in glycolysis and the pentose phosphate pathway. Although, the sucrose concentration increases as the internodes mature, both the genes responsible for synthesis and breakdown of sucrose, i.e. SPS and SuSy, were found to be expressed at very low levels (Carson *et al*., 2002; Casu *et al.,* 2003; Casu *et al.,* 2005). Although SPS enzyme activity was shown to be highest in those internodes where sucrose accumulation

is the highest, no correlation was observed between SuSy (sucrose synthesis direction) enzyme activity and sucrose content (Botha and Black, 2000). These studies have yielded an extensive annotated gene list and correlative data, however, the identity of key regulatory genes remains elusive (Watt *et al*., 2005).

Commercial sugarcane cultivars are interspecific poly-aneuploid hybrids with chromosome numbers usually in excess of 100 (Casu *et al*., 2005). In addition, sugarcane traits are polygenic and/or multi-allelic, and quantitatively inherited. Furthermore, the genome size of commercial cultivars is approximately 3000 Mbp, compared with 750 Mbp for its close relative, sorghum (Dufour *et al*., 1997; Grivet *et al.,* 1994). As a consequence of this extremely large and complex genome, the use of genomic research approaches to sugarcane involves many challenges (Grivet and Arruda, 2002).

Like sugarcane, sucrose is the photoassimilate translocated from the leaves and stored in the stalk of sorghum (Tarpley *et al*., 1994). In addition, as in sugarcane, sucrose is the dominant sugar in the developing and maturing internodes. Unlike sugarcane, sorghum is a diploid crop that has a chromosome number that ranges from 2n = 10-40 (Doggett, 1988). Comparative mapping of sugarcane and sorghum has shown considerable co-linearity and synteny between these genomes, implying conservation in the order of DNA sequences on chromosomes (Dufour *et al*., 1997; Glaszmann *et al*., 1997). In addition, sugarcane and sorghum EST databases show similar patterns of gene expression (Ma *et al.,* 2004). Therefore, the simple diploid genetics of sorghum makes it an attractive model organism for studying the polyploid sugarcane genome (Ming *et al.,* 1998; Asnaghi *et al.,* 2000).

The main aim of this study was to investigate the differential expression of genes in low and high sucrose accumulating sorghum genotypes/tissues at various developmental stages, which could potentially serve as a model system for studying primary carbon metabolism in sugarcane. Two sorghum varieties with

different sugar accumulating abilities were therefore identified and a sensitive and reproducible macroarray protocol, for the identification of differentially expressed genes during sorghum development, was developed. Differential gene expression was confirmed using semi-quantitative PCR analysis and, finally, genes that were confirmed to be differentially expressed were further investigated on protein level.

To conclude, an overview of all the aims of this study is presented in context of the various chapters in which they are dealt with.

Chapter 2*:* Literature review.

Aim To present the relevant background to carbohydrate metabolism in source and sink tissues, with special emphasis on sugarcane.

Chapter 3*:* Establishment of a protocol for transcript profiling in *Sorghum bicolor.*

Aim To develop an in-house protocol that is robust and reproducible for multiple transcript profiling during sorghum development.

Chapter 4: Analysis of gene expression in low and high sucrose accumulating sorghum genotypes.

Aim: To compare the sugar concentrations in the internodal tissues of two sorghum genotypes, at different developmental stages. Secondly, to identify differentially expressed genes and finaly to verify these differences on protein / activity level.

Chapter 5: General discussion and conclusions.

Aim: To critically discuss and integrate the observations of the experimental chapters. To conclude and suggest the potential focus of future research on this topic.

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Chapter 2

LITERATURE REVIEW

2.1 Carbohydrate assimilation in the source tissues of C4 plants

Carbon 4 (C4) plants include a number of economically important crop species such as sugarcane (*Saccharum* L. spp. hybrids), sorghum (*Sorghum bicolour* (L) Moenh) and maize (*Zea mays* L.) (Hatch, 1987). Their economic importance is due to their ability to produce high amounts of photoassimilates and accumulate these as carbohydrates such as sucrose and starch. Accumulation of carbohydrates in the leaves of C4 plants is achieved by two unique photosynthetic cell types; the mesophyll and bundle-sheath cells. Carbon dioxide (CO_2) is initially assimilated in the chloroplasts of leaf mesophyll cells and is carboxylated by phenolenolpyruvate (PEP) carboxylase into the organic acid oxaloacetate (OAA). The OAA is then converted into malate or aspartate, which diffuses through plasmodesmata into the bundle-sheath cells. The C4 plants have three ways to decarboxylate malate or aspartate to generate CO_2 in the bundle-sheath cells, i.e. (1) chloroplastic NADP-malic enzyme that decarboxylates malate into pyruvate, (2) mitochodrial NAD-malic enzyme, and (3) cytosolic phosphoenolpyruvate carboxykinase (Hatch, 1987). The decarboxylated CO_2 is transferred into the Calvin cycle and is refixed by ribulose bisphosphate carboxylase-oxygenase (Rubisco). The products of the Calvin cycle, in the form of triose-phosphate (triose-P), pyruvate or PEP, may return to the mesophyll cells and be used in other biosynthetic pathways, such as starch, lipid, or amino acid biosynthesis in chloroplasts, or sucrose and amino acid synthesis in the cytosol. The C4 plants saturate CO_2 in the Calvin cycle so that it inhibits photorespiration and increases the production of carbohydrates (Lunn and Furbank, 1999).

Sucrose is the primary product of carbon fixation during photosynthesis in the source leaves and the major transported form of carbohydrates to the rest of the plant. Triose-P exported from chloroplast is converted to hexose phosphates (hexose-P), which are in turn converted to sucrose in the cytosol (Winter and

Huber, 2000). Sucrose is synthesised by the action of sucrose-phosphate synthase (SPS), which catalyses the synthesis of sucrose-6-phosphate (suc-6-P) from fructose-6-phosphate (fru-6-P) and uridine diphosphate-glucose (UDP-Glc) (Lunn and Furbank, 1999). Finally sucrose-phosphate phosphatase (SPP) irreversibly hydrolyses suc-6-P to sucrose and phosphate (Lunn and ap Rees, 1990). Sucrose may also be synthesised by sucrose synthase (SuSy) (Geigenberger and Stitt, 1991). There is evidence from feeding experiments with labelled sugars that both pathways contribute to sucrose synthesis (Geigenberger and Stitt, 1993).

2.2 Transport of sucrose to sink tissues

2.2.1 Uploading in source tissues

The synthesised sucrose has two fates; it is either stored in the vacuoles in the leaves or loaded into the phloem sieve elements (Winter and Huber, 2000). Transport of sucrose into the vacuole is believed to be mediated by passive movement and/or active transport (Komor, 2000). Loading of sucrose into the phloem sieve elements may take two routes, either by symplastic movement across the plasmodesmata, and/or by energy-dependent apoplastic movement (Moore, 1995; Hellman *et al.,* 2000; Walsh *et al.,* 2005). Madore *et al*. (1986), using a tracer dye in *Ipomoea tricolour*, demonstrated that sucrose was symplastically loaded into the phloem. It was also shown that sucrose, after reaching the companion cells and sieve elements via apoplastic movement, is loaded into the phloem by a proton-sucrose symporter (Reismeier *et al*., 1994; Lalonde *et al*., 2003). Mutant plants with an insertion in the gene encoding this sucrose transporter were found to be unable to transport sucrose normally (Gottwald *et al*., 2000). However, in the sugarcane leaf, the conducting cells of the phloem are not connected to other cells of the leaf by plasmodesmata, suggesting that loading may occur by apoplastic movement (Robinson-Beers and Evert, 1991). In sugarcane source tissues, loading of sucrose causes the influx of

water and the resulting turgor pressure promotes movement away from the source (Rae *et al.*, 2005). Conversely, unloading sucrose from the phloem causes a reduction in osmotic pressure in sink phloem (van Bel, 2003).

2.2.2 Unloading in sink tissues

Unloading of sucrose from the phloem to the storage parenchyma cell may also occur apoplastically and/or symplastically (Patrick, 1997; Moore, 1995). However, the unloading of the photo-assimilate from the phloem depends on the sink strength of the tissue, and the developmental stage of the plant (Turgeon, 1989). The apoplastic route is necessary in filial and maternal tissue in developing seeds, where there is a symplastic discontinuity between two tissues (Patrick, 1997; Rae *et al.*, 2005). In vegetative tissues unloading of sucrose may proceed either apoplastically or symplastically (Rae *et al*, 2005). In *Solanum tuberosum* L. (potato), a study conducted using carboxyfluorescein dye showed unloading to be predominantly apoplastic in stolons. However, with the onset of tuberisation this switched to the symplastic route (Viola *et al*., 2001). Similarly, using tracer dyes in potato tuber and tomato fruit, it was shown that assimilates are loaded apoplastically (Oparka and Prior, 1988; Patrick, 1997).

Interestingly, loading of sucrose into parenchyma cells in sugarcane is also influenced by the structure of the culm (Walsh *et al*., 2005). Sugarcane stem vascular bundles are surrounded by a layer of thick-walled cells which become progressively lignified and suberised with development (Moore, 1995; Rae *et al*., 2005; Walsh *et al*., 2005). Hawker and Hatch (1965) found high amounts of sugars in the apoplastic space, leading them to conclude that sucrose is loaded directly into the apoplast, with uptake of invert sugars from the apoplast into the storage parenchyma cells. However, the lignified and suberised walls will form a physical barrier for the apoplastic movement of sucrose or invert sugars to the storage parenchyma cells (Welbaum and Meinzer, 1990; Jacobsen

et al., 1992; Walsh *et al*., 2005). Therefore, sucrose cannot reach the storage parenchyma cells from the phloem via the apoplastic route.

Using tracer dyes, plasmodesmata were observed in all cell types in the pathways from phloem to storage parenchyma cell (Welbaum *et al*., 1992; Rae *et al*., 2005). While sucrose movement may not be directly equatable to tracer dyes, it is possible that sucrose is able to move symplastically to the storage parenchyma cells (Rae *et al*., 2005; Walsh *et al*., 2005). The driving force for symplastic transfer from the phloem to the storage parenchyma may be diffusion along a concentration gradient and/or bulk flow by hydrostatic pressure (Komor, 2000). However, in order to maintain a concentration gradient, sucrose in sugarcane may be exported to the apoplast compartment and/or into the vacoule (Moore and Cosgrove, 1991; Walsh *et al*., 2005).

In the sugarcane culm a large proportion of the volume inside the parenchyma cells is occupied by the vacuole. It is thought that a sizeable quantity of the stored sucrose resides in this compartment (Rae *et al*., 2005). Compartmentation of sucrose into the vacuole aids to maintain low concentrations in the cytoplasm, thus promoting movement of sucrose into the parenchyma cells through uptake from the apoplast or symplastic connection (as discussed above).

2.3 Carbohydrate metabolism in sink tissues

Photo-assimilate partitioning is a highly integrated process which involves not only the transport of sugars for growth and development, but also the regulation of gene expression (Koch, 1996). Sugar signals that regulate gene expression act both at the transcriptional and translational levels and depend on the sugar status of the plant (Koch, 1996; Coruzzi and Zhou, 2001). Sugar-modulated genes have direct and indirect roles in sugar metabolism, which suggests that their altered expression may also influence the allocation of sugars to different compartments (Roitsch, 1999; Stitt *et al*., 2002; Koch, 2004). Furthermore, sugar

dependent modulation of gene expression appears to be involved in responses to biotic and abiotic stresses (Gibson, 2005). Koch (1992) showed that feeding plants exogenous sucrose or glucose at different concentrations had an effect on gene expression. Similarly, when *Arabidopsis thaliana* seedlings were fed with exogenous sucrose, a number of genes were shown to be differentially expressed (Gonzali *et al*., 2006).

In the last few years, gene expression has attracted a lot of attention, especially in high sucrose-storing tissues like *S. officinarum* culms, *Beta vulgaris* roots and the fruits of many plant species (Lunn and MacRae, 2003). Insight into the regulation of genes involved in sucrose metabolism during partitioning is not only important for understanding plant growth and development, but is also a prerequisite for the genetic manipulation of source-sink relations in transgenic plants to increase crop yield. The review below will therefore focus on enzymes that are directly involved in sucrose metabolising pathways and which have been implicated in carbon partitioning in sink tissues.

2.3.1 *Sucrose phosphate synthase*

Sucrose synthesis occurs mainly in the cytoplasmic compartment of plant cells. Sucrose phosphate synthase (SPS, EC 2.4.1.14) is involved in sucrose synthesis in both photosynthetic and non-photosynthetic tissues (Huber and Huber, 1996). The synthesis of suc-6-P from Fru-6-P and UDP-Glc by SPS is a freely-reversible reaction, but the rapid conversion of suc-6-P into sucrose by sucrose phosphate phosphatase (SPP, EC 3.1.3.24) renders the SPS reaction irreversible (Lunn and MacRae, 2003). The level of expression of SPS is regulated at the developmental and environmental level and by the sugar status of the plant (Hesse *et al.*, 1995; Huber and Huber, 1996; Winter and Huber, 2000). Exogenous glucose induced for example the expression of SPS mRNA in excised leaves of *Beta vulgaris* (Hesse *et al.* 1995). In the same study, exogenous sucrose slightly repressed the expression of SPS mRNA. In addition,

potato tubers with reduced ADP-Glc pyrophosphorylase (AGPase) activity have high soluble sugar (sucrose and glucose) concentrations and increased SPS mRNA levels (Muller-Rober *et al*., 1992). Klein *et al.* (1993) also observed that when spinach (*Spinacia oleracea* L) plants that have been grown at low irradiance are transferred to high irradiance this results in increased expression of SPS mRNA, followed by a steady increase in the SPS protein and carbon flux into sucrose.

In photosynthetic cells, the activity of SPS can be a limiting factor for *de novo* sucrose synthesis and also photosynthesis (Stitt *et al*., 1988; Huber and Huber, 1996). Leaf cytosolic SPS activity in a variety of plants, e.g. sugarcane, wheat and potato, correlates with leaf sucrose content (Stitt *et al*., 1988, Grof *et al.*, 1998; Trevanion *et al*., 2004). This critical role of SPS in leaf carbon-partitioning and sucrose accumulation is demonstrated by molecular genetic manipulation. Over-expression of the maize SPS gene in tomato (*Lycopersicon esculentum* L.) and tobacco resulted in reduced levels of starch and increased levels of sucrose in the leaves (Worrell *et al*., 1991; Galtier *et al*., 1993, 1995; Baxter *et al*., 2003). Conversely, antisense repression of SPS activity in potato resulted in an inhibition of sucrose synthesis and increased flow of carbon to starch (Krause, 1994; Geigenberger *et al*., 1999). Overall, these results strongly support the idea that SPS expression in photosynthetic tissue is important for sucrose biosynthesis.

In addition to the role of SPS in sucrose synthesis in source leaves, SPS is also expressed in non-photosythetic tissues (Huber and Huber, 1996; Im, 2004). The sucrose that is unloaded into the sink tissues is subjected to a futile cycle of synthesis (by SPS and SuSY) and degradation (by invertases and SuSy) (Wendler *et al*., 1990; Geigenberger and Stitt, 1991; Whittaker and Botha, 1997). Labelling experiments in potato (Geigenberger *et al*., 1999), sugarcane (Botha and Black, 2000) and *Ricinus communis* (Geigenberger and Stitt, 1991) showed that SPS is the major enzyme responsible for this re-synthesis of sucrose.

Banana (*Musa acuminate*) and kiwi (*Actinidia chinensis)* fruits store imported sucrose in the form of starch. During fruit ripening the stored starch is converted into sucrose and this increase in sucrose levels is preceded by an increase in the expression of SPS mRNA (do Nascimento *et al*., 1997). Similarly, during maturation (increase in sucrose, glucose and fructose) of kiwi fruit there is an increase in the expression of SPS mRNA (Langenkamper *et al*., 1998). In the same study it was also shown that exposure of the fruit to ethylene rapidly increases the expression of SPS mRNA. In maize kernels, where sucrose is unloaded, it is hydrolised into glucose and fructose by invertase (Cheng *et al*., 1996). Im (2004) also showed that there is an increase in the expression of SPS mRNA during the re-synthesis of sucrose in maize endosperm.

The complexity of SPS gene expression is confounded by the presence of several SPS isoforms which are differentially expressed in individual species (Castleden *et al*., 2004; Chen *et al*., 2005; Grof *et al*., 2006). In wheat (*Triticum aestivum*), the isoforms are grouped into four families; A, B, C and, D (Castleden *et al*., 2004). The C-family genes are highly expressed in the leaf, along with *SPSII* (family A) and *SPSIV* (family D). Similarly, in tobacco (*Nicotiana tabacum*) family C is exclusively expressed in source leaves, while families A and B show intermediate expression in several tissues (Chen *et al*., 2005). While silencing of the A isoform (*NtSPSA*) had no detectable influence on leaf carbohydrate partitioning, silencing of the C isoform (*NtSPSC*) led to an increase in leaf starch as reported earlier in similar studies (Krause, 1994; Geigenberger *et al*., 1999). Chen *et al* (2005) suggest that *NtSPSC* is specifically involved in the synthesis of sucrose during starch mobilisation. The expression of the SPS C has also been observed in *A. thaliana* leaves during starch mobilisation to sucrose (Gibon *et al.,* 2004). In sugarcane, as in wheat, four SPS families were found to be differentially expressed in different tissues (Grof *et al*., 2006). In contrast to wheat, the sugarcane B-family was found to be highly expressed in young and old leaves. The other *SPS* genes families were also expressed, but at low levels in both young and old leaves. Similarly, in sugarcane, Sugiharto (2004) found

that B-family *SoSPS1* gene is expressed in leaves. It therefore seems that more than one isoform is responsible for sucrose synthesis in sugarcane leaves.

In addition to the multiple SPS isoforms that are differentially expressed in the leaf, there is also evidence that different isofoms are expressed in non-photosynthetic tissues (Chen *et al*., 2005; Grof *et al*., 2006). In sugarcane, the four isoforms of SPS, A, B, C, and D are differentially expressed in the stem during sucrose accumulation (Grof *et al.,* 2006). Similarly, the D isoform family of SPS is expressed in culms of wheat, suggesting that these isoforms also contribute to sucrose synthesis during fructan remobilisation (Castleden *et al*., 2004). In sugarcane the SPS A and D families exhibit the highest level of expression in both immature (low sucrose) and mature (high sucrose) culms, in particular the D2 subfamily. Interestingly, the A family's relative expression increases as the sucrose concentration increases in the sugarcane culms and, in contrast, the expression of the B family decreases as the sucrose amount increases down the internodes (Grof *et al.,* 2006). These observations lead Grof and co-workers (2006) to speculate that the D family might play a role in sucrose accumulation in sugarcane culms. The functional significance of the differential expression of all these isoforms is not yet clear; more precise methods are required to unravel the role of specific SPS isoforms in sucrose metabolism in sugarcane.

2.3.2 *Sucrose synthase*

Utilisation of sucrose as a source of carbon and energy depends on its cleavage into the corresponding hexoses (Sturm, 1999). Sucrose may enter the cell via the symplast and/or the apoplast (as discussed above) and once in the cell, sucrose synthase (SuSy, EC 2.4.1.13) and invertase catalyse the breakdown of sucrose (Sturm, 1999). Although SuSy can also synthesise sucrose, the degradation reaction dominates *in vivo* (Geigenberger and Stitt, 1993; Botha and Black, 2000). SuSy resides in the cytoplasm of both photosynthetic and non-

photosynthetic tissue (Geigenberger *et al*., 1993; Nolte and Koch, 1993). Generally, SuSy activity is low in photosynthetic cells and high in sink organs (Sung *et al*., 1989) where it is thought to supply UDP-Glc and fructose for glycolysis and starch synthesis (Nguyen-Quoc and Foyer, 2001; Harada *et al*., 2005). In addition, a SuSy isoform associated with the plasma membrane is thought to contribute to growth by supplying sugars for cell wall biosynthesis (Amor *et al*., 1995; Carlson and Chourey, 1996; Carlson *et al*., 2002).

Expression of the SuSy gene is regulated at the developmental and environmental levels, as well as by the sugar status in the plant (Koch, 1996; Zeng *et al*., 1998; Barratt *et al*., 2001). In pea (*Pisum sativum)*, SuSy is expressed in young leaves but not in mature leaves (Barratt *et al*., 2001). Similarly, when roots of maize were exposed to hypoxia, expression of SuSy increased (Zeng *et al*., 1998). Furthermore, SuSy transcripts were differentially expressed when roots of maize were supplied with different glucose concentrations (Koch *et al*., 1992; Koch, 1996).

In monocotyledons such as maize and rice, SuSy is encoded by two or more differentially expressed isoforms (Huang *et al*., 1996; Chourey *et al.,* 1998; Carlson *et al.,* 2002). These isoforms exhibit distinct spatial and temporal expression patterns (Chourey *et al*., 1998; Carlson *et al.,* 2002). In maize, SuSy is encoded by three genes *Sh1*, *Sus1* and *Sus3* (Chourey *et al*., 1998; Carlson *et al.,* 2002). Similarly, rice has three isoforms; *RSus1, RSus2* and *RSus3* (Huang *et al*., 1996). Expression of rice *RSus1* and maize *Sus1* is up-regulated by an abundance of soluble sugars (Chourey *et al*., 1998; Liao and Wang, 2003), whereas *Sh1* and *RSus*2 are highly expressed at low sugar levels (Koch *et al.,* 1992; Chourey *et al.,* 1998; Liao and Wang, 2003).

Similarly, in dicotyledonous plants that store sucrose or hexose, such as carrot (*Daucus carot,* Sturm *et al*., 1999), sugarbeet (Haagenson *et al*., 2006) and citrus fruits (Komatsu *et al*., 2002) there also appear to be at least two or more

differentially expressed SuSy isoforms. In carrot, two SuSy isoforms were found to be differentially expressed (Sturm *et al.,* 1999). Sugarbeet also has two SuSy isoforms which are differentially expressed (Haagenson *et al*., 2006). In carrot and sugarbeet, Susy *Dc1* and *SBSS1* were found to be strongly expressed during development in the roots. This led Sturm *et al.* (1999) to suggest that Susy *Dc*1's function is to mobilise sucrose for growth in developing tissues.

The expression of SuSy isoforms exhibit different spatial and temporal expression patterns and are differentially regulated at the transcriptional and translational levels (Déjardin *et al*., 1999). Expression may also vary according to the tissue type and carbohydrate metabolic state (Winter and Huber, 2000). Sucrose-storing plants *Citrus unshiu* (Komatsu *et al*., 2002) and sugarcane (Buczynski *et al.,* 1993; Lingle and Dyer, 2001; Schäfer *et al*., 2004) have three and two SuSy isoforms respectively, which are differentially expressed during development. When sucrose levels were low in immature plants, the expression level of *CitSUS1* and *Susy1* isoforms were the most abundant but both isoforms declined when sucrose levels were high. Interestingly, in sugarcane as the plant matures (high sucrose internodes) SuSy activity in the breakdown direction also increased (Schäfer *et al*., 2004). Therefore SuSy activity could play a dual role by providing reducing sugars to actively growing internodes and creating a strong sink for the unloading of sucrose into the cytoplasmic space of mature internodes in sugarcane.

2.3.3 *Invertases*

As mentioned above two enzymes are responsible for sucrose hydrolysis, i.e. the invertases (INV, EC 3.2.1.26) and SuSY (Winter and Huber 2000; Koch, 2004). The INVs hydrolyse sucrose to fructose (fru) and glucose (glc) (Sturm and Tang, 1999; Koch, 2004). Plants possess three types of INVs, these isoenzymes can be distinguished based on their solubility, subcellular localization, pH-optima and isoelectric point (Sturm, 1999; Roitsch and González, 2004). Neutral or alkaline

invertase (NI) is located in the cytoplasm and functions at pH optima between 6.8 and 8, whereas insoluble acid or cell wall invertase (CWI) is bound to the cell wall and has pH optima between 3.5 and 5 and the soluble acid invertase (SAI) is localised in the vacuole and has pH optima between 5 and 5.5. NI hydrolyses sucrose in the cytoplasm, whereas CWI is involved in sucrose partitioning and signal transduction (González *et al.,* 2005). SAI plays an essential role in sugar storage, osmoregulation and abiotic stress response (Moore, 1995; Sturm, 1999; Roitsch and González, 2004). The expression of INVs is regulated at the developmental level and by diverse internal and external stimuli (Roitsch *et al.,* 2003; Rausch and Greiner, 2004).

2.3.3.1 *Cell wall invertase*

Transport of sucrose from source leaves into sink organs is driven by differences in osmotic potential (Moore, 1995). Cleavage of sucrose by CWI in the apoplastic space may thus control the sink strength by generating a sucrose gradient to support unloading of sucrose from the phloem (Roitsch *et al*., 2003). Isoforms of CWI are expressed differentially in an organ-specific manner in a variety of plant species (Weber *et al.,* 1996; Godt and Roitsch, 1997; Tymowska-Lalanne and Kreis, 1998; Kim *et al*., 2000). In fava beans (*Vicia faba*) and barley seeds, where sucrose is unloaded apoplasmically during development, CWI is expressed during seed development and its expression correlates with an increase in hexose levels in the apoplastic space (Weber *et al*., 1996; Weschke *et al.,* 2003). Similarly, the maize seed mutant *Miniature-1,* which is deficient in CWI activity, has low hexose levels and a slow growth rate (Miller and Chourey, 1992). Therefore, CWI expression during growth and development seems to contribute to establishing sink strength during sucrose unloading in the apoplastic space.

In tomato (*Lycopersicon esculentum* L.) fruits and *Chenopodium rubrum* stems, which transport sucrose into the apoplastic space, CWI isoforms were found to be differentially expressed (Roitsch *et al*., 1995; Godt and Roitsch, 1997). The

Lin6 (tomato) and *CIN1* (*C. rubrum*) isoforms are expressed in a tissue-specific manner and are also induced by glucose. Godt and Roitsch (1997) suggested that expression of CWI plays an important function in apoplastic cleavage of sucrose and in source-sink regulation.

Expression of an antisense CWI gene in carrot plants resulted in plants that had no tap roots and reduced soluble sugars (Tang and Sturm, 1999). By contrast, in sugarcane, the over-expression of CWI activity in the apoplastic space resulted in high levels of soluble sugars (Ma *et al.,* 2000). CWI activity could therefore play a dual role by providing reducing sugars to maintain sink metabolism and by creating a strong sink for the unloading of sucrose into the apoplastic space.

2.3.3.2 Soluble a*cid invertase*

In plants such as sugarcane, sucrose does not only serve as the transported form of photoassimilate but also as a long-term storage compound in the vacuole (Preisser *et al.,* 1992). In sugarcane, vacuolar SAI is developmentally regulated (Zhu *et al.,* 2000). Expression of SAI mRNA is relatively high in developing internodes of both high- and low-sucrose cultivars. In maturing internodes where sucrose is rapidly accumulating, however, the expression of SAI declines. While the decline was similar between the two cultivars, expression of SAI mRNA in the low-sucrose line was quantitatively higher than that of the high-sucrose storing line (Zhu *et al.,* 2000). Vacuolar SAI is also highly-expressed during the early stages of development of Japanese pear fruit (*Pyrus pyrifolia*), which also stores sucrose in the vacuole and similar to sugarcane, the levels of SAI mRNA decrease during the fruit maturation stage when sucrose is accumulated (Yamada *et al*., 2006).

Suppression of vacuolar SAI by antisense RNA technology has resulted in increased sucrose content in tomato fruit (Klann *et al*., 1996), potato tuber (Zrenner *et al*., 1996), carrot (Tang *et al*., 1999), and sugarcane (Ma *et al.,* 2000),

confirming the role of vacuolar SAI in the control of sugar accumulation. Surprisingly, although sucrose increases the total amount of soluble sugars allocated to the sink organ, i.e. tomato fruits, remains the same. Klann *et al.* (1996) suggested the presence of multiple genes of vacuolar SAI in tomato. Similarly, in sugarcane vacuolar SAI activity was reduced by only 65%, leading Ma *et al.* (2000) to suggest that the limited decrease in SAI activity might be related to the high polyploid level of sugarcane, i.e. a large SAI gene family. Improved understanding of expression of SAI might allow the exploitation of their properties to specifically alter sucrose partitioning in storage organs.

2.3.3.3 *Neutral invertase*

The sucrose unloaded into the cytosol of sink tissues is mobilised by NI and or SuSy (Komor, 2000; Nguyen-Quoc and Foyer, 2001). NI is considered to be a maintenance enzyme that hydrolyses sucrose when the activities of the acid invertases and SuSy are low (Winter and Huber, 2000). In sugarcane stem the unloaded sucrose undergoes a continuous cleavage (by NI and/or SuSy) and re-synthesis (by SPS and SuSy), this cycling is refer to as 'futile cycle' (Botha and Rower, 2001). Early findings on activity levels of NI in sugarcane internodes reported an increase in enzyme activity as the stem matures and the activity correlated weakly to hexose or sucrose levels (Batta and Singh 1986; Lingle and Smith, 1991). In contrast, Bosch *et al*., (2004) found that the immature, actively growing internodes of sugarcane contain high levels of NI activity, which correlates positively with high hexose content and conversely, internodes with high sucrose levels (mature internodes) have low NI activity.

Despite NI's central role in sucrose metabolism, transcript expressions patterns have been studied in only a few plants, e.g. poison rye grass (*Lolium temulentum*) (Gallagher and Pollock, 1998), carrot (Sturm *et al*., 1999) and sugarcane (Bosch *et al*., 2004; Rossouw *et al*., 2007). NI is expressed in all organs of sugarcane and carrot plants (Sturm *et al*., 1999; Bosch *et al*., 2004).

Down-regulation of NI in sugarcane suspension culture resulted in increased sucrose and the cell displayed impaired growth characteristics (Rossouw *et al*., 2007). NI therefore seems to play a role in supplying hexoses for growth and respiration in actively-growing tissues.

2.3.4 *Sugar transporters*

Sucrose can be downloaded in sink tissues either symplastically or apoplastically and is further facilitated by sugar transporters (Williams *et al*., 2000; Lalonde *et al*., 2003, Harrington *et al.,* 2005). There are two transporter families, i.e. the sucrose and the hexose transporters (Dibley *et al*., 2005; Reinders *et al.,* 2006).

2.3.4.1 *Sucrose transporters*

The first sucrose transporters (SUTs) identified from plants were isolated from spinach and potato (Riesmeier *et al*., 1992; Riesmeier *et al*., 1993). Sucrose transport across the plasma membranes of sieve elements and companion cells is important for partitioning of assimilates and the development of the plant (Sauer *et al*., 2004). Leaked assimilates are also transported from the apoplast into the phloem by sugar transporters (Rae *et al*., 2005). The expression of SUTs is regulated by developmental, biotic and abiotic factors, sugar levels and is tissue specific (Shakya and Sturm, 1998; Furbank *et al.,* 2001; Yao Li *et al*., 2003; Rae *et al*., 2005).

SUTs were also found to be expressed in leaves of *Arabidopsis*, carrot, *Plantago major*, sugarcane and tobacco (Gahrtz *et* al., 1994; Bürkle *et al*., 1998; Shakya and Sturm, 1998; Lemoine *et al*., 1999; Reinders *et al.,* 2006). The effect of antisense suppression of SUTs was analysed in potato and tobacco plants (Riesmeier *et al*., 1994; Bürkle *et al*., 1998). The capacity of these transgenic tobacco and potato lines to load assimilate from source leaves is greatly reduced and resulted in increased carbohydrate levels in their leaves, inhibition of

photosynthesis and reduced tuber yield in the potato plants. Furthermore, using *Arabidopsis* knockout mutants with a disrupted SUT, Gottwald *et al.* (2000) showed that the mutant plants had stunted growth. This led the authors to suggest that SUT is responsible for the loading of sucrose into the phloem. Reinders *et al.* (2006) expressed a sugarcane SUT, *ShST1*, in *Xenopus* oocytes and found that it was highly selective for sucrose.

Apart from their role in phloem loading in source tissues, SUT genes are also expressed in sink tissues such as developing seeds, stems, fruits, and roots (Shakya and Sturm, 1998; Weschke *et al.,* 2000; Yao Li *et al.,* 2003; Rae *et al*., 2005). In seeds there is an absence of symplastic linkage between the maternal and filial tissues and transport of sucrose across this apoplastic space therefore requires SUTs (Patrick and Offler, 2001). Expression of SUTs in developing barley and faba bean seeds coincides with increases in sucrose concentration (Weber *et al.,* 1997, Weschke *et al* 2000). Localisation studies of SUTs further support the role of SUTs in sucrose unloading from the maternal tissue and/or loading into the endosperm (Weschke *et al* 2000).

In carrot, *DcSUT2* mRNA transcripts are highly expressed in both the phloem and xylem tissues of the taproots (Shakya and Sturm, 1998). In contrast, the expression of *DcSUT2* is low in leaves, leading these authors to suggest that the high-levels of expression in storage tissue may play a role in importing sucrose into the parenchyma cells. In grape (*Vitis vinifera* L.) berries, three SUT genes are differentially expressed (Davies *et al*., 1999). *VvSUC27* is highly expressed in berries before ripening, whereas *VvSUC11* and *VvSUC12* transcripts are highly expressed during ripening when the berries are rapidly accumulating hexoses. This led Davies *et al.* (1999) to suggest that SUTs play an essential role in the partitioning of sucrose during ripening in grape berries.

In sugarcane, unloading of sucrose into the storage parenchyma cells is predominantly symplastic (Walsh *et al.,* 2005 and as discussed above) and

ShSUT1, a putative SUT, is differentially expressed in internodal tissues (Rae *et al.* 2005). In young, developing internodes that accumulate sucrose at a high rate, the *ShSUT1* transcript is present at high levels, whereas in mature internodes that accumulate sucrose at a slow rate the transcript is only expressed at low levels. Previously, a second putative SUT, i.e. *PST6*, was identified which is expressed only in maturing internodes (Casu *et al.* 2003). This led Rea *et al.* (2005) to suggest that SUTs may be involved in the retrieval of sucrose lost from the symplastic continuum or in supplying sugars to cells that undergo rapid cell wall suberisation.

2.3.4.2 *Hexose transporters*

The second sugar transporter family consists of the hexose transporters (HXTs) or monosaccharide transporters that are expressed in sink tissues (Sauer and Stadler, 1993; Truernit *et al.*, 1999). Expression of HXTs in sink tissues may support apoplastic phloem and post-phloem unloading (Sauer and Stadle, 1993). Sucrose released from the phloem into the apoplastic space is postulated to be hydrolysed by CWI (as discussed above) and the resulting hexoses are then transported by HXT's into sink cells (Sauer *et al.,* 1994; Hellman *et al.,* 2000). Expression of hexose transporters is regulated by phytohormones, abiotic stress and wounding, indicating an elastic system for the partitioning of carbohydrates depending on the demand (Sauer and Stadler, 1993; Ehneß and Riotsch, 1997).

The first higher plant HXT was cloned by heterologous hybridisation with HXT genes from the green alga *Chlorella kessleri* (Sauer and Tanner, 1989; Sauer *et al*., 1990). The *Arabidopsis* genome contains 14 putative HXT genes within a family of 50 closely-related genes, while multiple genes of HXT have also been isolated in other species (Lalonde *et al.,* 1999; Sherson *et al.,* 2003). In *Arabidopsis,* Sherson *et al.* (2000) showed that the HXT *AtSTP1* is expressed in leaves, roots, stems and seedlings. In the same study, it was also shown that a knockout mutant in the HXT *AtSTP1* gene resulted in a decrease in uptake of

exogenous hexose by *Arabidopsis* seedlings. Furthermore, in soybean (*Glycine max*) seedlings, two putative HXTs (*GmMST1* and *GmMST2*) were found to be differentially expressed in different tissues (Dimou *et al*., 2005). Interestingly, the expression of HXT *GmMST1* in soybean coincided with expression of CWI, leading the authors to suggest that the sucrose unloaded from the phloem may be hydolysed into hexoses before being transported by HXTs into the sink cells (as mentioned above).

In sugarcane, putative HXTs were found to be expressed in maturing internodes (Casu *et al.,* 2003; Watt *et al.,* 2005). Further analyses revealed that the HXT *PST2* is expressed in the phloem and associated parenchyma cells, but not the storage parenchyma of maturing and mature internodes (Casu *et al*., 2003). The specific localization of this HXT transcript in the phloem led Casu *et al.* (2003) to suggest that it might be involved in the translocation and maintenance of sugar fluxes in the sink during sucrose accumulation.

2.4 Sorghum as a model plant for sugarcane

Sugarcane belongs to the family *Poaceae* and the tribe *Adropogoneae,* like maize and sorghum (Grivet, *et al*., 1994). It has an extreme complex genome (D'Hont *et al*., 1996). Sugarcane plants are the result of a series of crosses and backcrosses derived from the domesticated species *S. officinarum* L. (2n=80) and the wild species *S. spontaneum* (2n=40-120) (Butterfield *et al.,* 2001). Commercial cultivars are interspecific poly-aneuploid hybrids with chromosome numbers usually in excess of 100. In addition, sugarcane traits are polygenic and/or multi-allelic, and quantitatively inherited (Butterfield *et al.,* 2001). Furthermore, the genome size of commercial cultivars is approximately 3000 Mbp, compared with 750 Mbp for its close relative, sorghum (Arumuganathan and Earle, 1991; Grivet *et al.,* 1994). As a consequence of this extremely large and complex genome, the use of genomic techniques in sugarcane poses many challenges (Grivet and Arruda, 2002).

Unlike sugarcane, *Sorghum bicolor* commonly known as sorghum is a diploid crop that has a chromosome number that ranges from 2n = 10-40 (Doggett, 1988). The molecular analysis of the complex polyploid genome of sugarcane might therefore be simplified by exploiting its close relationship with the genome of sorghum (Ming *et al*., 1998). During alignment studies of sugarcane and sorghum genomes, it was found that about 84% of the loci mapped by 242 probes were homologous (Ming *et al.,* 1998). Comparative genetic mapping studies between sugarcane, maize and sorghum also found that there was a complete co-linearity between sorghum linkage group G and the sugarcane linkage groups II and III (Dufour *et al*., 1996). Similarly, Guimaráes *et al.* (1997) also found a striking co-linearity between sorghum and sugarcane genomes based on restriction fragment length polymorphism analyses. Furthermore, comparative mapping of sugarcane and sorghum has shown considerable synteny between these genomes, implying conservation in the order of DNA sequences on chromosomes (Dufour *et al*., 1997; Glaszmann *et al*., 1997). When sugarcane expressed sequence tags (ESTs) were compared to a sorghum EST database similar expression patterns were revealed in various tissues (Ma *et al.,* 2004).

EST and micro- and macro-array analyses have also been used in the search for genes that control sucrose accumulation in sugarcane (Carson and Botha, 2000, 2002; Casu *et al.,* 2003, 2005; Watt *et al*., 2005). These studies have yielded an extensive annotated gene list and correlative data (Watt *et al*., 2005). However, the identities of key regulatory genes remain elusive. As in sugarcane, sucrose is the photoassimilate translocated from the leaves and stored in the stalk of sorghum (Tarpley et al. 1994). In addition, sorghum genotypes that accumulate various amounts of sucrose in their stalk parenchyma are available (Lingle, 1987; Vietor and Miller, 1990).The simple diploid genetics of sorgum and its co-linearity and synteny with sugarcane therefore makes it an attractive, potential model system for the identification of differentially expressed genes in the sink tissues of genotypes that accumulate different amounts of sucrose.

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Chapter 3

Establishment of a protocol for transcript profiling in *Sorghum bicolor*

Abstract

To date, genomic research into sucrose accumulation in sugarcane has focused on genes that are expressed in association with stalk development/maturation, with the aim of identifying key regulatory steps in sucrose metabolism. However, the identification of possible controlling points is complicated by the polyploid nature of the plant and these have therefore remained elusive. A close relative of sugarcane, sorghum, is diploid, has a small genome size and also accumulates sucrose in the stalk parenchyma. Here we provide a robust and reliable method for profiling multiple transcripts using sorghum as a model for sugarcane for studying carbohydrate metabolism. To determine the sensitivity of probe-target hybridisation, cDNA (the target) derived from young internodal tissue was probed with four randomly selected ESTs that were immobilised on nylon membranes in a macroarray format. The hybridisation signal increased as expected with increasing amounts of probe until the hybridisation signals reached maximum levels at 0.05 pmol probe. Using this as the probe concentration, 48 ESTs were arrayed in duplicate and hybridised with cDNA from pooled internodal tissues. Inter-array and intra-array variability was found to be low. Additionally, to test the reliability and sensitivity of the method, the target mRNA was spiked with an *in vitro* transcribed bacterial gene before cDNA synthesis. Variation in the spiked mRNA's concentration was reliably detected. Under the conditions described here the technology is reproducible, robust and can reliably detect as little as two-fold changes in the levels of a specific transcript.

Introduction

There are many reasons why the analysis of transcript expression in plant cells is an essential tool in molecular biology. By comparing the different levels of transcripts present in samples originating from different genotypes, developmental stages or growth conditions, differentially expressed gene(s) can be identified (Kuhn, 2001). In the past, analysis of gene expression through the measurement of steady state levels of mRNA was conducted one gene at a time. Northern blot, dot blot, differential display and serial analysis of gene expression (SAGE) were the methods of choice for investigating changes in gene expression. In northern blot analyses, total RNA populations are resolved according to size via agarose gel electrophoresis, transferred to positively-charged membranes and probed with a labelled gene-specific sequence (Alwine *et al.,* 1977). Similar to northern blot analyses, dot blot analyses also identify a single gene but differ in that the RNA molecules are not separated electrophoretically but spotted directly onto the membrane (Lennon and Lehrach, 1991). Both techniques suffer from the same problem, i.e. the analysis of a single gene per experiment, and the analysis of multiple transcripts will therefore be time consuming.

Differential display (Liang and Pardee, 1992) and SAGE (Velculescu *et al.,* 1995) were subsequently developed to increase the throughput of gene expression analyses. Differential display employs low stringency PCR. The combination of oligo-(dT) 3'-primers and short, arbitrary 5′-primers allow the random amplification of transcripts. The resulting cDNA fragments are separated electrophoretically and visualised on a denaturing polyacrylamide gel. The advantages of the differential display technique in comparison to RNA blots include parallel profiling of transcripts from two samples and the minimal use of mRNA. However, the technique suffers from the inability to capture rare transcripts, and tends to be biased towards the identification of abundant transcripts (Bertioli *et al*., 1995; Callard *et al*., 1994). Contamination with

neighbouring and overlapping fragments in the gel is also a major problem (Bertioli *et al*., 1995; Callard *et al*., 1994).

In contrast to differential display, SAGE has the advantage of being more quantitative (Velculescu *et al.,* 1995). The SAGE method is based on the isolation of unique sequence tags (9-11 base pairs in length) from mRNA. The tags are amplified once by PCR, concatenated and cloned into a plasmid and sequenced. The frequency of each tag in the sequence directly reflects the abundance of that mRNA in the tissue. However, SAGE is laborious for routine analysis of gene expression. Although all the previous techniques have been applied successfully for identification of differentially-expressed genes, they suffer from the same limitations in capturing rare transcripts (Schena *et al*., 1995).

The development of DNA arrays for large-scale transcript profiling and their ability to identify rare transcript species was a major achievement in functional biology (Schena *et al*., 1995). Expression levels of multiple transcripts can be studied by reverse transcribing, labelling and hybridising the full complement of messengers (the targets) from a particular tissue to specific DNA fragments (the probes) arrayed/immobilised on nylon or glass (Lockhart and Winzeler, 2000). In contrast to the other techniques, the advantage of DNA arrays is that they yield signal intensities that are relative to the level of each mRNA in that population (Schena *et al*., 1995; Desprez *et al*., 1998). DNA arrays have been used successfully in plants for the identification of genes that show significant changes when exposed to different growth conditions or stimuli, or by comparing different developmental stages (Desprez *et al*., 1998; Kurth *et al.,* 2002, Casu *et al.,* 2005; Watt *et al*., 2005).

The generation of expressed sequence tags (ESTs) for major crops and the availability of the complete genome sequences of model plants, e.g. *Arabidopsis thaliana* and *Oryza sativa,* further facilitates the analysis of large numbers of

genes in closely related species because it greatly increased the number of sequences that could potentially be used as probes (The Arabidopsis Genome Initiative, 2000; Sequencing Project International Rice Genome, 2005; Casu *et al.,* 2005).

In general, nylon membrane DNA arrays are called macroarrays whereas the glass-imprinted arrays are said to be microarrays because of the size of the individual spots in which the probes are applied onto the arrays. Currently, double-stranded DNA clones, PCR amplicons or oligonucleotides are arrayed as probes onto membranes and glass slides (Schena *et al.,* 1995; Desprez *et al*., 1998). In macroarrays, the target is prepared through reverse transcription of a specific target poly(A)$^+$-RNA population in the presence of a radioactive isotope and is then allowed to hybridise with the denatured immobilised probes on a membrane. In microarrays, various targets, i.e. various poly(A)$^+$-RNA populations, are reverse transcribed and then labelled with different fluorescent dyes, which are allowed to hydridise with the immobilised probes on a slide. Although microarrays are expensive to prepare and cannot be re-hybridised, the fluorescent scheme allows multiple samples, e.g. treatment and control, to be hybridised on the same glass slide (Freeman *et al.,* 2000). A further advantage of microarrays is their higher sensitivity and reproducibility (Desprez *et al*., 1998). Although microarrays are superior, a macroarray system is desirable as it can be set up in most molecular biology laboratories. A further advantage of macroarrays is their relatively cheap preparation, since the membranes can be prepared in-house and re-hybridised up to ten times (Kuhn, 2001).

Although macroarrays have the potential to enhance our understanding of gene expression, there are technical challenges to overcome. The most important of is ensuring sufficient sensitivity, a linear response between signal strength and target concentrations and reproducibility (Hanano *et al*., 2002; van Bakel and Holstege, 2004). Here, we describe the establishment of a macroarray protocol

for quantifying gene expression in sorghum. The protocol was found to be robust, reproducible and can reliably detect variation in transcripts levels.

Results and Discussion

Calibration of target-probe hybridisation

To assess the efficiency of target-probe hybridisation, e.g. the saturation levels of signal intensity and the prevalence of non-specific interactions, increasing amounts, i.e. 0.005, 0.01, 0.05 and 0.075 pmol, of four randomly selected *Oryza sativa* ESTs were arrayed in duplicate on a positively-charged membrane as probes. All hybridisation experiments were performed independently with three sets of membranes. All probes were prepared by reverse transcription of boot stage, sorghum mRNA (internode 1-3) in the presence of [α-^{33}P]-dCTP. Ideally, the amount of probe DNA spotted onto membrane should be in excess so that the intensity of the hybridisation signal depends only on the amount of target cDNA and should not be limited by the amount of the spotted probe DNA. Conversly, the amount of probe should not be too high because this could lead to the steric hindrance of hybridisation or non-specific binding.

The first step in data normalisation is background subtraction, which ensures that any non-specific binding of the probe to the membrane is deducted from the detected signal. In this case the average signal intensity of two independent spots was therefore normalised by subtracting the average background of two negative control spots of buffer only, i.e. contained no DNA. As expected there was a linear correlation between the hybridisation signal intensity and probe amount when the amount of probe was limiting, i.e 0.005-0.05 pmol (Figure 1). At 0.05 pmol all the hybridisition signal intensity reached a plateau, indicating that the probe amount is not limiting for the target concentration used here. Therefore, variation in signal intensities at this probe amount should directly reflect target concentration, i.e. the amount of a particular cDNA.

Similar observations were also demonstrated in macroarray analyses with *Saccharum* spp hybrid *cv* N19 in immature and maturing internodes (Watt *et al*., 2005). Probes arrayed at 0.05 pmol should therefore ensure optimal target-probe hybridisation efficiency and therefore enable the detection of variations in the concentration of a specific cDNA species. In other words, by using this amount of probe the signal intensity should represent the abundance of the particular target mRNA in the population. Consequently, probes were arrayed at 0.05 pmol to be able to detect differences in transcript expression.

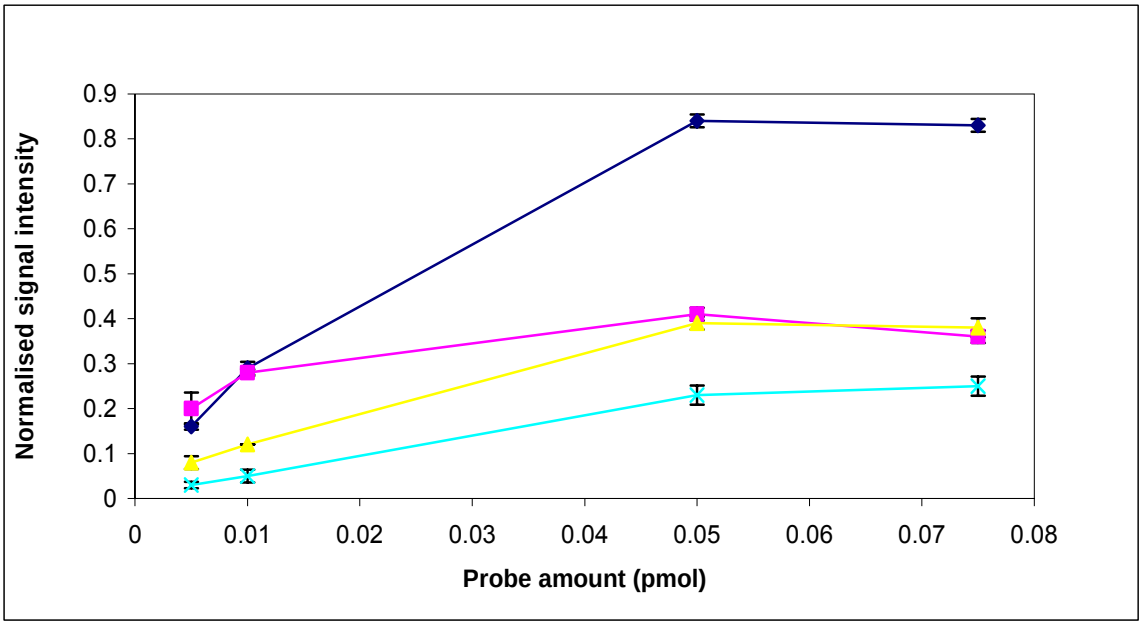

Figure 1: Normalised target-probe hybridisation signal intensities for increasing amounts of four EST derived probes. Labelled cDNA targets were prepared from 1 µg *Sorghum bicolor* boot stage mRNA of internodes 1-3.

Linearity and sensitivity of target-probe interactions

The linearity and sensitivity of the detection system was assessed by preparing cDNA from *S. bicolor* poly(A)$^+$-mRNA (1µg), spiked with increasing amounts of *in vitro*-transcribed RNA, i.e. the *Giardia lamblia* transcript for PFP. Similarly, *in vitro*-transcribed human gene RNA has been spiked in the poly(A)$^+$-mRNA of plants as an external control to test the sensitivity of macroarrays (Kurth *et al.,* 2002; Hanano *et al*., 2002). The *in vitro*-transcribed *Giardia lamblia* PFP RNA

was spiked to represent 0.06, 0.25 and 0.5% respectively of the total amount of poly(A)$^+$-mRNA used for cDNA synthesis. It has been shown that a great majority of transcripts are represented at level between 0.08-0.5% of the total mRNA pool (Desprez *et al.,* 1998). Therefore sensitivity of macroarray was tested with 0.06-0.5% of spiked *Giardia lamblia* PFP RNA. The *G. lamblia* PFP gene sequence was also used as a probe and arrayed in triplicate. The normalised hybridisation signal intensities generated by the *in vitro*-transcribed *G. lamblia* PFP RNA exhibited a linear relationship with the concentrations of transcript tested here, i.e. 0.06-0.5% (Figure 2). One-way analysis of variance (ANOVA), also confirmed that the mean signal intensities produced by the spiked *G. lamblia* PFP RNA at the various concentrations were significantly different from each other ($p < 0.05$).

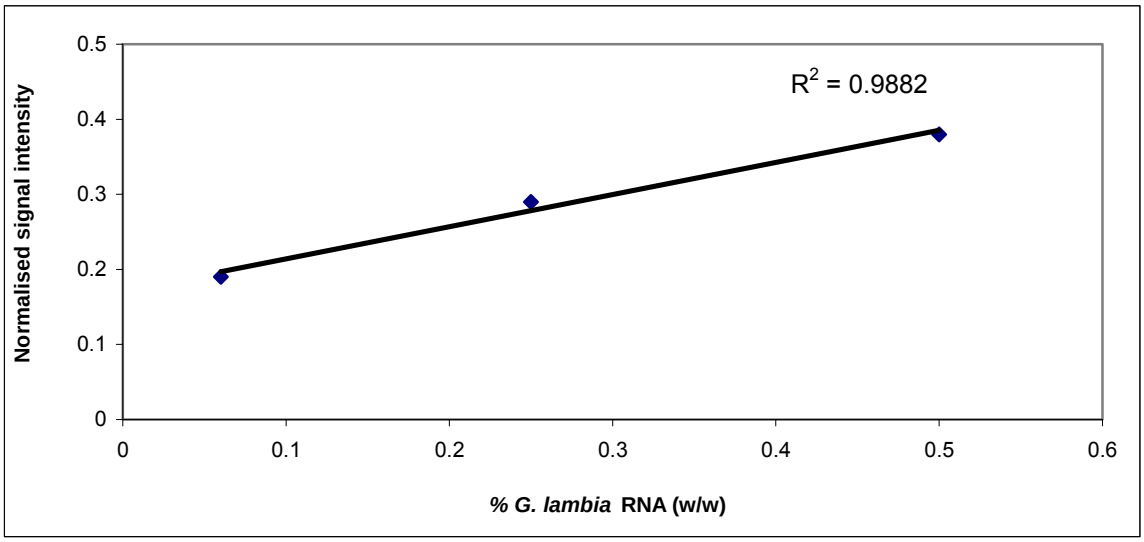

Figure 2: Correlation between the normalised signal intensities and the relative concentration of *G. lamblia* RNA. *In vitro* transcribed *G. lamblia* PFP RNA was added to 1 µg poly(A)$^+$ RNA from *S. bicolor* before cDNA synthesis. The resulting ^{33}P-labelled cDNA was hybridised with 0.05 pmol of the probe DNAs on the macroarray filters.

These results indicates that the protocol has the ability to specifically identify individual species of mRNAs, including rare transcripts which only represent as little as 0.06% of the total mRNA pool, and detect these mRNAs quantitatively between 0.06% and 0.5% of the total RNA pool. Similar sensitivity and linearity relationships has been shown for other macroarray studies (Desprez *et al.,* 1998; Girke *et al*., 2000; Hanano *et al*., 2002; van Bakel and Holstege., 2004; Radonjic

et al., 2005). This level of sensitivity is sufficient to detect variations in the transcript levels of a large proportion of genes expressed in developing *S. bicolor.*

Reproducibility

To assess the reproducibility of the macroarray hybridisation results, 0.05 pmol of 48 EST derived probes were arrayed in duplicate and queried in two separate experiments with radioactively-labelled target cDNA populations from pooled sorghum internode 1-3 poly(A)$^+$-mRNA. The first step in the analysis was to evaluate intra-array reliability by comparing the signal intensities of duplicate spots in the same membrane. The hybridisation signal intensities of the duplicate spots on the same membrane were firstly visually inspected and thereafter a coefficient of variation (CV) was determined for each sample (Equation 1, see material and methods). The hybridisation signal intensities of 95% (46/48) of the duplicate spots were found to have a CV of less than 5% [Figure 3(a)]. The CVs of the other two duplicate spot was above 10% and were excluded from futher analysis. The variation observed between duplicated spots is most probably due to local membrane variability or artefacts caused by the gridding pin. For this reason all probes were spotted in duplicate on each membrane and the mean of their signal intensities were used to increase the accuracy of the data (Desprez *et al*., 1998; Dale *et al.,* 2001; Watt *et al*., 2005).

Secondly, the inter-array reproducibility of the hybridisation results was evaluated by determining the variation between two independent hybridisation experiments using the probes and cDNA described above. One of the major prerequisites for the accurate assessment of macroarray signal intensities is the normalisation of the data, between different membranes (van Bakel and Holstege, 2004). Normalisation is necessary because of the varying efficiencies of RNA preparation, reverse transcription, probe purification, hybridisation and membrane quality. Although housekeeping genes such as actin, tubulin and

glyceraldehyde-3-phosphate dehydrogenase have been used to normalise array data, these were often found to be differentially expressed (Thellin *et* al., 1999; Warrington *et al.,* 2000*)***.** In this study, the actin and tubulin genes were also found to be differentially-expressed in the young and old internodes in both stages of developments and were therefore unsuitable for data normalisation (data not shown). Consequently, *in vitro* transcribed, control *G. lamblia* PFP RNA was added to the poly(A)$^{+}$-mRNA and used to normalise the data. Spiking of the control with an external control early during sample preparation helps in monitoring of downstream steps (van Bakel and Holstege, 2004). Similar external standards have previously been used to normalise data (Lockhart *et al*., 1996; Eickhoff *et al.,* 1999; van Bakel and Holstege., 2004).

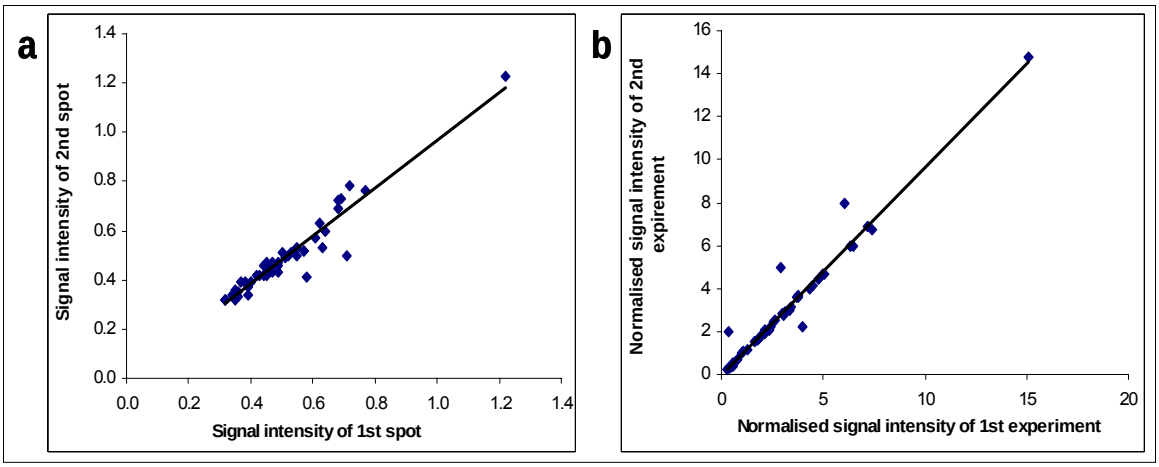

Figure 3: Assessment of intra- and inter-membrane hybridisation signal reproducibility. (a) Correlation between the signal intensities measured at two different locations on a membrane. (b) Normalised signal intensities of two independent membranes, prepared using the same mRNA targets.

The average signal intensity of two independent spots was normalised by subtracting the average background of two negative control spots (buffer containing no DNA) and then expressing it relative to the average signal intensity of the *G. lamblia* PFP signal on that membrane (Equation 2, see material and methods). Inter-array reproducibility was assessed by plotting the normalised signal intensities (NSIs) of the two independent membranes against each other [figure 3(b)]. One-way ANOVA analyses confirmed that 43 of the 48 NSIs (90%) gave similar values ($p \geq 0.05$), whereas only 5 sample NSIs were significantly

different from each other ($p \leq 0.05$). The 43 samples NSIs ($p \geq 0.05$) agree with the null hypothesis that all the mean values drawn from the same population (same tissue, internode 1-3) are equal, based on the degree of similarity between the two independent experiments. The variation in five of the sample NSIs could have been cause by varying efficiencies of RNA preparation, reverse transcription, probe purification, hybridisation and membrane quality. Together the above presented data therefore suggest that the established macroarray protocol is a robust and highly-reproducible tool for profiling multiple transcripts in developing *S. bicolor*.

Material and Methods

Plant material and growth conditions

All experiments were conducted using plants that were grown in a tunnel. *Sorghum bicolor* seeds of genotypes GH1 and SH2 were grown in 5 L plastic pots, containing soil and vermiculite (1:1, v/v), fertilised once in a month with N, P and K (3:2:1) and watered every second day of a week. Plant material was harvested at the boot stage, i.e. when the flag leaf collar appears, and the soft dough stage, ie. when the seeds are soft and a white milk-like liquid is obtained when they are squeezed. The panicle and peduncle were excised and the rind and nodal tissues were discarded. Internodal tissues were then pooled into two developmental/sugar accumulation phases, i.e. immature internodes (internodes 1-3) and maturing internodes (internodes 5-8). The pooled internodes were frozen in liquid nitrogen and ground to a fine powder using an A11 Basic Analysis Mill (IKA®). The powder was stored in 50ml polypropylene centrifuge tubes (Corning ®) at -80°C until use.

Bacterial clones, PCR amplification and macroarray production

Thirty-four *Oryza sativa* ESTs, identified as homologous to genes potentially playing key roles in carbohydrate metabolism were received as lypholised, recombined Bluescript plasmid DNA from the MAFF DNA Bank in Tsukuba, Japan. A further four ESTs were obtained from the South African Sugarcane Research Institute collection, while ten genes were obtained from an in-house collection. Lypholised plasmids were resuspended in 30 µl TE buffer. Then 10 µl plasmid DNA was transformed into 100 µl *E. coli* DH5α. The cells and plasmid DNA were incubated on ice for 45 min, heat shocked at 42°C for 45 seconds and then incubated on ice for 2 min. One millilitre of Luria Bertani (LB) medium was added to the transformation mixture, followed by shaking at 200 rpm for 1 h at 37°C. One hundred microlitres of the transformation reaction was plated onto an LB agar plate supplemented with 100 µg ml^{-1} ampicillin. Plates were then incubated overnight at 37°C. Single colonies were picked using sterile toothpicks, added to 50µl sterile water, and incubated for 15 min at 95°C. A 5µl aliquot was then subjected to a Polymerase Chain Reaction (PCR) using 3 µM of M13 forward and reverse primers (Inqaba Biotec), in the presence of 2.5 mM $MgCl_2$. PCR reactions were done with a Perkin & Elmer 9600 thermal cycler with the following cycling protocol: 5 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 45 sec at 55°C, 105 sec at 72°C and terminated by 7 min at 72°C. The PCR products were verified by loading 10 µl of the PCR reaction onto a 1% (w/v) agarose gel to assess insert size, and quantified using DyNA Quant 200 flourometer (Hoefer). Although non-specific bands were obtained in some EST clones during PCR, this problem was solved by excising the correct size band. The fragment was cloned into the pGEM® T Easy vector (Promega) and the identity of the clone was confirmed by sequencing. Prior to array immobilisation onto a positively-charged nylon Hybond-N+ membrane® (Amersham), PCR products were denatured with 0.2M NaOH. A 96-pin manual gridding device (V&P Scientific Inc., San Diego) was used to transfer 2 µl aliquots (containing 0.05pmol) of the denatured ESTs in duplicate. The size and the molecular weight

of the fragment were taken into consideration when the amount of probe to be immobilised onto the membranes was calculated. The following controls were used: pBluescript II KS (-) plasmid, pGEM-T Easy plasmid (negative control), Actin (positive internal control) and bacterial *G. lamblia* PFP gene (positive external control). Membranes were air-dried and the probe DNA cross-linked by means of short-wavelength UV-radiation (120 KJ cm^{-1}) for 1.5 min per side (Hoefer UV-Crosslinker). The DNA arrays were then wrapped in filter paper, sealed in foil and stored at room temperature until required.

RNA extraction, *in vitro* transcription and target synthesis

Total RNA was extracted according to a method modified from Bugos *et al.* (1995). Approximately 5g internodal tissue was transferred into 50 ml Corning tubes containing 10ml of 25:24:1 (v/v/v) phenol: chloroform: isoamyl alcohol and 10ml of homogenisation buffer (0.1M Tris-Cl (pH 7.5); 1mM EDTA; 0.1M NaCl; 1% (w/v) SDS). Samples were mixed using a vortex set at high speed for 1-2 min. Sodium acetate (pH 5.2) was added to each sample to a final concentration of 0.1 M and samples were further homogenised for 30 sec, followed by storage on ice for 15 min. After centrifugation at 12,000 g for 15min at 4°C, the upper aqueous phase of each sample was transferred to a fresh 50 ml Corning tube containing an equal volume of isopropanol to precipitate the RNA. Samples were incubated overnight at -20°C and centrifuge at 10,000 g for 10 min to recover the precipitated total RNA. The supernatant was discarded and the white RNA pellets washed with 5 ml 70% (v/v) ethanol, followed by centrifugation at 10,000 g for 5 min and brief drying of the pellets after removing the ethanol. The pellet was resuspended in 800 µl DEPC-treated water, insoluble material was removed by centrifugation at 10,000 g for 5 min at 4°C. The supernatant was subsequently transferred to a sterile microfuge tube containing 250 µl 3 M lithium chloride. The precipitated total RNA was recovered by centrifugation at 12,000 g for 20 min at 4°C and the supernatant discarded. The pellet was washed with 1 ml 70%

ethanol, centrifuged at 10,000 g for 5 min and the dried briefly after discarding the ethanol. Total RNA pellets were resuspended in 100-200 µl DEPC-treated water, and stored at -80°C. Total RNA concentrations were calculated from UV spectrophotometric absorbance measurements at 260nm, quality was expressed as the ratio of absorbance measurements (260:280), and confirmed via gel electrophoresis. For electrophoretic analysis, 5 µg of total RNA was diluted to a final concentration of 0.5 µg/µl in denaturing buffer (20 mM MOPS (pH 7); 2.2 M formaldehyde; 50 % (v/v) formamide), heated for 5 min at 65°C and snap cooled on ice. RNA loading buffer (3µl) (0.9% (w/v) bromophenol blue; 0.09% (w/v) xylene cyanol; 25% (w/v) ficoll) was added and the samples briefly centrifuged. Samples were loaded on a 1.2% (w/v) agarose gel (1X Tris/Borate/EDTA buffer) and electrophoresed at 150 V until the dye front had run 8 cm. The RNA was then visualised by trans-illumination with short-wavelength UV light at 302nm. A polyA$^+$ RNA isolation kit (Dynal® Oligo $(dT)_{25}$ mRNA Purification kit) was used to extract polyA$^+$-RNA (mRNA) from 100 µg total RNA, according to the supplier's instructions. The polyA$^+$ mRNA was eluted in a volume of 10 µl and was quantified spectrophotometrically.

The LabelStar™ Array Kit cDNA Labelling Module (Qiagen) was utilised to generate single-stranded cDNA fragments from mRNA. One microgram mRNA was adjusted to 18 µl with RNase-free water, 2 µl denaturation solution plus was added to the solution, and incubated at 65°C for 5 min. The solution was then snap cooled on ice and the following components were added to give the final volume of 50 µl: 5 µl reverse transcriptase buffer (10X), 5 µl dNTP (0.5 mM) mix, 5 µl oligo-dT primers (20 µM), 0.5 µl RNase inhibitor (40 units/µl), 12 µl RNase-free water, 2.5 µl LabelStar Reverse Transcriptase and 5 µl (α-^{33}P)-dCTP. The reaction mixture was incubated at 40°C for 120 min, after which 2 µl Stop Solution was added to stop the reaction. The labelled cDNA target was purified using the Cleanup Module of the LabelStar Array Kit (Qiagen) and eluted in 10 µl (10mM Tris-Cl, pH 8). A *Giardia lamblia* PFP bacterial gene, cloned in pBluescript II KS, was linearised (1.5 µg) by incubating with 2 U *Sal*I, 2 µl 10X

Buffer (Fermentas) and RNase-free water to a total volume of 20µl at 37°C for 4 hours. The linearised *G. lamblia* PFP gene was transcribed *in vitro* from the T7-promoter, according to the instructions provided by the supplier of the T7 mMessage mMachine (Ambion®). The *in vitro*-transcribed *G. lamblia* PFP gene was polyadenylated at the 3' end
using the Poly(A) Tailing Kit (Ambion®). Different amounts of *in vitro*-transcribed *G. lamblia* PFP mRNA were mixed with 100 µg of *Sorghum bicolor* total RNA before target synthesis. The percentage of the spiked *G. lamblia* PFP mRNA was calculated assuming that mRNA represents 2% of the total RNA pool.

Hybridisation procedure

The macroarray filters were prehybridised for 10-16 hours in 20 ml Church and Gilbert buffer (0.5 M sodium phosphate (pH 7.2); 7% (w/v) SDS; 0.94 mM EDTA) containing 10 µg/ml denatured fragmented salmon sperm DNA, and incubated at 65°C in hybridisation bottles within a rotary hybridisation oven (Hybaid). For hybridisation, the cDNA target was first denatured at 95°C for 10 min and added to the prehybridisation buffer. Following overnight hybridisation at 65°C, the filters were washed with 2X SSC (155 mM tri-sodium citrate; 150 mM NaCl), 0.1 (w/v) SDS for 15 min at 65°C and in 1X SSC, 0.1% SDS at 65°C for 15 min to remove traces of unbound target. The membranes were sealed in plastic and exposed to a high-resolution phosphor screen (Cyclone™) for 16-24 hours. The image on the phosphor screen was captured by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard).

Data analysis

Macroarray raw image data were analysed using Spot Densitometry Analysis Software (Alpha Image). The hybridisation signal intensity data generated was exported to Microsoft Excel for further manipulation. The average signal intensity of duplicate spots was normalised by subtracting the average background of two

negative control spots, i.e. blank area without the target DNA (buffer only). Duplicate spot signals were quantitatively compared and excluded for further analysis if the coefficient of variation (CV) yielded a value of greater than 10 % (Equation 1). Furthermore, in order to standardise the signal intensity for inter-array comparison, the data was expressed relative to a spiked external standard (*G. lamblia* PFP) [Equation 2].

Equation 1

$$CV = \left[\frac{SD}{\left(\frac{S1 + S2}{2} \right)} \right] \times 100\%$$

Where:

CV = coefficient of variation

SD = standard deviation between spots

S = spot

Equation 2:

$$NSI = \left[\frac{\left(\frac{S1 + S2}{2} \right) - \left(\frac{B1 + B2}{2} \right)}{\left(\frac{ES1 + ES2}{2} \right)} \right]$$

Where:

NSI = normalised signal intensity

S = Spot

B = Background

ES = External standard, i.e. *G. lamblia* PFP.

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Chapter 4

Expression analysis of genes involved in primary carbohydrate metabolism in low and high sucrose accumulating sorghum genotypes

Abstract

The hypothesis of this study was that differences in sucrose accumulation in the internodal tissues of different sorghum genotypes are based on differences in gene expression. Sucrose and hexose concentrations of two sorghum genotypes, GH1 and SH2, were therefore determined in immature and maturing internodal tissues at the boot and soft dough stages. Total sugar concentrations were high during softdough stage in both genotypes in comparison to the boot stage and sucrose was the dominant sugar in both the immature and maturing internodes at both stages. Hexose concentrations were high during boot stage in both immature and maturing internodes and decreased significantly at the softdough stage while sucrose concentration levels increased. Based on these differences in the sugar concentrations between different internodal tissues, developmental stages and genotypes, the gene expression patterns in the various tissues were compared using macroarrays to identify potential differentially expressed genes. A number of genes encoding enzymes involved in sugar metabolism were found to be differentially expressed. Fourteen of these genes were randomly selected to confirm the macroarray data by semi-quantitative RT-PCR. Only seven of the 14 genes (50%) showed similar expression patterns to that observed in the macroarrays. Finaly, the activities of these seven enzymes were determined to establish if there was a direct correlation between the changes in the transcript levels and enzyme activity. Only the activities of soluble acid invertase, sucrose synthase and alcohol dehydrogenase, i.e. 43% of the genes investigated, correlated with their respective transcript levels. This study demonstrated that some genes are differentially expressed in sorghum tissues with varying capacities to accumulate sucrose.

4.1 Introduction

Sorghum (*Sorghum bicolor* (L) Moench) is a globally-cultivated crop that is called a 'Life Saver' in some areas, due to its ability to survive in semi-arid and arid regions under hot and dry environmental conditions (Mullet *et al*., 2002). The grain of sorghum is used as a food source, and other parts of the plant are used as substrates for alcoholic beverages, as well as for colourants, medicines, cosmetics, and textiles. Most importantly, some genotypes have the ability to store sugars in high concentrations in their stems, which is used as a source of sucrose (Lingle, 1987).

Sucrose is the photoassimilate translocated from the leaves and stored in the stalks (internodes) of sorghum (Tarpley *et al*., 1994). Sorghum internodes start to accumulate sucrose when the panicle (inflorescence) has formed, all leaves are fully expanded and the flag leaf is the last leaf to emerge at the boot stage; approximately 60 days after planting (Vanderlip and Reeves, 1972; McBee *et al*., 1982). During the boot stage there is no competition between the stalk and grain for sugars (Lingle, 1987). The total sugar concentration of sorghum internodes is lowest at the boot stage and highest at the soft dough stage. Soft dough stage begins after flowering when the seed is soft and a white milky fluid appears when the seed is squeezed, approximately 120 days after planting (Vanderlip and Reeves, 1972; Lingle, 1987; Vietor and Miller, 1990; Tarpley *et al.,* 1994). In addition, sucrose is the dominant sugar at all the stages. Interestingly, sorghum internodes are the dominant sink for photoassimilates during seed development (Vietor and Miller, 1990). The extent of sucrose accumulation in the internodes of sorghum genotypes varies (Tarpley *et al.,* 1994). Sweet genotypes accumulate higher amounts of non-structural carbohydrates, i.e. sucrose, glucose and fructose, in comparison to non-sweet genotypes during all stages of development (Lingle, 1987; Vietor and Miller, 1990; Miller and McBee, 1993; Tarpley *et al.,* 1994). In comparison to non-sweet genotypes the increased sucrose concentrations in sweet genotypes have been suggested to depend on differences in assimilate partitioning (Lingle, 1987).

Expressed sequence tag (EST) and DNA array analyses have been the methods of choice in the search for coarse regulatory mechanisms in plants (Carson *et al*., 2002; Casu *et al.,* 2005; Watt *et al*., 2005). Macroarray technology offers a potentially more efficient alternative to traditional library screening for the detection of differentially expressed genes, as the expression profiles of multiple cDNA fragments are generated simultaneously through a single hybridisation event. Successful application of this approach has been demonstrated in the identification of differentially expressed gene patterns in plants when they are, for example, exposed to different growth conditions or stimuli, or by comparing different developmental stages (Desprez *et al*., 1998; Kurth *et al.,* 2002; Casu *et al.,* 2005; Watt *et al*., 2005).

In this study, the sugar concentrations of various internodal tissues of two sorghum genotypes (low and high sucrose accumulating genotypes), at different developmental stages (boot and softdough stages), were first compared to verify possible differences in sucrose concentrations. Thereafter, tissues with significantly different sucrose concentrations were subjected to macroarray based gene expression analyses to identify potential, differentially expressed genes. The macroarray data was validated by semi-quantitative RT-PCR and, finally, changes in transcript levels were compared to changes in enzyme activity levels. We demonstrated that a combination of macroarray and semi-quantitative RT-PCR analyses is an effective technique to identify differentially expressed genes in sorghum, but also that these changes in transcript levels are not necessaraly reflected in the related enzyme activities.

4.2 Materials and Methods

4.2.1 Plant material

All experiments were conducted using plants that were grown in a tunnel. *Sorghum bicolor* seeds of genotypes GH1 and SH2 were grown in 5 L plastic pots, containing soil and vermiculite (1:1, v/v), fertilised once in a month with N, P and K (3:2:1) and watered every second day of a week. Plant material was harvested at the boot stage, i.e. when the panicle has formed, and all leaves are fully expanded and the flag leaf has emerged approximately 60 days after planting, and at the soft dough stage, i.e. after flowering, when the seed is soft and a white milky fluid appears when the seed is squeezed at approximately 120 days after planting (Vanderlip and Reeves, 1972). The panicle and peduncle were excised and the rind and nodal tissue discarded. Internodal tissues were then pooled into two-sugar accumulation phases; immature (internodes 1-3) and maturing internodes (internodes 5-8). The pooled internodes were frozen in liquid nitrogen and ground to a fine powder using an A11 Basic Analysis Mill (IKA®). The fine internodal tissue powder was stored in 50 ml polypropylene centrifuge tubes (Corning ®) at -80°C.

4.2.2 Sugar extraction and concentration determination

Soluble sugars (sucrose, glucose and fructose) were extracted in a buffer containing 70% (v/v) ethanol and 50 mM Tris-HCl (pH 7). Tissue samples (100 mg) were incubated at 70°C overnight in 1 ml (1:10 w/v) of freshly prepared buffer. Insoluble cell components were removed by centrifugation at 16000 $\boldsymbol{g}$ for 10 min at 4°C. Sucrose and hexose concentrations in the supernatant were determined using standard enzymatic methods as described by Bergmeyer and Bernt (1974).

4.2.3 Probe and macroarrays preparation

Expressed sequence tags (ESTs) identified as homologous to genes playing key roles in carbohydrate metabolism were used as probes in all hybridisation experiments. Thirty-four *Oryza sativa* ESTs were received from the MAFF DNA Bank in Tsukuba, Japan, four EST's were obtained from the South African Sugarcane Research Institute collection and ten genes from an in-house collection of the Institute for Plant Biotechnology. Probes and macroarrays were prepared as described in Material and Methods (Chapter 3; Bacterial clones, PCR amplification and macroarray production).

4.2.4 RNA extraction, *in vitro* RNA and target synthesis

Total RNA was extracted from pooled internodal tissues samples (internodes 1-3 and 5-8) collected from the GH1 and SH2 sorghum genotypes respectively during both the boot and softdough stages, as described in Materials and Methods of Chapter 3, RNA extraction, *in vitro* RNA and target synthesis. In addition, *in vitro* RNA synthesis and target synthesis were performed as described in Materials and Methods (Chapter 3, Bacterial clones, PCR and Macroarray production.

4.2.5 Hybridisation and data analysis

The macroarray membranes were prepared by immobilising the probe DNA fragments onto a positively-charged nylon Hybond-N+ membrane® (Amersham). These fragments were then denatured in 0.2 M NaOH and a 96-pin manual gridding device (V&P Scientific Inc., San Diego) was used to transfer 2 µl aliquots (containing 0.05pmol) of the denatured ESTs in duplicate to the membrane. The following controls were used: pBluescript II KS (-) plasmid, pGEM-T Easy plasmid (negative control), and bacterial *G. lamblia* PFP gene (positive external control). Membranes were air-dried and the probe DNA cross-linked by means of

short-wavelength UV-radiation (120 KJ.cm^{-1}) for 1.5 min per side (Hoefer UV-Crosslinker). Pre-hybridisation and hybridisation were performed as described in the Materials and Methods of Chapter 3, Hybridisation procedure. Data analysis was performed as described in Materials and Methods of Chapter 3, Data analysis.

Equation 3: $$ABC = \left[NSI1 - NSI2\right]$$

Where:

ABC = Absolute change

NSI = Normalised signal intensity

Equation 4: $$R = \left[\frac{NSI1}{NSI2}\right]$$

Where:

R = Ratio between signal intensities

NSI = Normalised signal intensity

4.2.6 Semi-quantitative RT-PCR

Total RNA (1 µg) was reverse transcribed to first-strand cDNA using a SuperScript™ III reverse transcriptase kit, according to the manufacturer's instructions (Invitrogen™). The synthesis was primed with oligo(dT) using SuperScript™ III reverse transcriptase in a total reaction volume of 20 µl. Two microlitre aliquots of this cDNA were subsequently used as a template for PCR amplification with gene-specific primers under stringent conditions. Annealing temperature and the number of cycles were optimised to ensure linearity for the quantification of the amplification product. Initially the actin gene was used as an internal control for data normalisation. Spot densitometry was conducted by ascertaining the integrated density value (IDV-score) of each PCR fragment, using the "AUTO BACKGROUND" option of AlphaEase image-analysis software (AlphaInnotech, San Leandro, CA, USA). Primer sequences and the specific PCR conditions for each gene are presented in Table 1.

Table 1. Sequences of the specific primers and the annealing temperatures used for semi-quantitative RT-PCR. The number of cycles used was optimised for each gene to ensure linearity between the template concentration and the IDV-score.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Number of cycles
Soluble acid invertase	GTGCTCATCTGCATTGCTGT	CTTGTGCCAATTGTTTGTGG	56 °C	34
Sucrose synthase	ATGGTATTCTCCGCAAGTGG	CCTGCGATTTCTTGGAATGT	56 °C	25
Sucrose transporter 2	GTGCTCATCTGCATTGCTGT	CTTGTGCCAATTGTTTGTGG	56 °C	28
Cellulase	TCAGGTGGGTGTAGGTGACA	GCTGTGGCGACTGTGAGATA	56 °C	30
Sucrose transporter 1	GTGCTCATCTGCATTGCTGT	CTTGTGCCAATTGTTTGTGG	56 °C	25
Sucrose phosphate phosphatase	GAAGGTGATGTCCGTGTGTG	TTACGCACCAAATCCTCTCC	56 °C	35
Alcohol dehydrogenase	GGATTCCCGTAGTGGGATTT	ATCGCACATGTTGCTCTCTG	56 °C	28
Fructose 1,6 bisphosphatase	AAAAACTGGGATGGGCCTAC	GATCTCCTCCACGTCATCGT	56 °C	30
Phosphoglucom utase	AGAGTTGGTTGAACGGATGG	CCAGTGCCAAAGCTTTCTTC	56 °C	40
Actin	TCA CAC TTT CTA CAA TGA GCT	GAT ATC CAC ATC ACA CTT CAT	52 °C	25-40

4.2.7 Enzyme assays

4.2.7.1 Protein extraction and quantification

Crude protein extracts were made from internode 1-3 and 5-8 tissues during both boot and softdough stages of GH1 and SH2 sorghum genotypes. The extraction buffer consisted of 50 mM Tris-HCl, (pH 8.0), 2 mM EDTA and 5 mM dithiothreitol (DTT, Roche Diagnostics, Mannheim, Germany). Extracts were centrifuged for 2 min at 16000 ***g*** and 4°C. Supernatants were transferred to new tubes and again centrifuged as before. For desalting the crude protein extracts, supernatants were transferred to Sephadex G-50 (Sigma-Aldrich Fine Chemicals, St. Louis, Missouri, USA) spin columns pre-equilibrated in extraction

buffer and centrifuged for 2 min at 2000 ***g*** at 4 °C. Protein concentration was determined according to Bradford (1976), using a commercially available protein assay solution (Bio-Rad, Hercule, California, USA). Bovine albumin (Fraction V, Roche) was used as the protein standard.

4.2.7.2 Alcohol dehydrogenase

Alcohol dehydrogenase activity was measured according to a previously published protocol (Russel *et al*., 1990). Desalted protein (20 µl) was added to an assay buffer which consisted of 100 mM Tris-HCl (pH 8.5) and 5 mM absolute ethanol. The reactions were started by adding 2 mM NAD^+ (Roche) in a final volume of 250 µl. The reduction of NAD^+ was monitored at 340 nm in a PowerWaveX spectrophotometer (Bio-Tek Instruments, Winooski, VT).

4.2.7.3 Sucrose synthase in the breakdown direction

The activity of SuSy was assayed according to Schäfer *et al.* (2004). The extraction buffer consisted of 100 mM Tri-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM EDTA and 10 mM DTT. Complete® protease inhibitor cocktail tablets (Roche) were added to the extraction buffer just prior to use, according to manufacturer's instructions. Crude protein and Sephadex G50 columns were prepared as before (4.2.7.1).

4.2.7.4 Sucrose synthase in the synthesis direction

Desalted crude protein (20 µl) was added to 230 µl assay buffer (100 mM Tri-HCl [pH 7.5], 15 mM $MgCl_2$, 10 mM UDPGluc and 10 mM Fructose) (Zeng *et al.,* 1998). The reaction was incubated for 30 min at 35 °C and stopped by adding 5 mM KOH with subsequent incubation at 100 °C for 10 min to destroyed unutilised hexoses. The sucrose concentration was determined by adding 200 µl anthrone reagent (0.14% anthrone in 14.6 M H_2SO_4) to 50 µl sample and measuring absorbance at

620 nm. Absolute amounts of sucrose were calculated from a standard curve containing a range of sucrose between 0 and 200 nmol.

4.2.7.5 Soluble acid invertase

Soluble acid invertase activity was assayed at 37 °C using a protocol modified from Zhu *et al*. (1997). Desalted crude protein (50 µl) was added to an assay buffer (1 M sodium acetate [pH 4.5]) and the assay was started by adding 5 µl (0.5 M the final concentration) sucrose in total reaction of 250 µl. Fifty microlitres were removed at 30 min intervals for 1.5 hr and the assay was stopped by the addition of 3 M Tris base, followed by heating for 3 min at 100 °C. Hexose determinations were performed as described above (4.2.2).

4.2.7.6 ADP-glucose pyrophosphorylase

AGPase activity was determined according to Beckles *et al*. (2001). The spectrophotometric assay contained 100 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 1.5 mM NaPPi, 1 mM ADP-Gluc, 1.3 mM NADP, 4 units phosphoglucomutase, 10 units of Gluc-6-P dehydrogenase and 20 µl desalted crude protein in a total volume of 250 µl.

4.2.7.7 Fructose 1, 6-bisphosphatase

Fructose 1, 6-bisphosphate activity was quantified spectrophotometrically at 340 nm in an enzyme-coupled assay, by monitoring NADPH production (Rashid *et al.,* 2002). The assay mixture (250 µl) contained: 100 mM Tri-HCl (pH 8.0), 0.4 mM NADP, 20 mM $MgCl_2$, 0.5 units phosphoglucose isomerase, 0.5 units glucose 6-phosphate dehydrogenase and 20 µl desalted crude protein.

4.3 Results and Discussion

4.3.1 Sugar accumulation in selected sorghum genotypes

The sugar concentrations of two different tissues types, i.e. immature (internodes 1-3) and maturing internodes (internodes 5-8), of two sorghum genotypes were determined at two different developmental stages, i.e. boot and softdough stage. The total sugar concentrations, i.e. that of sucrose, glucose and fructose, of genotype SH2, in both immature and maturing internodes were higher than that of genotype GH1 during boot stage (Figure 1[a] and [b]). Vietor and Miller (1990) reported a similar pattern for total sugar concentrations when they compared a sweet and grain genotype during the boot stage. During softdough stage total sugar concentrations in the immature internodes were highest in genotype SH2 (Figure 1[c]), but in the maturing internodes the opposite was true, i.e. GH1 had the highest total sugar concentrations (Figure 1[d]). It has been shown that the concentrations of non-structural carbohydrates in the upper and lower internodes of sweet genotype were 2 and 1.3 times higher than the grain genotype (Vietor and Miller, 1990). The differences in stem non-structural carbohydrates between sweet and grain genotypes before physiological maturity have been associated with large differences in grain (seed) yields in the two genotypes (Vietor and Miller, 1990).

The patterns of sucrose, glucose and fructose accumulation between the two different genotypes, the two developmental stages and the two tissue types were also investigated. Sucrose was the predominant sugar in all the tissues at both developmental stages (Figure 1). At boot stage, the immature internodes of SH2 had higher sucrose concentrations than the GH1 immature internodes (Figure 1[a]). In SH2 immature internodes, the ratio between sucrose and the invert sugars was slightly different, sucrose represented about 37% of the total sugars with glucose and fructose representing 33% and 28% respectively. While sucrose concentration in immature GH1 internodes represented about 65% of

the total sugars. Similar patterns in non-structural carbohydrate accumulation in immature internodes (internodes 1-3) of sorghum genotypes have been reported (Lingle, 1987; Vietor and Miller, 1990; Tarpley *et al*., 1994). Lingle (1987) found sucrose (50%) to be predominant at boot stage in sweet genotype, with glucose and fructose making up the remainder.

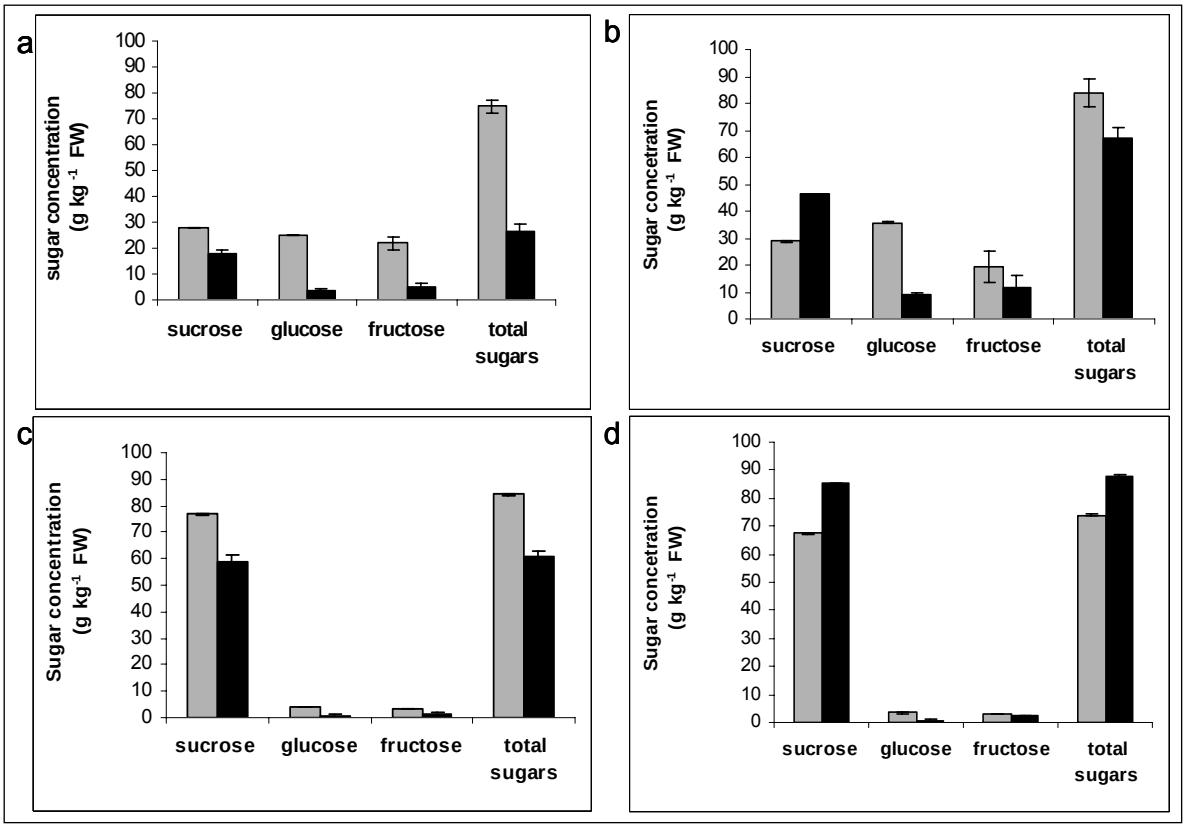

Figure 1: Internodal sugar concentrations of two *Sorghum bicolor* genotypes at different developmental stages. Sucrose, glucose and fructoses concentrations were determined in the immature and maturing internodes of two sorghum genotypes, SH2 and GH1, during the boot (a & b) and softdough (c & d) stages. Values represent the average of three independent samples and error bars represent the standard deviation. Figures (a) represents sugar levels during the boot stage in immature internodes of SH2 (■) & GH1 (■) and (b) maturing internodes of SH2 (■) & GH1 (■). Figures (c) represents sugar levels during the softdough stage in immature internodes of SH2 (■) & GH1 (■) and (d) maturing internodes of SH2 (■) & GH1 (■).

In the maturing internodes during the boot stage, SH2 had low sucrose concentrations compared to GH1 (Figure 1[b]). Sucrose represented about 67% of the total soluble carbohydrate in the maturing tissue of GH1, with glucose (14%) and fructose (17%) making up the remainder. Interestingly, in the SH2 genotype's maturing internodes, glucose was the main sugar, making up about 42% of the total sugars, with sucrose and fructose representing 34% and 23% respectively. In the lower internodal tissues (maturing internodes) of sorghum genotypes during the boot stage, differences in accumulation of non-structural carbohydrates i.e. sucrose, fructose and glucose were observed (Vietor and Miller, 1990; Tarpley *et al*., 1994). In contrast to our findings, sucrose was the predominant soluble carbohydrate, with glucose slightly higher than fructose.

At softdough stage, the immature internodes of SH2 had the highest sucrose concentrations (77 g/kg FW) compared to GH1 (59 g/kg FW) immature internodes (Figure 1[c]). In the same immature internodes, the glucose and fructose concentrations of the SH2 genotype were significantly higher that that of the GH1 genotype. Similar differences in sugar concentrations and composition has previously been reported for sorghum immature internodes at the softdough stage (Ferraris and Charles-Edwards, 1986; Lingle, 1987; Tarpley *et al*., 1994; Dolciotti *et al*., 1998). Interestingly, the ratio of sucrose in the immature internodes of genotype SH2 was slightly higher (77%) compared to the maturing internodes (67%) (Figure 1[c] and [d]). In contrast, Tarpley *et al*., (1994), found non-structural carbohydrates to be the same in the lower (maturing) and upper (immature) internodes of sweet and grain sorghum genotypes during the softdough stage. This slight difference in sucrose ratio in the SH2 genotype's immature and maturing internodes in this study could be ascribed to environmental factors, i.e. cold temperatures and rainy weather during the time of harvesting. These could influence sugar concentrations based on a fresh weight level, reduced growth (cold temperatures) in the immature tissues will increase sugar concentrations and the uptake of water will dilute sugars in the maturing internodes. Conversely, the GH1 genotype probably had a faster growth rate

(harverst early before onset of cold temperatures and rainy weather) compared to the SH2 genotype, sucrose concentration was high in the maturing internodes compared to immature internodes (Figure 1 [c] and [d]). Similar trends of growth and higher sucrose concetration were observed when sweet sorghum genotypes were planted in high light intensity and warmer temperature in the green house (Lingle, 1987).

The results of this study suggest that non-structural carbohydrate concentrations in the internodes of sorghum genotypes SH2 and GH1 vary between the boot and softdough stages. At the boot stage, the total sugar levels, i.e. sucrose, fructose and glucose, were low in both immature and maturing internodal tissues, whereas at the softdough stage there was an increase in total sugar levels in the same internodal tissues of both genotypes (Figure 1). Interestingly, in both genotypes the increase in total sugars at the softdough stage was due to an increase in sucrose concentrations, whereas invert sugars decreased (Figure 1[c] and [d]). When immature internodal tissues of genotypes SH2 and GH1 were compared during the boot and soft dough stages, genotype SH2 had a higher sucrose concentration than genotype GH1 at both stages. In contrast, when maturing internodes of both genotypes were compared, GH1 had higher sucrose concentrations than genotype SH2 in both stages. Therefore results of this study suggest that during vegetative growth (boot stage) and at the reproductive growth (softdough stage), the immature internodes of SH2 and maturing internodes of GH1 genotypes respectively are efficient in assimilating sucrose. These differences in the patterns of non-structural carbohydrates accumulation provide a platform to investigate the differential expression of genes during sugar accumulation.

4.3.2 Differential expression of genes in sorghum internodal tissues with different sugar accumulation patterns

The hypothesis of this study was that differences in the abilities of internodal tissues of different sorghum genotypes to accumulate sucrose during

development may be based on differences in gene expression. Based on differences in sucrose concentrations found during the boot and soft-dough stages, between the different internodal tissues (immature and maturing internodes) and the different sorghum genotypes (GH1 and SH2), differential gene expression in these different tissues was therefore assessed using macroarrays.

The precision of any macroarray is dependent on its technical reproducibility, i.e. low technical variation. Consequently, the hybridisation signals generated were normalised by subtracting the background and expressing the data relative to spiked *in vitro*-transcribed *G. lamblia* PFP RNA (external control) as described in Chapter 3. The coefficient of variation (CV) was also assessed for all membranes. To evaluate intra-array reliability, the signal intensities of duplicate spots on the same membrane were compared. If the hybridisation signals of the duplicate spots generated a CV above 10%, they were omitted for further analysis. On this basis, each array tested yielded an average of 96% (92 out of 96 spots) valid duplicate spots (CV less than 10%), with the lowest being 91%. Generally, a coefficient of variation of less than 15% between duplicate spots is thought to be good indicator of reproducibility of a macroarray (Watt *et al*., 2005; Clarke and Zhu, 2006).

To unmask possible coarse regulatory events involved in the different patterns of sugar accumulation in sorghum internodal tissues and genotypes, differentially expressed genes were assessed during the boot and softdough stages. To evaluate the differential expression of genes between two different arrays (inter-array), the means of the normalised hybridisation signals were subjected to statistical analysis using one way ANOVA, with p-values below 0.05 being taken as an indication or probability of a gene being differentially expressed. In addition, for a gene to be said to be "significantly differentially expressed", the difference in normalised hybridisation signal intensities of a gene being compared between two arrays should be greater than or equal to one (Equation

3), and the ratio of the hybridisation signals should be greater than or equal to two (Equation 4). Generally, for a gene to be identified as being differentially expressed, the hybridisation signal intensities should differ 1.5-2-fold (Watt *et al*., 2005; Clarke and Zhu, 2006). The problem with the fold condition as the only measure for differential expression is that many false positives are identified among weak hybridisation signals (Clarke and Zhu, 2006). Therefore, a second condition, i.e. that the absolute change in the hybridisation signal intensity should be more than one, was included in these analyses.

When the immature internodal tissues of the two genotypes were compared at boot stage, 14 genes were found to be significantly differentially expressed (Table 2). All the genes were up-regulated in the immature internodes of genotype GH1 relative to the same tissue of genotype SH2. The genes that were significantly differentially expressed encode for enzymes in a number of enzymatic pathways, i.e. sucrose metabolism (e.g. SPP, SPS and SAI); cell wall synthesis (e.g. CWI and UGPase); glycolysis (e.g. hexokinase and pyruvate kinase) and the citric acid cycle (e.g. malate dehydrogenase).

Table 2: Genes with significantly different expression levels in the immature internodal tissues of sorghum genotypes SH2 and GH1 during boot stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
5	pANSI 510 (NI)	up-regulated in GH1	1.0	5.1	0.0132
6	malate dehydrogenase	up-regulated in GH1	1.1	1.9	0.0061
7	Pyruvate kinase	up-regulated in GH1	1.4	2.5	0.0047
15	Cellulase	up-regulated in GH1	1.1	2.0	0.0051
21	Cell wall invertase (CWI)	up-regulated in GH1	1.3	2.4	0.0019
23	Hexokinase	up-regulated in GH1	1.4	2.2	0.0032
30	Sucrose phosphate phosphatase (SPP)	up-regulated in GH1	1.4	1.9	0.0033
37	Sucrose transporter 1 (sut1)	up-regulated in GH1	1.6	2.5	0.0009
41	Soluble acid invertase (SAI)	up-regulated in GH1	2.8	2.7	0.0072
42	Sucrose transporter (sut2)	up-regulated in GH1	2.3	2.6	0.0042
43	UDP-glucose pyrophosphorylase (UGPase)	up-regulated in GH1	1.0	2.1	0.0022
46	Fructose transporter (ght6)	up-regulated in GH1	1.9	2.2	0.0003
48	pASE510 (enolase)	up-regulated in GH1	1.0	3.7	0.0019
18	Sucrose phosphate synthase (SPS)	up-regulated in GH1	1.0	2.3	0.0023

When the same comparison was done between the maturing internodal tissues of the two genotypes four genes were found to be significantly differentially expressed (Table 3). Three genes, e.g. sut2, SuSy and trehalose phosphatase, were up-regulated in the maturing internodes of genotype SH2 relative to GH1, and one gene, e.g. cellulase, was up-regulated in GH1 relative to SH2.

Table 3: Genes with significantly different expression levels in the maturing internodal tissues of sorghum genotypes SH2 and GH1 during boot stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
42	Sugar transporters2 (sut2)	up-regulated in SH2	1.0	2.0	0.0240
13	Sucrose synthase (SuSy)	up-regulated in SH2	1.0	2.0	0.0025
15	Cellulase	up-regulated in GH1	1.0	3.0	0.0235
2	Trehalose phosphatase	up-regulated in SH2	1.0	2.0	0.0110

When the immature and maturing internodal tissues of genotype GH1 were compared for differential gene expression as described above, seven genes were significantly differentially expressed (Table 4). All seven genes were up-regulated in the immature internodes of genotype GH1. Two of these genes, hexokinase and enolase, are involved in glycolysis; one in gluconeogenesis (fruc-1.6- bisphosphatase) and a sucrose degrading gene (SAI), and two are sucrose transporters (sut1 and sut 2).

Table 4: Genes with significantly different expression levels in the immature and maturing internodal tissues of sorghum genotype GH1 during the boot stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
23	Hexokinase	up-regulated in 1-3	1.0	2.0	0.0410
25	Sucrose transporter 1 (sut1)	up-regulated in 1-3	1.4	2.0	0.0001
38	Aconitate hydratase	up-regulated in 1-3	1.0	2.0	0.0083
41	Soluble acid invertase (SAI)	up-regulated in 1-3	2.1	2.0	0.0111
42	Sucrose transporter 2 (sut2)	up-regulated in 1-3	1.8	2.0	0.0171
44	Fructose-1,6-bisphosphatase	up-regulated in 1-3	1.1	2.0	0.0197
48	pASE510 (enolase)	up-regulated in 1-3	1.0	2.0	0.0028

Similarly, at the softdough stage, immature internodal tissues of the two genotypes were compared for differentially expressed genes. Seven genes were significantly differentially expressed in the immature internodal tissues of SH1 genotype (up-regulated) (Table 5). These significantly differentially expressed genes encode enzymes that are involved in a number of pathways: sucrose synthesis (e.g. SPS and SPP); cell wall synthesis (e.g. CWI and cellulase) and glycolysis (e.g. pyruvate kinase and hexokinase).

Table 5: Genes with significantly different expression levels in the immature internodal tissues of sorghum genotypes SH2 and GH1 during the softdough stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
7	Pyruvate kinase	up-regulated in SH2	1.4	2.0	0.0149
8	Alcohol dehydrogenase (ADH)	up-regulated in SH2	1.7	2.0	0.0019
15	Cellulase	up-regulated in SH2	1.1	2.0	0.0030
18	Sucrose phosphate synthase (SPS)	up-regulated in SH2	1.0	3.0	0.0073
21	Cell wall invertase (CWI)	up-regulated in SH2	1.3	3.0	0.0009
30	Sucrose phosphate phosphatase (SPP)	up-regulated in SH2	2.6	3.0	0.0047
23	Hexokinase	up-regulated in SH2sweet	1.0	2.0	0.0035

When maturing internodal tissues of the two genotypes were compared, eleven genes were significantly upregulated in SH2 (Table 6). These genes encode enzymes involved in a number of pathways: cell wall metabolism (e.g. CWI and UGPase); gluconeogenesis (e.g. fructose-1-6-bisphosphatase); glycolysis (e.g. alcohol dehydrogenase) and the citric acid cycle (e.g. malate dehydrogenase and aconitate hydratase).

Table 6: Genes with significantly different expression levels in the maturing internodal tissues of sorghum genotypes SH2 and GH1 during the softdough stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
6	malate dehydrogenase	up-regulated in SH2	1.3	2.0	0.0504
7	Pyruvate kinase	up-regulated in SH2	1.3	2.2	0.0110
8	Alcohol dehydrogenase (ADH)	up-regulated in SH2	1.7	2.2	0.0005
25	Sugar transporters 1 (sut 1)	up-regulated in SH2	1.3	2.5	0.0368
26	AGPase (SC)	up-regulated in SH2	1.3	3.8	0.0210
15	Cellulase	up-regulated in SH2	1.3	4.5	0.0106
16	Alcohol dehydrogenase(SC) (ADH)	up-regulated SH2	1.1	1.9	0.0006
21	Cell wall invertase (CWI)	up-regulated in SH2	1.0	3.1	0.0006
38	Aconitate hydratase	up-regulated in SH2	1.4	2.4	0.0502
43	UDP-glucose pyrophosphorylase (UGPase)	up-regulated in SH2	1.4	2.2	0.0296
44	Fructose-1,6-bisphosphatase	up-regulated in SH2	1.6	3.9	0.0048

Internodal tissues of GH1 genotypes were compared for significantly differentially expressed gene during the softdough stage and two were significantly differentially expressed in the immature internodal tissues of GH1 genotype (up-regulated in internodes 1-3) (Table 7). These genes encode enzymes involved in cell wall metabolism (e.g. cellulose) and starch metabolism or glycolysis (e.g. phosphoglucomutase).

Table 7: Genes with significantly different expression levels in the immature and maturing internodal internodes tissues of sorghum genotype GH1 during the softdough stage

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
14	Phosphoglucomutase	up-regulated in 1-3	1.0	2.0	0.0016
15	Cellulase	up-regulated in 1-3	1.0	2.8	0.0187

When genotype SH2 internodal tissues were compared for differentially gene expression, six genes were significantly expressed in the maturing internodal tissues of SH2 (up-regulated in internodes 5-8) (Table 8). These significantly differentially expressed genes encode enzymes that are involved in a number of pathways: sucrose transporters (e.g. sut1); starch synthesis (e.g. AGPase); cell

wall metabolism (e.g. cellulose synthase); gluconeogenesis (e.g. fructose-1,6-bisphosphatase) and glycolysis (e.g. alcohol dehydrogenase).

Table 8: Genes with significantly different expression levels in the immature and maturing internodal internodes tissues of sorghum genotype SH2 during the softdough stage.

Clone number	EST (gene)	Relative change	Absolute change	Ratio between signal intensities	p-value
25	Sugar transporters (suct1)	up-regulated in 5-8	1.6	3.8	0.0197
11	AGPase (SC)	up-regulated in 5-8	1.5	5.8	0.0061
16	Alcohol dehydrogenase (SC) (ADH)	up-regulated in 5-8	1.5	2.9	0.0033
19	Cellulase synthase	up-regulated in 5-8	1.0	2.4	0.0114
26	ADP-glucose pyrophosphorylase (AGPase)	up-regulated in 5-8	1.0	2.0	0.0264
44	Fructose-1,6-bisphosphatase	up-regulated in 5-8	1.0	1.9	0.006

Inter-array analyses of hybridisation signal intensities provided a means of identifying potentially differentially expressed genes between different sorghum genotypes and internodal tissues that have different sugar accumulation properties. During the boot and softdough stages, 25 and 22 genes were found to be significantly differentially expressed between different internodal tissues and genotypes respectively. Although these genes were only identified as significantly differentially expressed after rigorous criteria were met, in most cases the trend was always the same, i.e. genes were always up- or down-regulated in one of the tissue types that were compared. This and the well documented error-prone nature of high throughput technologies (Czechowski *et al*., 2004; Le Gall *et al*., 2005; Jolly *et al*., 2005; Clarke and Zhu, 2006; Nakano *et al.,* 2006) necessitated the validation of the DNA array data by an independent method such as quantitative RT-PCR or northern blot analysis.

4.3.3 Verification of macroarray data with semi-quantitative RT-PCR

As discussed above, a number of genes were found to be significantly differentially expressed (up- or down regulated) in one particular tissue type, e.g. immature internodes or genotype GH1, only. This raised some concerns about

the validity of the macroarray data. Fourteen significantly differentially expressed candidate genes were therefore arbitrarily selected for semi-quantitative RT-PCR analyses. Gene-specific primer sets were designed based on the nucleotide sequence of the corresponding gene in the *O. sativa* sequence database. First strand cDNAs were reverse-transcribed from total RNA isolated from the same tissues used in the macroarray analyses. The relative band intensities of the semi-quantitative RT-PCR results are displayed in Figure 3. The expression patterns (up- or down-regulation) of only 7 of the 14 genes (50%) were the same as in the macroarray analyses. Five of seven genes that gave different results than in the macroarray analyses, showed no significant differences in gene expression levels with the semi-quantitative RT-PCR analyses (Figure 2 a, b, d, g and m) and, the other two genes gave exactly the opposite results than in the macroarray analyses (Figure 2 f and j). Five of the seven (71%) experiments that gave different results in the macroarray and semi-quantitative RT-PCR analyses were analyses where tissues from the two different genotypes were compared to each other. For the seven experiments that gave similar results for the two methods, only three (43%) involved inter-genotype comparisons, suggesting that the variability might be bigger between genotypes than between different tissues within a particular genotype.

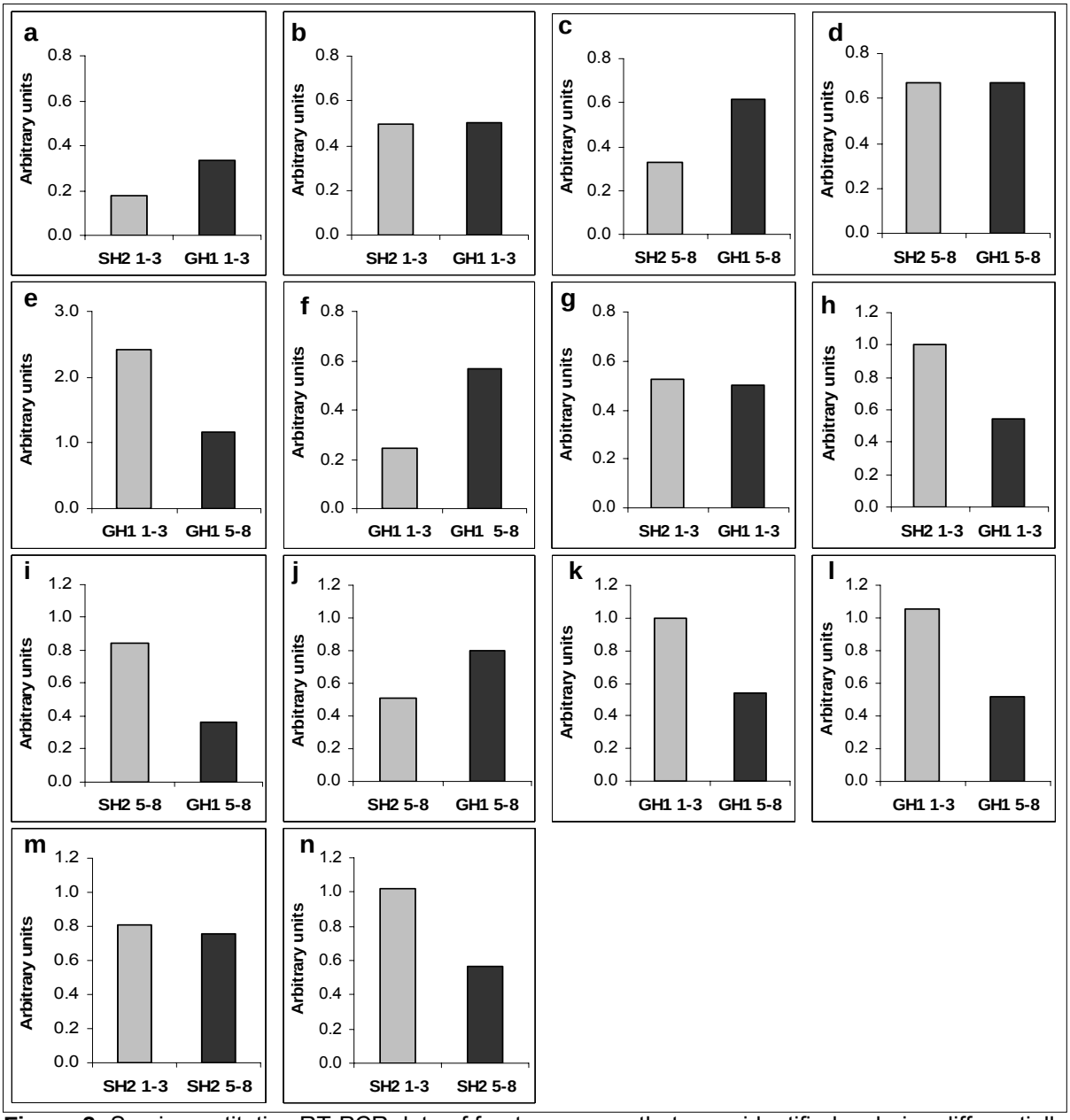

Figure 2: Semi-quantitative RT-PCR data of fourteen genes that were identified as being differentially expressed using macroarrays. Relative signal intensities are plotted in the order as listed: (a) SAI SH2 1-3 vs. GH1 1-3 (b) suct2 GH1 1-3 vs. SH2 1-3 (c) SuSy GH1 5-8 vs. SH2 5-8 (d) Cellulase GH1 5-8 vs. SH2 5-8 (e) SAI GH1 1-3 vs. GH1 5-8 (f) suct1 GH1 1-3 vs GH1 5-8 (g) ADH GH1 1-3 vs. SH2 1-3 (h) SPP GH1 1-3 vs. SH2 1-3 (i) ADH GH1 5-8 vs. SH2 5-8 (j) Fruc-1-6-bisphosphate GH1 5-8 vs. SH2 5-8 (k) Cellulase GH1 1-3 vs.GH1 5-8 (l) Phosphoglucomutase GH1 1-3 vs. GH1 5-8 (m) AGPase SH2 1-3 vs. SH2 5-8 (n) suct1 SH2 1-3 vs. SH2 5-8.

Similar contradictory results for DNA array and semi-quantitative RT-PCR analyses have been observed previously (Czechowski *et al*., 2004; Le Gall *et al*., 2005; Jolly *et al*., 2005 Nakano *et al.,* 2006). The results from these methods may be affected by the selected sequence of the probes or primers and

biological variation of the samples. With only the rice and Arabidopsis genome sequence completed and published preparations of other plant DNA arrays often rely on ESTs from these two genomes. In this study, both rice and sugarcane sequences were used as probes. Therefore, the possibility exists that sequences coding for different isoforms of the same enzyme can cross-hybridise with the probe sequence (Harai *et al*., 2003). This cross-hybridisation may lead to identification of differentially expressed genes, which can be considered to be false positives. In contrast to macroarrays, which are less sensitive to minor mismatches in sequences (isoforms), semi-quantitative RT-PCR primers are usually gene specific and offer a higher sensitivity.

The variability in the data could also be confounded by using different samples for the preparation of cDNA for RT-PCR as this could introduce biological variation (Clarke and Zhu, 2006). In this study, different samples (although taken from the same tissues and harvested on the same day) were used for the preparation of target cDNA for the macroarray hybridisation experiments and for the RT-PCR analyses. Therefore, biological variation of the samples could also have contributed to the dissimilar expression pattern of the macroarray and RT-PCR data.

The precision of DNA-array detection is estimated by technical reproducibility and a good experiment should have a CV less than 15 % among technical replicates (Close *et al*., 2004; Clarke and Zhu, 2006). Macroarray data was normalised relative to an external control (spiked *in vitro*-transcribed *G. lamblia* PFP), which was added at a constant level (equal amounts in all arrays) in the target mRNA. In theory, values obtained from the external control should therefore have the same CV when hybridisation signal intensities (inter-array analysis) are compared. However, in this study, some arrays had a 50% CV for the external control when different arrays were compared. The high CV might mean that the cDNA synthesis, labelling and or hybridisation of the arrays were not reproducible enough. Therefore, the high CV of the external control between

arrays might have prejudiced the macroarray data and increased the chances of identification of false positives in the macroarrays.

Macroarray analysis requires familiarity with data generated in a high-throughput fashion and to acceptance of the fact that data are not always error-free. There are factors which can contribute to identification of false positives, e.g. probe cross-hybridisation, biological variation and data normalisation by an external control. To truly identify differentially expressed genes, probe and target should be hybridized under high stringency conditions to minimize cross-hybridisation between similar sequences. While sample pooling was used to reduce the effect of intra-sample variation and environmental effects, replicates of macroarrays should be used to estimate biological variation. Additionally, the high CV of the external control between arrays might have prejudiced the macroarray data and increased the chances of identifying false-positive differentially expressed genes in the macroarrays. A low CV (below 10%) between the external controls' intensities on different arrays is therefore a prerequisite for macroarray data normalisation and inter-array comparison to minimise the chances of identifying false-positive differentially expressed genes. Although the different methods also gave contradictory results, the combination of macroarray and semi-quantitative RT-PCR analyses enabled the identification of seven differentially expressed genes.

4.3.4 Changes in transcript levels and enzyme activities

An understanding of metabolic networks requires quantitative data about transcript levels, protein levels or enzyme activities and metabolite levels. Interactions between these three functional levels will depend on the structure of the metabolic and signalling network, and on the dynamics of transcript, protein and metabolite turnover (Gibon *et al.,* 2006). To verify whether a change in mRNA transcript levels, based on macroarray and semi-quantitative RT PCR analyses, lead to a similar change in enzyme activity, seven significantly

differentially expressed candidate genes were selected, for enzyme activity assays. The enzyme activity of three enzymes, i.e. SAI: GH1 1-3 vs. GH1 5-8 boot stage, SuSy: GH1 5-8 vs. SH2 5-8 boot stage and ADH: GH1 5-8 vs. SH2 5-8 softdough stage, were related to the changes of the respective transcript levels in the tissues compared (Figure 5 b, c and e). However, four enzyme activities, i.e. SAI: GH1 1-3 vs. SH2 1-3 boot stage; ADH: GH1 1-3 vs. GH1 1-3 softdough stage; fruc-1-6-bisphosphate: GH1 5-8 vs. SH2 5-8 softdough stage and AGPase: SH2 1-3 vs. SH2 5-8 softdough stage did not correlate to the change in the transcript level (Figure 5 a, d, f and g).

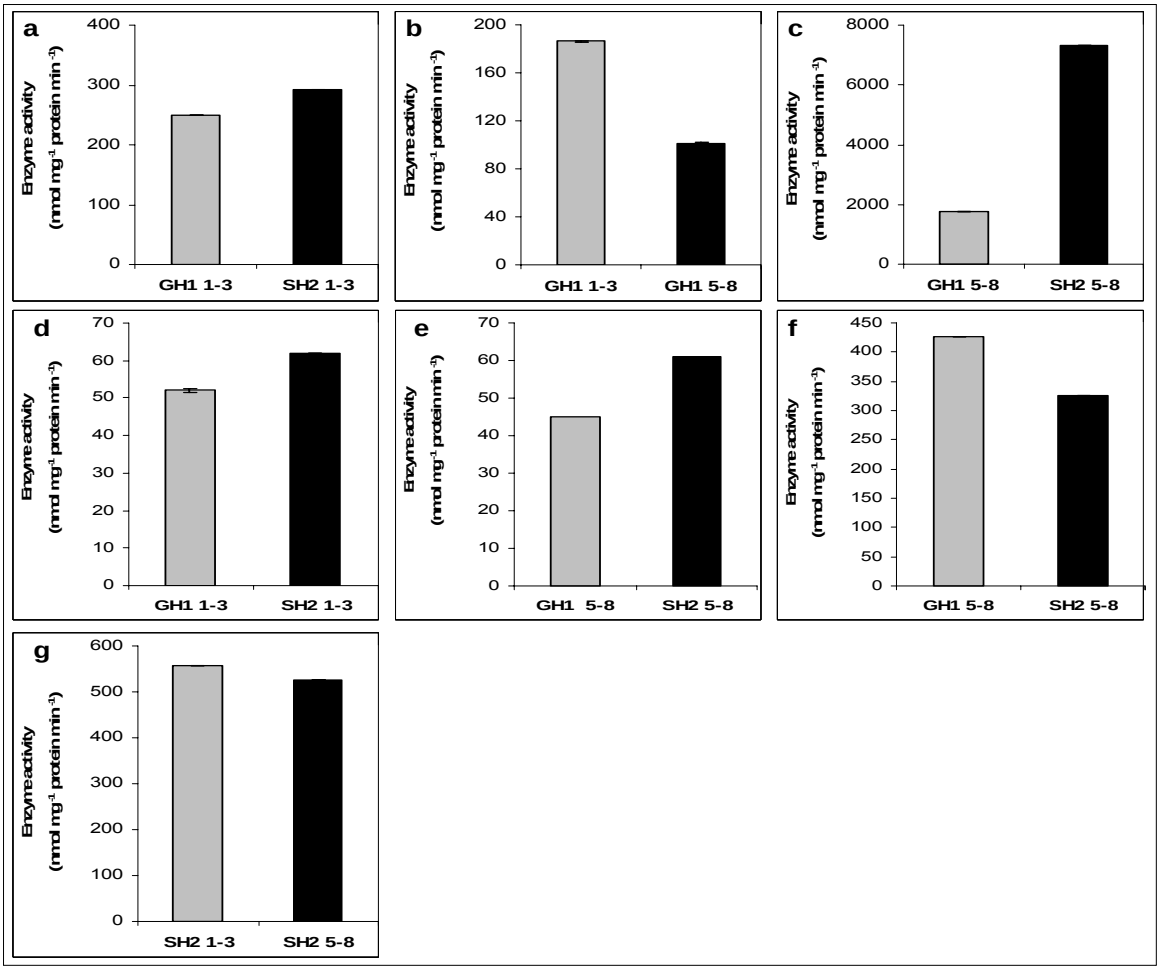

Figure 3: Enzyme activity of seven selected differentially expressed genes during the boot and softdough stages of two sorghum genotypes. Enzyme activity of (a) SAI GH1 1-3 vs. SH2 1-3 boot stage (b) SAI GH1 1-3 vs. GH1 5-8 boot stage (c) SuSy GH1 5-8 vs. SH2 5-8 boot stage (d) ADH GH1 1-3 vs. SH2 1-3 softdough stage (e) ADH GH1 5-8 vs. SH2 5-8 softdough stage (f) Fruc-1-6-bisphosphatase GH1 5-8 vs. SH2 5-8 softdough stage (g) AGPase SH2 1-3 vs. SH2 5-8 softdough stage are presented.

Similar trends were observed in Arabidopsis mutants compared to wild type during diurnal cycles (Gibon *et al.,* 2004; 2006) and in pepper (*Capsicum annuum* L.) during different developmental stages (García-Pineda *et al*., 2004; Rius *et al*., 2006). The reason for these dissimilarities may be that there is a delay before a change in transcript levels leads to a change in enzyme activity. Gibon *et al.* (2004) illustrated this by monitoring transcript (mRNA) expression and enzyme activity over time intervals. Furthermore, these dissimilarities may also suggest that enzyme activity is not entirely controlled at the transcriptional level, but rather post-transcriptionally (Gibon *et al.,* 2006). Therefore, caution should be exercised in interpreting transcript expression levels because these only represent one of the regulatory mechanisms of enzyme activity.

4.4 Conclusion

In concluding, seven differentially expressed genes were identified based on differences in the sucrose content of the internodal tissues of two sorghum genotypes, SH2 and GH1. Interestingly, the enzymatic activities of three genes (SAI, SuSy and ADH), correlated with their respective transcript levels. This study demonstrated that some genes are differentially expressed in sorghum genotypes tissues with varying capacities to accumulate sucrose. In addition, a combination of macroarrays and semi-quantitative RT-PCR is an effective technique to identify differentially expressed genes. The biological relevance of the findings will be discussed in Chapter 5.

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Chapter 5

General discussion and conclusions

The main aim of this study was to identify differentially expressed genes in low- and high-sucrose accumulating sorghum tissues/genotypes. Total sugar, i.e. fructose, glucose and sucrose concentrations vary in sorghum genotypes during development (Tarpley *et al.,* 1994). The highest total sugar concentrations in sorghum genotypes occur at the softdough and physiologically mature stages, whereas the lowest sugar concentrations occur at the boot stage (Ferraris and Charles-Edwards, 1986; Lingle, 1987; Tarpley *et al*., 1994). In addition, the stalk of sorghum is the dominant sink and sucrose is the dominant sugar (Vietor and Miller, 1990; Tarpley *et al*., 1994). Therefore, based on these sugar concentration differences in low and high-sucrose sorghum genotypes and immature and mature tissues it was hypothesised that these differences could be based on differential gene expression.

Various internodal tissues from two sorghum genotypes, GH1 and SH2, were assayed to determine their sugar concentrations at the boot and softdough stages. Similarly to previous studies, total sugar levels were high in internodal tissues at the softdough stage and low at the boot stage in both sorghum genotypes, with sucrose being the dominant sugar (Figure 1, Chapter 4). Sucrose concentrations were highest in genotype GH1 in the maturing internodes (internodes 5-8) at both stages of development. In contrast, the sucrose concentrations of the immature internodal tissues (internodes 1-3) of genotype SH2 were slightly higher in comparison to the maturing internodes during the softdough stage. These relative high sucrose concentrations in immature internodal tissues may have been influenced by changes in environmental conditions. Genotype SH2 grew much slower than genotype GH1, which resulted in it being harvested much later in the season when temperatures were low (cold) and rainy during the time of harvesting. Accumulation of sugars

in sorghum genotypes have been shown to vary due to environmental conditions such as higher light intensity and warmer temperatures (Lingle, 1987). Care should therefore be taken in future studies on the pattern of sugar accumulation in sorghum genotypes to ensure that plants are grown under identical conditions, preferably in a controlled environment such as a greenhouse.

Based on these differences in sugar concentrations in sorghum internodal tissues, a sensitive macroarray protocol was developed for the identification of differentially expressed genes (Chapter 3). There are several technical factors that need to be considered when designing macroarrays, including sensitivity, fold-changes in the levels of transcripts and reproducibility (Hanano *et al*., 2002; van Bakel and Holstege, 2004). Knowing the detection sensitivity and the linear range of target-probe hybridisation of a macroarray is essential to prevent misinterpretation as a result of signal noise or saturation. In this study, the target-probe hybridisation signal intensity was not limiting at 0.05 pmol when 1 µg target cDNA was used (Figure 1, Chapter 3). Therefore, variation in signal intensities at this probe amount should reflect target concentration, i.e. the amount of a particular cDNA in the pool. Consequently, probes were arrayed at 0.05 pmol to be able to detect differences in transcript expression.

To assess the ability of the macroarray to specifically detect an individual mRNA species and to determine the sensitivity of the system, increasing amounts, i.e. 0.06, 0.25 and 0.5% (m/m), of an *in vitro*-transcribed RNA from a bacterial gene was added to 100 µg mRNA from sorghum internodal tissues. Similar spiked *in vitro*-transcribed genes have previously been used to investigate sensitivity and fold-changes in transcript levels (van Bakel and Holstege, 2004, Close *et al*., 2004). The hybridisation signal intensities of the spiked *in vitro*-transcribed RNA exhibited a linear correlation with the amounts of transcript tested here, i.e. 0.06-0.5% when hybridised with 0.05 pmol probe DNA on the array filters (Figure 2, Chapter 3). Based on this result, the protocol has the ability to specifically identify individual mRNAs, eight fold-changes in transcripts levels and detect rare

transcripts at approximately 0.06% of the total cDNA pool. This level of sensitivity is sufficient to detect a large proportion of the genes expressed in developing *S. bicolour* (Desprez *et al*., 1998).

The usefulness of macroarrays to detect differentially expressed genes depends also very strongly on its technical reproducibility, i.e. low technical variation. Consequently, intra- and inter-array reproducibility was investigated in independent hybridisation experiments using the same target sample under the same labelling and hybridisation conditions. The hybridisation signal intensities of duplicate spots (same probe) on the same membrane were compared and the great majority of spots (95%) were found to have a coefficient of variation of less than 5% (Figure 3a, Chapter 3). Generally, a coefficient of variation of less than 15% between duplicate spots is thought to be a good indicator of reproducibility of a macroarray (Watt *et al*., 2005; Clarke and Zhu, 2006). The results obtained here therefore more than fulfil the minimum requirements set for intra-array reproducibility.

Secondly, to test inter-array reproducibility, the data from each of two independent membranes were first normalised relative to a spiked *in vitro* transcribed RNA to yield normalised signal intensity (NSI) values. The average NSI values, of genes with an intra-membrane coefficient of variation (CV) of less than 5%, from the two membranes were then subjected to a one-way ANOVA. Forty-three out of 48 probes (90%) produced p-values ≥ 0.05, meaning that the null hypothesis was satisfied, i.e. the means drawn from the different membranes for the same population genes (target cDNA) were equal (Figure 3b, Chapter 3). Based on this degree of similarity between the two independent experiments, the macroarray system developed in this study was judged to be highly reproducible and suitable for profiling transcript levels.

In general, normalisation of data is the process by which non-biological variation (technical variation) is minimised and standardised (Clarke and Zhu, 2006).

Technical variation could be the result of membrane preparation, as well as varying efficiencies of RNA preparation, reverse transcription, probe purification, hybridisation and membrane quality. Housekeeping genes that were thought to be constitutively expressed, e.g. β- and γ-actins, α-, β-tubulins and G3PDH, have been used in the past to normalise DNA array data, but they were found to be differentially expressed and unsuitable for data normalisation (Thellin *et* al., 1999; Warrington *et al.,* 2000*)*. Similarly, in this study both actin and tubulin were showed to be differentially expressed during sorghum development and were therefore not suitable for the normalisation of data. All the macroarray data was therefore normalised by using a spiked *in vitro*-transcribed and polyadenylated non-plant gene. Spiking of the external control early into the total RNA sample helped with the assessment of the efficiency of target preparation (synthesis and labelling of cDNA), as well as the efficiency of hybridisation and the performance of the scanner (Tong *et al*., 2006). Most importantly an external control will aid in the normalisation of data between arrays (inter-array) prepared for separate experiments (van Bakel and Holstege, 2004).

Having developed a robust and highly reproducible technique for multiple transcript profiling, internodal tissues of sorghum genotypes GH1 and SH2 at different stages of development and with different sugar accumulating profiles were analysed for differential gene expression (Chapter 4). To do this, intra-array reliability was first evaluated by comparing the signal intensities of duplicate spots and excluding all data with a CV of greater than 10%. Secondly, in order to be able to compare different membranes (different target cDNA samples) the data was normalised as explained above and subjected to one way ANOVA analyses. For a gene to be considered to be significantly differentially expressed, it had to meet three criteria: (i) the absolute difference between the NSI of the two-sample being compared should be greater than or equal to one, (ii) the ratio between their signal intensities should be greater than or equal to two and (iii) the difference between their NSI should be statistically significant ($p < 0.05$).

A number of genes, which are either closely or directly related to sucrose metabolism, were found to be significantly differentially expressed during the boot and softdough stages when the different internodal tissues of genotypes GH1 and SH2 were compared. In immature internodes during boot stage, SAI, SPS and sut2 were found to be up-regulated in genotype GH1 (Table 2, Chapter 4). In the maturing internodes sut2, SuSy and Trehalose phosphatase were up-regulated in genotypes SH2 and cellulase in genotypes GH1 (Table 3, Chapter 4). When genotype GH1's immature and maturing internodes were compared, hexokinase, enolase, aconitate hydratase, FBPase, SAI, sut1 and sut2 were expressed at significantly higher levels in the immature internodal tissues (Table 4, Chapter 4). Similarly, during the softdough stage, 33 genes, e.g. ADH, SPP, sut1, sut2, malate dehydrogenase and AGPase, were found to be significantly differentially expressed when immature and/or maturing internodes of genotypes GH1 and SH2 were compared (Tables 5, 6, 7 and 8, Chapter 4). In order to validate the reliability of macroarrays analyses, fourteen genes that were shown to be significantly differentially expressed during the boot and softdough stages were arbitrarily selected for semi-quantitative RT-PCR.

First strand cDNA was reverse transcribed from total RNA isolated respectively at the boot and softdough stages and the selected genes were quantitatively amplified using specific primers. Only seven out of 14 (50 %) genes showed a similar trend of expression in both the macroarray and semi-quantitative RT-PCR experiments (Figure 3, Chapter 4). Similar disagreement between the two techniques has been observed previously (Le Gall *et al*., 2005; Jolly *et al*., 2005; Nakano *et al*., 2006). The semi-quantitative RT-PCR method is gene-specific and usually offers a higher sensitivity in comparison to macroarray analyses (Balczun *et al*., 2005; Nakano *et al*., 2006). In this study, for macroarrays, DNA probes from rice and sugarcane were used for probing sorghum. Therefore, the probability of cross-hybridisation between the probes and various isoforms homologous to sorghum sequences is high. In addition, the apparent variation in

expression patterns could also be introduced by technical and biological variation (Churchill, 2002; Zakharkin *et al*., 2005; Clarke and Zhu, 2006).

Biological variation in DNA arrays results from tissue heterogeneity, genetic polymorphisms, pooled samples and changes in mRNA levels within different cell types and genotype-environment interactions (Kendziorski, 2003; Zakharkin *et al*., 2005). The RNA used in these experiments was extracted from pooled samples that were harvested on the same day and during the same stage of development, to compensate for biological variation. Pooling of samples does not allow statistical comparison of changes between samples (individual) and can result in a loss of information (Jolly *et al*., 2005). Some of the dissimilarities between macroarrays and semi-quantitative RT-PCR gene expression patterns might therefore be ascribed to biological variation between samples. In future, a number of biological replicates should be performed to estimate the extend of biological variation in an experiment. Although replicates cannot remove biological variation, it will delineate the minimum amount of difference that is required between two samples before a gene could be considered as differentially expressed. It will also give an estimate of the number of replicates that are required to identify truly differentially expressed genes.

The detection of changes in mRNA expression levels requires the normalization of the data from different arrays (inter-array variation). This should counter non-biological variation, such as differences in labelled material, local array differences and hybridisation efficiency. The high CV (up to 50% in some arrays) of the external control between arrays in this study might have prejudiced macroarray data and increased the chances of identifying false positives. Normalisation of macroarray data in future may require a number of external controls, i.e. (1) an external control for normalising variation in mRNA isolation and labelling and (2) an external control for normalising variation in hybridisation and local array differences. In this study membranes with low CV (10%) of the external control during inter-array comparison gave better result e.g. GH1

immature vs maturing internodes, GH1 vs SH2 maturing and GH1 vs SH immature. Interestingly, three genes (SAI, SuSy and SPP) from membranes with low CV (10%) were also confirmed by semi-quantitative RT-PCR to be differentially express. Therefore, it is proposed that a good external control CV for data normalisation between arrays should be less than 10% to ensure that the external control does not influence the results.

An understanding of metabolic networks requires quantitative data about transcript levels, protein levels or enzyme activity and metabolite levels (Gibon *et al*., 2006). Genomic techniques have been used extensively to characterise differential gene expression with the aim of identifying transcripts that correlate with a specific phenotype. This direct correlation between transcript levels and a particular phenotype assumes that transcript levels are directly reflected in protein levels and catalytic activity and do not take any post-transcriptional and –translational regulatory mechanisms into account.

To verify that changes in the levels of a transcript are also reflected in changes on enzyme activity, seven candidate genes that were differentially expressed were evaluated on enzyme activity level. Only three out of seven genes (43 %), i.e. SAI, SuSy and ADH, showed enzyme activities similar to the relative transcript levels (Figure 5, Chapter 4). Not surprisingly, it was shown recently that transcript levels are not necessarily reflected in the extractable activity levels of most enzymes in carbohydrate metabolism (Glanemann *et al*., 2003; Gibon *et al*., 2004; 2006). Gibon *et al.* (2006) suggest that changes in transcript levels may or may not immediately lead to similar changes in enzyme activity. In addition, these results clearly suggest that enzyme activity is controlled at both transcriptional and post-transcriptional levels.

The three genes, SAI, SuSy and ADH, were not only shown to be differentially expressed between two tissues with contrasting sugar profiles, but the activities of their related enzymes were also shown to be similarly coarsely regulated.

Given the additional fact that the genes were initially selected because of their roles in carbohydrate metabolism it would therefore be fair to argue that these genes could contribute to the contrasting phenotypes of the two relevant tissues. So, finally, the potential biological relevance of these will be discussed in more detail.

SAI transcript expression decreased in genotype GH1 as the tissue matured (from internodes 1-3 to 5-8) during the boot stage (Figure 2 e, Chapter 4). The sugar content of the same two tissues differ predominantly in terms of their sucrose content, i.e. there is an inverse correlation between SAI activity and sucrose content in these tissues. SAI may play a role in the remobilization of stored sucrose from the vacuole (Sacher *et al*., 1963) and is also believed to regulate hexose levels in certain tissues (Singh and Kanwar, 1991). In sugarcane, SAI activity shows striking seasonal variation, where it is high when growth is rapid and *vice versa* (Venkataramana *et al.* 1991). Zhu *et al*. (1997) reported that the level and timing of sucrose accumulation in the whole stalk and within individual internodes was correlated with the down-regulation of SAI activity. SAI activity is extremely low in the mature internodes of sugarcane varieties with a high capacity to accumulate sucrose (Sacher *et al*., 1963; Venkataramana et al., 1991). In contrast, Botha *et al.* (2001) found no phenotype in transgenic sugarcane plants with reduced SAI activity. The fact that SAI was significantly differentially expressed in these specific sorghum tissues therefore fits well with the above mentioned arguments in that: (i) SAI activity and sucrose contents were inversely correlated. (ii) Activity decreased in the more mature, less metabolically active internodes, although (iii) at boot stage there was still suffieciently high SAI activity to ensure differentiation between the tissue using the methods described in this thesis.

SuSy was found to be differentially express between the maturing internodal tissues of the two different genotypes during boot stage, more specifically SuSy activity was significantly higher in the SH2 genotype (Figure 2 c, Chapter 4).

Although the SH2 genotype had lower sucrose content than the GH1 genotype its total sugar content was higher and, consequently, it has a much smaller ration between sucrose and the hexoses. SuSy is thought to supply UDP-Glc and fructose for glycolysis and starch synthesis (Nguyen-Quoc and Foyer, 2001; Harada *et al*., 2005). In addition, a SuSy isoform associated with the plasma membrane is thought to contribute to growth by supplying hexoses for cell wall biosynthesis (Amor *et al*., 1995; Carlson and Chourey, 1996; Carlson *et al*., 2002). Similar, SuSy transcript and enzyme activity level (in the break-down direction) have been observed in actively maturing sugarcane internodal tissues (Botha and Black, 2000; Schafer *et al*., 2004; Watt *et al.,* 2005). The fact that SuSy was expressed significantly higher levels in SH2 genotype and correlated with high hexoses concentrations in these specific sorghum tissues therefore fits well with the view that SuSy is responsible for providing hexoses to actively growing cells for glycolysis and as precursors for other growth processes.

ADH was found to be differentially express in the maturing internodal tissue of SH2 genotype (Figure 2 i, Chapter 4). ADH supplies NAD^+ by reducing acetaldehyde to ethanol, in the absence aerobic respiration and therby permits continued glycolysis without cyptoplasmic acidosis (Dolferus *et al.,* 1994). In higher plants, e.g. rice, arabidopsis, maize and sugarcane, alcohol fermentation is essential for survival under some environmental stress situations (Dolferus *et al.,* 1997; Nogueira *et al.,* 2003; Peters and Frenkel, 2004). Dolferus *et al.,* (1997) found that arabidopsis plants, exposed to cold, has higher ADH transcript levels and enzymatic activity than untreated plants. In sugarcane, when plantlets are exposed to low temperatures there is also an increased in relative number of ADH ESTs (Nogueira *et al.,* 2003). The fact that ADH was expressed at significantly higher levels in the SH2 genotype, which was harvested during a cold spell, therefore fits well with this data.

In conclusion, the potential of a combination of macroarray and semi-quantitative RT-PCR analyses, to identify differentially expressed genes, was demonstrated.

In addition, differences were observed in the expression levels of particular genes when sorghum tissues with different sugar concentrations were compared. In particular, the transcript and enzyme activity levels of SAI, SuSy and ADH showed patterns similar to those of sugarcane tissues during sucrose accumulation. Because a direct correlation between a specific enzyme's activity and a particular phenotype can only be directly illustrated through reverse genetic approaches, a combination of information on differential gene expression as generated in this thesis and the relative simple genetics of sorghum could contribute to the elucidation and enhancement of the sucrose accumulation phenotype in this family.

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