THE DEVELOPMENT OF AN *IN SITU* HYBRIDISATION TECHNIQUE TO DETERMINE THE GENE EXPRESSION PATTERNS OF UDP-GLUCOSE DEHYDROGENASE, PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE AND UDP-GLUCOSE PYROPHOSPHORYLASE IN SUGARCANE INTERNODAL TISSUES

by

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DECLARATION

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

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ABSTRACT

The cellular expression of the enzymes implicated in regulating sucrose metabolism and accumulation in sugarcane is poorly understood. The present study was therefore aimed at the development of an *in situ* hybridisation (ISH) technique to study differential gene expression among the various cell types of the sugarcane culm. This technique in conjunction with northern and western blotting was then used to determine the sites of cellular and tissue specific expression of the cytosolic enzymes, UDP-Glc dehydrogenase, pyrophosphate dependent phosphofructokinase and UDP-Glc pyrophosphorylase, involved in sucrose metabolism.

This study revealed that the determination of the influencing parameters associated with the development of an ISH protocol was essential for the successful detection of the endogenous RNA sequences in sugarcane internodal tissues. The parameters that were investigated included the type of embedding medium, duration of fixation period, pre-treatment procedures and hybridisation temperature. It further revealed that fresh internodal tissue sections, fixed for a period of 24 h and thereafter exposed to pre-treatment and hybridisation, facilitated the analysis of cytological gene expression at all stages of sugarcane development.

The second part of this study revealed very localised transcript expression for UDP-Glc DH, PFP and UGPase in the different internodal tissue and cell types. The UDP-Glc DH and UGPase transcripts were localised to the phloem elements, whilst xylem tissue only expressed the UDP-Glc DH transcript. Transcripts of UDP-Glc DH, PFP and UGPase were all expressed in the parenchyma cells that were associated with the vascular bundles and the stem storage compartment, suggesting that the parenchyma cells distributed throughout the stem in the different tissue types complement each other in function for the purposes of phloem loading, unloading and assimilate transport processes.

Complimentary northern and western hybridisations demonstrated that internode 7 represents a shift in the sink from utilisation to storage. This is evident by the observed decline in both the relative transcript and protein abundances of UDP-Glc DH, PFP and UGPase at this stage of development. The relative mRNA and protein abundances for the three enzymes showed a similar trend. Higher levels of the gene transcripts and translated products were observed in the younger sucrose importing tissues, than in the older sucrose accumulating internodes. At a cellular level, it was found that the sites of cellular UDP-Glc DH, PFP and UGPase expression differed marginally. Whilst UDP-Glc DH was expressed in the phloem, xylem

and parenchyma cells of the vascular complex and in storage parenchyma cells, PFP was expressed exclusively in parenchyma cells that were associated with the vascular bundles and those serving a storage function in the stem pith and UGPase was found to be localised in the phloem and parenchyma of the vascular bundles and the storage parenchyma cells. Such findings have demonstrated an increase in resolution with which gene expression can be examined at a cellular level. Hence, the results from this study have demonstrated that the knowledge of metabolic compartmentation between different tissue and cell types is a requisite to understanding the function(s) of individual enzymes within complex structures such as the sugarcane culm.

OPSOMMING

Die sellulêre lokalisering van die ensieme wat geïmpliseer word in die regulering van sukrose metabolisme is onbekend. Met dit in gedagte, was hierdie studie gefokus op die ontwikkeling van 'n *in situ* hibridisasie (ISH) tegniek om differensiële geenuitdrukking in die verskillende seltipes van die suikerrietstingel te ondersoek. Hierdie tegniek, tesame met RNA-en proteïen gel blots, is volgens aangewend om die areas van sellulêre-en weefselspesifieke uitdrukking van die sitosoliese ensieme UDP-glukose dehydrogenase, pirofosfaat-afhanklike fosfofruktokinase en UDP-glukose pirofosforilase, wat almal betrokke is by sukrosemetabolisme, te bepaal.

Dit het duidelik geword gedurende die studie dat die bepaling van die optimale parameters van die ISH protokol vir suikerriet van deurslaggewende belang sou wees vir die opsporing van endogene RNA volgordes. Die parameters wat ondersoek is het ingesluit die tipe inbeddingsmedium, die tydsduur van fiksering, voorafbehandelings- en hibridisasiemetodes. Dit het duidelik geword dat vars internodale weefselsnitte wat vir 24 h gefikseer is en daarna voorafbehandeling en hibridisasie ondergaan het, die bepaling van geenuitdrukking tydens alle fases van suikkerrietontwikkeling moontlik gemaak het.

Die tweede fase van hierdie studie het aangetoon dat al drie ensieme spesifiek gelokaliseerde uitdrukkingspatrone gehad het in verskillende internodale weefsels en seltipes. Al drie gene is konstitutief uitgedruk in internodes. Die UDP-glukose dehydrogenase en UDP-glukose pirofosforilase transkripte is gelokaliseer na die floeëm elemente, terwyl xileem slegs die UDP-glukose dehydrogenase transkripte bevat het. Al die gene is in die parenchiemselle uitgedruk wat geassosieer is met die vaatbondels en die stingel stoorkompartement, wat moontlik beteken dat die parenchiem selle wat deur die stingel versprei is 'n sentrale netwerk vorm wat direk of indirek koolstofassimileringsprosesse beïnvloed.

RNA-en proteïen gel blots op dieselfde internodes het gewys dat internode sewe 'n verskuiwing, van koolstofverbruik na berging, verteenwoordig. Dit word geïllustreer deur die afname in beide transkrip en proteïen vlakke van die drie ensiem in hierdie stadium van ontwikkeling. Alhoewel beide mRNA en proteïen vlakke vir al die ensieme 'n soortgelyke tendens getoon het, het die sellulêre uitdrukking van die ensieme volgens ISH verskil, wat die krag van die tegniek illustreer. Die resultate van hierdie studie het gedemonstreer dat begrip van die kompartementalisasie van metabolisme tussen verskillende weefsel-en seltipes 'n

voorvereiste is om die funksie/s van individuele ensieme in komplekse strukture soos die suikerrietstingel te bepaal.

To my parents

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"Remember the minute that has fled is no longer yours, the minute that is approaching cannot be counted as yours, the minute that is with you, this alone is truly yours. Make the best use of it, for it may be your last!" **BABA**

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LIST OF ABBREVIATIONS AND ACRONYMS

UDP-Glc DH (UGD)	uridine diphosphoglucose dehydrogenase
PFP	pyrophosphate dependent phosphofructokinase
UGPase	uridine diphosphoglucose pyrophosphorylase
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
ISH	*in situ* hybridisation
Susy	sucrose synthase
SPS	sucrose phosphate synthase
SP	sucrose phosphatase
UDP-Glc	uridine diphosphoglucose
PFK	ATP-dependent phosphofructokinase
FBPase	fructose 1,6-bisphosphatase
UDP-Glc UA	uridine diphosphoglucose glucuronate
PPi	inorganic pyrophosphate
Pi	pyrophosphate
NAD^+	oxidised and reduced nicotinamide-adenine dinucleotide
kDa	kilodalton
ATP	adenine triphosphate
UTP	uridine triphosphate
RNase	ribonuclease
UV	ultraviolet
DIG	digoxigenin
NTE	NaCl, Tris-HCl (pH 8) and EDTA
NBT/BCIP	Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl-phosphate
TBST	Tris buffer containing NaCl and tween 20

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CHAPTER 1
GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids), a member of the grass family Graminaceae, is a crop of socio-economic importance and is ranked as one of the world's top ten food crops (Gallo-Meagher and Irvine, 1996). Sucrose derived from sugarcane plays a vital role in the food manufacturing industry and as the primary agricultural source for ethanol production (Hawker, 1985; John, 1992). Commercial sucrose is derived almost entirely from sugarcane and sugarbeet with the former accounting for more than 60% of world production (Aftab and Iqbal, 1999).

South Africa is one of the world's leading cost competitive producers of high quality sugar (Trikam, 2003). Sale of sucrose is an important source of revenue in South Africa. Over the past year, the South African Sugar Association (SASA) produced over 2.8 million tons of sugar, which contributed an estimated R 6.5 billion to the country's foreign exchange earnings (Trikam, 2003). In order to maintain its international status, SASA needs to improve profit margins for sucrose production. Conventional breeding programmes have already exploited the parental germplasm and further improvements are becoming increasingly difficult (Gallo-Meagher and Irvine, 1996). Therefore, the emergence of new techniques such as those that allow for the genetic manipulation at the molecular level may provide an adjunct to conventional breeding programmes (Snyman, 1992). Such molecular techniques could be able to improve product yield through the manipulation of key enzymes involved in the alteration of resistance to biotic and abiotic stresses and/or sucrose metabolism. However, the functions and regulation of many of these enzymes in plant tissues are poorly understood. In addition, our understanding of how metabolic processes are compartmentalised between and within the different tissues is extremely limited (Walker *et. al.*, 2001). Attempts to study the compartmentation of key processes in plants have demonstrated that the technique of immunohistochemistry may be successfully employed as a tool in determining the localisation of enzymes in the different tissue types (Famiani *et al.*, 2000; Walker *et al.*, 2001). The molecular technique of *in situ* hybridisation has proven to be the most direct way of answering questions with regard to the site of gene expression and modulation during the different developmental transitions at the cellular level (Harrison *et al.*, 1973). It has further been successfully employed to detect the location of specific nucleic acid sequences (Caeto-Anolles and Trigiano, 1997). It has been reported that the nucleic acid sequences of genes encoding enzymes implicated in sucrose metabolism had been identified (Botha *et al.*, 1986;

Tenhaken and Thulke, 1996). Such information offers the potential to demonstrate when and where specific genes involved in sucrose metabolism are expressed, so that we can begin to understand the complexity associated with both plant metabolism and transport processes.

Knowledge of the control of sucrose metabolism within the storage parenchyma of sugarcane internodes is still very limited (John, 1992; Stitte and Sonnewald, 1995). Sucrose accumulation in the sugarcane culm has been the subject of extensive research in the fields of both physiology and biochemistry. Initial studies were conducted on whole plants and stalk tissues (Sacher *et al.*, 1963). However, the complexity at this level was too great to reveal detailed information about how sucrose accumulation is regulated at the enzyme, cell and molecular levels. Results obtained from those and subsequent studies suggest that the cycle of sucrose synthesis and degradation plays an important role in the regulation and control of sucrose accumulation in the storage parenchyma cells of sugarcane internodal tissue (Sacher *et al*, 1963; Komor, 1994). This cycle functions in a manner that supports the activity of sucrose phosphate synthase in the direction of sucrose synthesis, and invertase and sucrose synthase mediating its degradation (Wendler *et al.*, 1990). Consequently enzymes that have been implicated in the sucrose cycle have become a focal point for much research into the regulation of sucrose accumulation in sugarcane (Sacher *et al.*, 1963; Glasziou and Gaylor, 1972; Veith and Komor, 1993; Komor, 1994). These enzymes include the suite of cytosolic enzymes involved in the various sucrose metabolic pathways especially UDP-Glc dehydrogenase, pyrophosphate-dependent phosphofructokinase and UDP-Glc pyrophosphorylase. These enzymes have been associated with regulating the flow of carbon towards cell wall polysaccharide synthesis (Robertson *et al.*, 1995), glycolysis (Mahajan and Singh, 1989) and UDP-Glc synthesis (Kleczowski, 1994) respectively. Research into the regulatory activity of these enzymes is still in its infancy and hence there is at present no defined view of the regulatory pathway of sucrose accumulation in sugarcane. In an attempt to further understand this metabolic pathway, the regulation of sucrose synthesis and degradation during carbon partitioning needs to be further explored.

The main goals of this project were therefore to develop the methodology of RNA *in situ* hybridisation for the purpose of examining the expression of the cytosolic enzymes, UDP-glucose dehydrogenase, the pyrophosphate dependent phosphofructokinase and UDP-glucose pyrophosphorylase. These enzymes are responsible for the partitioning of cytosolic carbon into the synthesis of cell wall polysaccharides, regulating the carbon flux in the glycolytic and gluconeogenic pathway, and regulating UDP-Glc synthesis respectively. The results obtained

in this study can be used for the development of new tools to increase sucrose yield in sugarcane.

CHAPTER 2
LITERATURE REVIEW

In addition to its socio-economic importance, sucrose is also the major transport form of reduced carbon and plays a central role in all aspects of plant metabolism (Hawker, 1985). It forms the substrate for the synthesis of structural polysaccharides and storage polymers and respiration (Sung *et al.*, 1988). Sucrose also forms the major storage component in several plant species including sugarcane (Avigad, 1982; Hawker, 1985). The partitioning of this photoassimilate between competing sinks of vegetative growth, fibre and stored sucrose in sugarcane is under complex regulation. A detailed understanding of these processes is urgently required for the development of genetic engineering strategies in order to improve sucrose yield in this crop.

This chapter is primarily aimed at introducing the carbon partitioning enzymes, uridine diphosphoglucose dehydrogenase (UDP-Glc-DH, E.C 1.1.1.22), pyrophosphate dependent phosphofructokinase (PFP, E.C. 2.7.1.90) and uridine diphosphoglucose pyrophosphorylase (UGPase, E.C. 2.7.7.9) that have been implicated in the control of sucrose accumulation and metabolism in sugarcane. The review presented therefore outlines aspects of sugarcane anatomy, sucrose transport processes, sucrose biochemistry and accumulation. An integration of this knowledge with the gene expression patterns of UDP-Glc DH, PFP and UGPase can be used to develop a hypothesis of their gene function during sugarcane culm development and sucrose accumulation.

2.1. Sugarcane stem anatomy

The sugarcane stem is a complex organ composed of epidermal, vascular, meristematic and parenchyma tissues (Moore, 1995) (Figure 2.1). The vascular bundles are scattered throughout a matrix of parenchyma cells (Moore and Maretzki, 1996). These vascular bundles increase in number and decrease in size from the centre towards the periphery of the stem. The peripheral bundles are so small that they contain only xylem and usually lack phloem, which when present, exists as metaphloem elements (Moore and Maretzki, 1996). At the centre, both the vascular bundles and parenchyma cells are relatively large. The vascular bundles possess two large metaxylem vessels, a large protoxylem lacuna, crushed protophloem, complete metaphloem and two schlerenchymous caps on each pole of the vascular bundle. This whole structure is surrounded by schlerenchymous sheaths (Moore and Maretzki, 1996) (Figure 2.1). The gradient

of tissue maturation down the length of the sugarcane stalk involves a successive increase in lignification and suberisation of cells in the stem. In young internodes, the protoxylem elements are the only lignified cells. As the internodes mature, lignification spreads to the metaxylem, then to the bundle sheath cells and a few parenchyma cells. In mature and old internodes, lignification continues from the vascular bundles into the storage parenchyma cells (Moore and Maretzki, 1996). Suberisation occurs concomitantly. This pattern of increasing lignification and suberisation parallels the increase in sucrose content in the stem parenchyma cells. At the stage of maturity, most of the storage parenchyma cells are lignified and suberised, however the deposition of these secondary wall products is irregular along the cell wall surfaces (Jacobsen *et al* ., 1992). Such cells are suited for the exchange of water and metabolites between the symplast and apoplast (Moore and Maretzki, 1996) during sucrose transport.

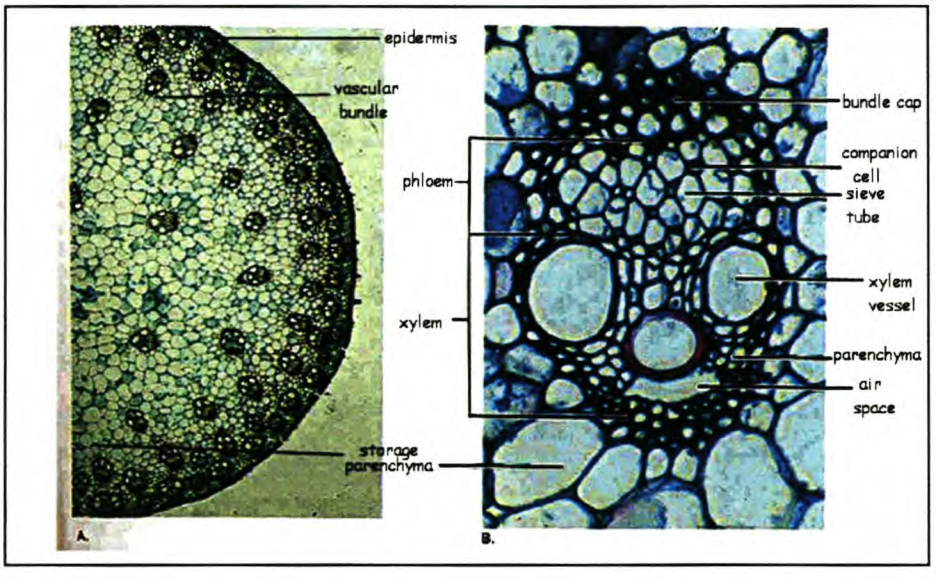

Figure 2.1: Diagram showing a typical sugarcane stem (A) including the different cell types (B) (from Ellmore, 2000).

2.2. Phloem loading and unloading

In the sugarcane stem, phloem loading and unloading for the movement of sucrose from the source to the sink cells may occur either through the plasmodesmata (symplasm) or cell wall free space (apoplasm) or both (Moore and Maretzki, 1996). On the basis of the pattern of lignification and suberisation of the cells in the stem tissue, the particular path followed may be dependent on the developmental stage of the stem. But, sucrose is also regarded as an important reserve carbohydrate and can accumulate in the vacuole rather than be exported (Kruger, 1997).

The exact uptake mechanism of sucrose by the vacuole however, remains unclear. Although a sucrose/H^+ anti-port system has been implicated (Frommer and Sonnewald, 1995), another argument involves the cleavage and hydrolysis of sucrose prior to its uptake (Kruger, 1997).

2.3. Sucrose degradation and synthesis

Upon arrival of sucrose into the stem, sucrose can be catabolised by sucrose synthase (Susy) or one of the three invertases: soluble acid invertase, cell wall bound acid invertase and neutral invertase (Moore and Maretzki, 1996) . On entry into the metabolic compartment of the parenchyma cells, the hexoses may be metabolised or resynthesised into sucrose by sucrose phosphate synthase (SPS) and sucrose phosphatase (SP). Potentially Susy could synthesise sucrose, however its equilibrium is usually in the direction of degradation (Moore and Maretzki, 1996). Initially it was thought that sucrose uptake from the apoplast was dependent on its hydrolysis via acid invertase, prior to transfer into the storage compartment of the parenchyma cells (Sacher *et al.*, 1963). Cellular uptake of the reducing sugars, and not sucrose, in sugarcane cell suspension cultures (Komor *et al.*, 1981) supported the development of the hypothesis that sucrose is not taken up directly by parenchyma cells. Both supporting and contradicting this hypothesis, was the radiolabelling study undertaken by Lingle (1989). Although, 25 % of ^{14}C was found in the apoplast as fructose, 15 % of [^{14}C] fructosyl sucrose fed to the tissue discs was randomised in the sucrose stored in the tissue. Hence, it was deduced that sucrose cleavage was not necessary for its transport into the cell.

2.4. Sucrose Accumulation

Sugar accumulates in the sugarcane internodes during the phase of elongation. This phase has been associated with high acid invertase activity and sucrose cleavage. Glucose and fructose are therefore abundant. Sucrose begins accumulating almost exclusively following elongation (Lingle, 1997). A large proportion of the daily sucrose production is transported rapidly to elongating internodes whilst a smaller quantity is added to older internodes. The stem internodes of sugarcane are growth sinks and as full elongation is reached, the imported photoassimilates remain in storage instead of being metabolised for growth (Moore and Maretzki, 1996). This phenomenon of sucrose accumulation in maturing stem tissue has been intimately associated with a decrease in the partitioning of carbon into the biosynthesis of insoluble matter, amino acids and phosphorylated intermediates and respiration with a simultaneous redirection of substrate towards sucrose storage (Whittaker and Botha, 1997). That study further demonstrated that although

proportionally less carbon was allocated to respiration, total cellular respiration increased. Such an increase in total cellular respiration might actually be a requirement for the accelerated sucrose accumulation in older internodes. Sugarcane culm development can therefore be defined as a continuous process resulting in a gradient of maturation and sucrose accumulation down the stalk to a point where full maturity and a stable, high sucrose concentration are reached (Moore and Maretzki, 1996).

Recently, however, increased attention has been focused on the synthesis of sucrose and its catabolism in sink tissues (Quick and Schaffer, 1996). This is because of the various aspects associated with sink sucrose metabolism, including growth metabolism, starch synthesis, and fructan, hexose and sucrose accumulation. In sink tissues, there is a partitioning of imported assimilates towards the biosynthetic pathways (Quick and Schaffer, 1996). UDP-glucose (UDP-Glc), formed when Susy cleaves sucrose, can be utilised as a substrate by enzymes involved in different metabolic pathways (Kruger, 1997). UDP-Glc can be used in the pathway leading to cell wall polysaccharide synthesis via the action of UDP-Glc DH, towards sucrose synthesis via Susy again or mediated into the glycolytic pathway by UGPase (Figure 2.2). In the glycolytic pathway, the abundance of the hexose moieties is under the strict control of the regulatory enzyme, PFP. This enzyme directs carbon either towards gluconeogenesis and sucrose synthesis or glycolysis and cellular intermediate biosynthesis, depending on the metabolic demands of the cell (Quick and Schaffer, 1996).

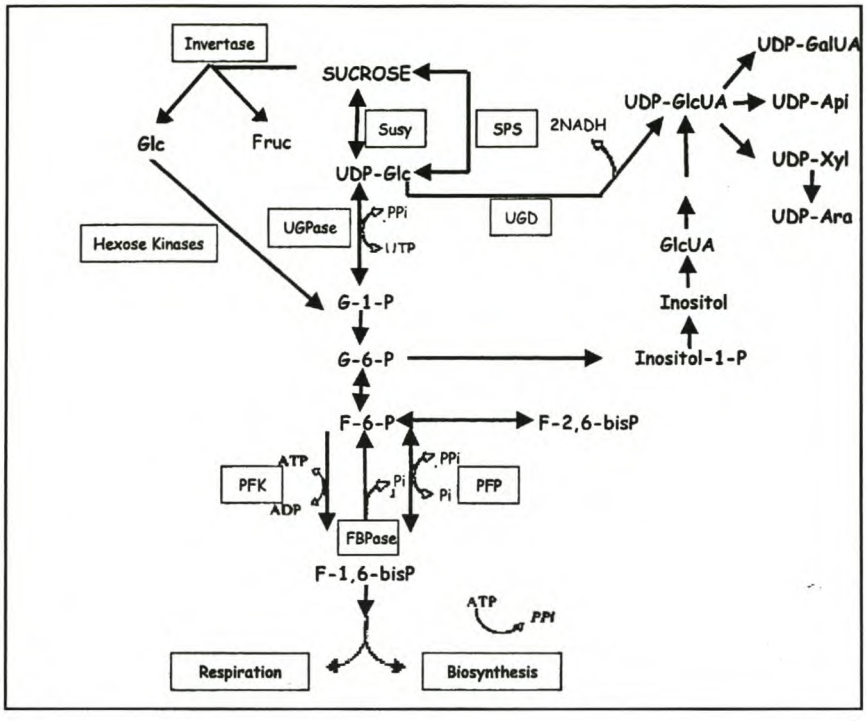

Figure 2.2: Schematic representation of sucrose metabolism in the cytosol. UGD: UDP-glucose dehydrogenase; Susy: Sucrose synthase; SPS: Sucrose phosphate synthase; UGPase: UDP-glucose pyrophosphorylase; PFK: ATP-dependent phosphofructokinase; PFP: Pyrophosphate-dependent phosphofructokinase; FBPase: Fructose 1,6-bisphosphatase

2.5. Carbon partitioning

Central to carbon partitioning and cycling is the flux through the pool of UDP-Glc, the metabolite that forms the substrate for sucrose and cell wall polysaccharide synthesis and respiration (Turner and Botha, 2002). This metabolite forms a significant pool in the cytosol following the cleavage of sucrose via Susy. UDP-Glc can be metabolised further in one of several ways (Stewart and Copeland, 1998). It may serve both as a glucosyl donor in glycosylation reactions or as the substrate to be oxidised by UDP-Glc DH to UDP-glucuronate (UDP-Glc UA) (Stewart and Copeland, 1998).

In squash hypocotyls (Wakabayashi *et al.*, 1989) and soybean nodules (Stewart and Copeland, 1998) the oxidation of UDP-Glc is important for the provision of the necessary precursors required by the plant for structural polysaccharides. Most cell wall polysaccharide precursors are derived from UDP-Glc through a series of sugar nucleotide reactions (Kruger, 1997). The

principle hexose and pentose residues required for pectin and hemicellulose synthesis can be produced via the following reactions: 1) the oxidation of UDP-Glc to UDP-Glc UA via the action of UDP-Glc DH; 2) the decarboxylation of UDP-Glc UA to UDP-xylose; and 3) the epimerisation of these three compounds to form UDP-galactose; UDP-galacturonate and UDP-arabinose respectively (Kruger, 1997). Biochemical investigations revealed that one of the major controlling factors in the quantitative production of these structural polysaccharides is the complement of synthases that are found in the endomembrane system (Robertson *et al.*, 1996).

Studies on UDP-Glc metabolism in *Riella helicophylla* revealed that during differentiation, the amount of UDP-Glc increased from the meristem to the expanding cells and decreased in mature cells (Witt, 1992). That study aimed at determining the amount of UDP-Glc and the activities of enzymes involved in hemicellulose metabolism in liverwort tissue fragments at different stages of development. It was observed that the UDP-Glc DH activity was positively correlated to the amount of UDP-Glc in the different tissue types, which was suggested to be the substrate regulator of the UDP-Glc DH reaction.

However, the demand for UDP-Glc by UDP-Glc DH is relatively insignificant when compared to the amount of UDP-Glc produced by the reaction involving Susy (Kruger, 1997). Instead a vast percentage of UDP-Glc is probably converted to hexose phosphates (Kruger, 1997). The most likely route for the conversion of UDP-Glc to hexose phosphates is through the reaction catalysed by UGPase generating glucose-1-phosphate (Figure 2.2).

These pathways, although divergent operate on a demand and supply principle (Singh and Malhotra, 2000) and secondary to this principle is the requirement for inorganic pyrophosphate (PPi) (Kruger, 1997). Although PPi is produced during protein, isoprenoid lipid and structural polysaccharide biosyntheses, it is doubtful that such pathways could provide the sufficient levels of PPi required to meet the demands of the sucrose synthesis pathway. An alternative is that PPi could be produced by PFP operating in the direction of fructose-6-phosphate synthesis (ap Rees, 1988) (Figure 2.2). However this suggestion now seems irrelevant. Subsequent studies comparing the relative levels of metabolic intermediates with the appropriate mass action ratio indicate that PFP acts in a net glycolytic direction in developing tubers (Frommer and Sonnewald, 1995). This direction of activity was further reported to occur at precisely the time when the demand for PPi by the sucrose metabolic pathway was greatest. Hence, instead of

providing a source of PPi under these conditions, PFP now seems likely to generate an additional demand for this metabolite (Kruger, 1997). Such discrepancies with respect to PFP activity in plant cells still remain unresolved. However, it does seem that the activity of this enzyme is divorced from the concentration of its carbon substrates, and that the role of PFP in the plant cytosol is to maintain a precisely regulated concentration of PPi (Dancer and ap Rees, 1989).

UDP-Glc therefore plays a central role in the sucrose metabolic pathway. However, this metabolite is also crucial for the optimal functioning of several other pathways, including cell wall polysaccharide synthesis, glycolysis and sucrose synthesis. These pathways are mediated by the activities of the cytosolic enzymes, UDP-Glc DH, PFP and UGPase, respectively. Hence, the dehydrogenation of UDP-Glc is an important step in the regulation of carbon flux within a plant cell (Robertson *et al.*, 1996).

2.6. Uridine diphosphoglucose dehydrogenase

Plant cell walls undergo profound changes under diverse conditions such as growth and differentiation and in response to stress and pathogenic attack (Robertson *et al.*, 1995). These changes in the cell wall appear to be regulated by the changes in the underlying synthetic systems. Moreover, the accumulation of cell wall polymers such as hemicelluloses and pectins represent a significant quantitative sink for the flow of carbon. Both hemicelluloses and pectins are key components of cell walls and form the matrix that strengthens and cements the cell wall structure (Johansson *et al.*, 2002). The precursor to these cell wall components is UDP-Glc UA, which is either directly or indirectly responsible for the total polysaccharide content of the cell walls (Johansson *et al.*, 2002).

The synthesis of UDP-Glc UA occurs spontaneously following the oxidation of UDP-Glc by UDP-Glc-DH (Stewart and Copeland, 1998). This essentially irreversible reaction also facilitates the concomitant reduction of two molecules of NAD^+ and is potentially subject to a feedback inhibition by the level of UDP-xylose (Dalessandro and Northcote, 1997a). Kinetic and characterisation studies on UDP-Glc DH from sugarcane revealed that this enzyme has a pH optimum of 8.4, with a K_m and V_{max} for the substrate, UDP-Glc of 18 μM and 2.17 $U.mg^{-1}$ respectively (Turner and Botha, 2002). Moreover the UDP-Glc DH protein was reported to be a single polypeptide of 52 kDa. Biochemical studies have demonstrated that the production of

UDP-Glc UA may be the rate-limiting step in the reaction that provides the precursors for cell wall synthesis (Tenhaken and Thulke, 1996).

The product of the UDP-Glc DH reaction in animals has been implicated in the synthesis of the connective tissue, glycosaminoglycans (Stewart and Copeland, 1998) that have been suggested to play a key role in the detoxification of xenobiotics (Tenhaken and Thulke, 1996). In plants UDP-Glc UA has been implicated in structural polysaccharide and nucleotide sugar synthesis (Stewart and Copeland, 1998; Robertson *et al.*, 1996). The production of UDP-Glc UA can be achieved in one of two ways in plants. The first route is the direct conversion of the ubiquitous UDP-Glc to UDP-Glc UA via UDP-Glc DH and the other via the inositol oxidation pathway (Loewus *et al.*, 1973) (Figure 2.2). The regulatory step(s) that are associated with the latter pathway are speculative and it is assumed that inositol is irreversibly oxidised to glucuronic acid, which is subsequently activated to form UDP-Glc UA (Seitz *et al.*, 2000). For the purposes of the present study, the pathway to UDP-Glc UA production via UDP-Glc DH will only be considered.

Recently, an investigation involving cell wall matrix polysaccharide precursor synthesis revealed that UDP-Glc DH is differentially expressed in *Arabidopsis*. This was examined using two different reporter genes (GUS and GFP) under the control of the UDP-Glc DH promoter, *Ugd*. Furthermore northern and western blot analyses demonstrated that *Ugd* transcript and protein expression respectively were highest in actively growing plant tissues such as the primary roots and expanding leaves (Seitz *et al.*, 2000). Separate northern blot analyses on tissue extracts from soybean revealed that the root tips and lateral roots exhibited high mRNA expression whilst the epicotyl and expanding leaves showed moderate expression (Tenhaken and Thulke, 1996). In contrast, much lower UDP-Glc DH expression was observed in the older parts of the main root, hypocotyl and mature leaves.

In *Riella helicophylla* the activity of UDP-Glc DH increased from the meristem to expanding cells. However, its activity became drastically lowered in the mature cells (Witt, 1992). Studies on sycamore showed that the specific activities and the units of enzyme activities per cell of UDP-Glc DH increased sharply from cambial cells to differentiated xylem cells (Dalessandro and Northcote, 1977a). Complementing this study was the immunohistochemical investigation undertaken to determine the sites of UDP-Glc DH expression in French bean (*Phaselous vulgaris* L.). That study revealed that UDP-Glc DH was immunolocalised in the vascular tissue of the

hypocotyls (Botha and Small, 1987). In a separate study, UDP-Glc DH activity was associated with the immature xylem and phloem cells but it was the larger phloem cells of *Zinnia* that expressed the highest levels of UDP-Glc DH activity (Demura and Fukuda, 1993). Those studies indicate that UDP-Glc DH plays an important role in the provision of the hemicellulose precursors in expanding tissues and its expression in vascular tissue was correlated with an increased demand for UDP-Glc UA and UDP-xylose during secondary thickening in angiosperms (Dalessandro and Northcote, 1977b). Lowered expression was attributed to the lack of demand of UDP-Glc UA derived sugars in already differentiated and mature cells (Tenhaken and Thulke, 1996).

Measurements of UDP-Glc DH activity at the cellular level, however, have proven rather difficult possibly due to the low activity of UDP-Glc DH compared to the activities of other enzymes involved in hemicellulose and pectin synthesis (Dalessandro and Northcote, 1977a; Dalessandro and Northcote, 1977b). This was observed in maize, barley and *Brassica* spp (Roberts and Cetorelli, 1973). One explanation for the observed lower levels of UDP-Glc DH expression might be the existence of the alternate route to UDP-Glc UA production in such tissue types, that is the inositol oxidation pathway (Figure 2.2).

A review of the literature on cell wall polymer synthesis iterates the existence of changes associated with the composition of the cell wall during growth and differentiation (Dalessandro and Northcote, 1977a). This observation explains the differences in UDP-Glc DH activity in the different tissue types at various stages of development. In older sugarcane internodal tissues, radiolabeling studies revealed that internode growth and development coincided with a redirection of carbon away from cell wall polysaccharide synthesis and respiration towards sucrose synthesis (Whittaker and Botha, 1997). It may therefore be that UD-Glc DH exerts a coarse control over carbon flux into cell wall polymer biosynthesis (Robertson *et al.*, 1995)

In summary, UDP-Glc DH has been shown to be very active in tissues where there is a demand for the synthesis of structural polysaccharides (Dalessandro and Northcote, 1977a). Although, much information is available at the biochemical and molecular level of the reaction that UDP-Glc DH catalyses, only a speculative understanding exists with respect to the regulation of carbon flux into polysaccharide cell wall synthesis (Robertson *et al.*, 1995) and the expression pattern(s) of this enzyme, in the different plant tissues.

2.7. Pyrophosphate dependent phosphofructokinase

An important regulator of photosynthetic carbon metabolism, as proposed by Stitt (1990) is fructose 2,6-bisphosphate which contributes to both the co-ordination of sucrose synthesis with the rate of carbon dioxide fixation and the partitioning of photosynthate between sucrose and starch (Scott *et al.*, 1995; Stitt, 1997; Truesdale *et al.*, 1999). This metabolite has been demonstrated to activate the cytosolic enzymes PFP and fructose bisphosphatase at nanomolar concentrations (Nielson, 1995; Fernie *et al.*, 2001). PFP catalyses the highly regulated yet reversible inter-conversion of fructose-6-phosphate and PPi to fructose-1,6-bisphosphate and Pi. This inter-conversion is also catalysed by two other enzymes that function irreversibly, namely PFK in the glycolytic direction and fructose 1,6-bisphosphatase in the gluconeogenic direction (Murley *et al.*, 1998) (Figure 2.2). Characterisation of PFP from *Brassica* species revealed that this enzyme has a pH optimum of 7.5 and consists of two subunits, the α and β subunits, of sizes 66 and 60 kDa respectively (Theodorou and Plaxton, 1996). The V_{max} value for the forward reaction was 26 $U.mg^{-1}$ with K_m values of 0.05 mM and 15 μM for fructose-6-phosphate and PPi respectively. The V_{max} for the reverse direction was 24 $U.mg^{-1}$ with K_m values for the substrates fructose 1,6-bisphosphate and Pi being 9 μM and 0.25 mM respectively. Hence PFP catalyses a reaction close to equilibrium *in vivo*, and assigning a precise role to this enzyme is therefore difficult (Stitt, 1990; Kruger, 1997). However, PFP has been suggested to play a regulatory role in glycolysis, gluconeogensis, PPi formation and Pi removal (Murley *et al.*, 1998). The hypothesis that has received much support is that proposed by Stitt (1989): Although PFP acts as a PPi- stat and its activity is dependent on the demand of PPi in the cytosol, it is also the regulator that partitions carbon between sucrose, starch and amino acids. Hence due to its physical and kinetic properties, PFP has been labelled as an adaptive enzyme, equilibrating the existing hexose and pentose phosphate pools in the cytosol (Dennis and Greyson, 1987) according to the demands presented by environmental, developmental and tissue-specific signals (Plaxton, 1996).

The cytosolic enzyme, PFP has been reported to occur abundantly in sink tissues and tissues associated with extensive biosynthesis, where sucrose import and degradation occurs (Krook *et al.*, 2000). It was further reported that changes in the total PFP activity occurred during developmental processes such as leaf development, as observed in barley (Nielson, 1992) and leaf maturation (Black *et al.*, 1987). Changes in PFP activity was also observed during periods of

cell growth in liquid media (Ashihara and Horikosi, 1987) and banana ripening (Beaudry *et al.*, 1987). Subsequent research by Botha and co-workers (1992) showed that PFP activity was strongly correlated with hexose utilisation in bean cells. In addition, at the stages of cell division and carbohydrate utilisation, maximum activity for this enzyme was reported. In wheat endosperm, the observed high PFP activity was in the direction of fructose-6-phosphate and PPi synthesis (Mahajan and Singh, 1989). Such high PFP activity was correlated with increased sucrose utilisation for the biosynthesis of cellular intermediates such as amino and organic acids and storage polymer (starch) synthesis (ap Rees, 1985; Dennis and Greyson, 1987; Hatzfeld *et al.*, 1990; Tobias *et al.*, 1992; Ashihara and Sato, 1993; Enomoto and Ohyama, 1994). The alternate proposal supported the hypothesis presented by Stitt (1989), and argued further that PFP functions to provide the PPi necessary for the metabolism of UDP-Glc by UGPase during incoming sucrose utilisation (Black *et al.*, 1987). It would therefore appear that PFP works in concert with other cytosolic enzymes such as UGPase and Susy, to ensure the quick metabolism of sucrose in biosynthetic tissues. Hence, the presence of PFP in tissues could act as indicators of sink tissue and sink strength and is selectively induced depending on the metabolic status of the cell (Black *et al.*, 1987; Ashihara *et al.*, 1988).

Recently it has been demonstrated that PFP activity is closely associated with sucrose accumulation although its activity has been reported to decrease as tissue maturity continued. Furthermore, it is not as yet obvious as to why PFP is negatively correlated with sucrose content and positively correlated to carbon partitioning towards respiration (Whittaker and Botha, 1999). It was reported further that there was an appreciable amount of PFK activity in conjunction with that of PFP in sugarcane culm tissue suggesting that both these enzymes contribute significantly to fructose-6-phosphate utilisation and respiratory carbon flow and may in fact be related to a long-term requirement for sucrose utilisation rather than storage in these varieties. This was observed across both commercial varieties and segregating F1 populations (Whittaker and Botha, 1999). In transgenic sugarcane tissue transformed with an untranslatable form of the PFP-β gene, the hexose concentrations increased significantly in young internodes whilst its sucrose content remained unchanged (Groenewald and Botha, 2002). In older internodes, it was reported that the hexose content was slightly higher than the control plants although the sucrose content did indeed increase significantly (Groenewald and Botha, 2002). Those results illustrate that the down-regulation of PFP activity does indeed influence sucrose metabolism in transgenic sugarcane

plants. However its influence on other phenotypic traits and its underlying mechanism requires further investigation.

2.8. Uridine diphosphoglucose pyrophosphorylase

UDP glucose pyrophosphorylase is one of the key enzymes involved in the sucrose metabolic pathway (Borovkov *et al.*, 1997). It catalyses the reversible conversion of glucose-1-phosphate and UTP into PPi and UDP-Glc. The latter product is a central metabolite and a precursor to carbohydrate metabolism including sucrose, cellulose, starch, glycogen and β-glucan synthesis in all cells (ap Rees, 1988; Kleczowski, 1994). Although this enzyme exists predominantly as a soluble protein in the cytosol, a membrane-bound isoform has been reported for both plants and animals (Kleczowski, 1994). In plants, UGPase activity is believed to be primarily involved in either sucrose synthesis or its degradation, depending on the metabolic status of the tissue (ap Rees, 1988; Kleczowski, 1994). This enzyme is strongly inhibited by its substrate, UDP-Glc (Kleczowski, 1994). This is significant in that the content of this metabolite varies in the different tissue types. Other inhibitors of UGPase include ATP and UDP (Neuhaus *et al.*, 1990). Kinetic studies on UGPase extracted from barley revealed that the K_m and V_{max} values for substrates in the synthesis direction were 74 μM and 228 $U.mg^{-1}$ for glucose-1-phosphate and 93 μM and 255 $U.mg^{-1}$ for UTP respectively (Elling, 1996). In the pyrophosphorylysis reaction the observed K_m and V_{max} values were 0.172 mM and 345 $U.mg^{-1}$ for PPi and 0.191 mM and 350 $U.mg^{-1}$ for UDP-Glc respectively. It was further reported that UGPase in barley exists as a 51.6 kDa polypeptide with a pH optimum of 8.35. From such kinetic investigations, it appears that the status of PPi in the cytosol influences the direction of UGPase activity (Kleczowski, 1994).

The PPi content is probably influenced by the gluconeogenic reaction of PFP, the enzyme that has been implicated in regulating PPi levels in the cytosol (Neuhaus *et al.*, 1990; Dancer and ap Rees, 1989). Removal of this inorganic substrate may result in a channelling of the substrates required by UGPase towards UDP-Glc synthesis. This phenomenon is thought to occur in source tissues where UGPase is involved in sucrose biosynthesis (Kleczkowski, 1994). However the directional flux of carbon through UGPase might rather depend on the demand determined by the stage of tissue development. In actively growing potato tubers for example, imported sucrose was degraded by the cytosolic Susy to form fructose and UDP-Glc, which was utilized by UGPase to form the precursors for amyloplastic starch biosynthesis (Sowokinos, 1990). In the

sprouting tuber, where sucrose synthesis is prone to occur, UGPase facilitated UDP-Glc formation, for the synthesis of sucrose, pectins, hemicelluloses, glycolipids and other assorted glycosylated molecules (Goodwin and Mercer, 1983; Sung *et al.*, 1988; Sowokinos, 1990). In transgenic potato plants with a 50 % reduction in the UGPase activity, a decrease in sucrose concentration was reported (Borovkov *et al.*, 1996), consistent with the important role UGPase plays in sugar synthesis.

In non-photosynthetic tissues and developing leaves, UGPase has been linked to sucrose degradation, providing carbon skeletons for starch synthesis (Ciereszko *et al.*, 2001). It was further stated by other researchers such as Sowokinos and co-workers (1997) that during periods of environmental stress, the potato UGPase could impose control at the catalytic level by restricting the flow of carbons in the gluconeogenic direction.

In rice scutella tissue, biochemical studies have shown that UGPase plays a significant role in sucrose synthesis by producing UDP-Glc as a donor for glucose residues (Kimura *et al.*, 1992). Studies conducted by researchers such as Sowokinos *et al.* (1993) revealed that during periods of elevated hexose-phosphate levels, a coarse metabolic control via UGPase may actually supply varying levels of UDP-Glc, hence directly affecting the velocity of the rate limiting SPS reaction in potato (Sowokinos *et al.*, 1993).

A review article further highlighted the use of UGPase as an excellent model to study the pyrophosphorylases at the protein/enzyme level (Kleczowski, 1994). This is possible due to the identified characteristics of UGPase, namely: it is a single-gene-encoded protein; it is very active both in source and sink tissues; and it is stable both in its crude and purified form. Moreover, due to its strategic positioning at the crossroads of several sucrose metabolic pathways, UGPase may also represent a suitable target for transcriptional regulation under different conditions (Kleczowski, 1994). This hypothesis is substantiated by the discovery of membrane-bound UGPase activity in barley (Eimert *et al.*, 1996). It was further suggested that there was either a distinct gene for the membrane-bound UGPase protein or some post-translational modification mechanism for an otherwise soluble enzyme (Eimert *et al.*, 1996). At present, however, no evidence distinguishing these two possibilities has been reported. An avenue that could potentially answer this question is the determination of the site(s) of UGPase expression in the different plant tissue types.

2.9. Concluding Remarks

Much research has been directed at understanding and determining the biochemistry and physiological functions of these enzymes in both plants and animals. However, to date, nothing to our knowledge has been reported to locate the sites of expression of these enzymes *in situ*. The determination of the sites of UDP-Glc DH, PFP and UGPase cellular expression in the different cell types in the sugarcane stem will enhance our knowledge of the underlying metabolic and regulatory mechanisms associated with sucrose accumulation. Such information may ultimately lead to the development of new tools for improving sucrose yield in sugarcane.

CHAPTER 3
DEVELOPMENT OF AN *IN SITU* HYBRIDISATION TECHNIQUE FOR SUGARCANE

3.1. Abstract

The aim of this project was to determine the parameters required for the examination of cellular gene expression in sugarcane using the technique of *in situ* hybridisation (ISH). It was deduced that tissue fixed for a period of 24 h, pre-treated by de-proteinization and acetylation were essential for the minimization of background signal and the visualization of the purple-pink precipitate in tissues treated with the DIG-labeled anti-sense probes. Moreover, it was found that different probes require different hybridisation temperatures. The UDP-Glc dehydrogenase, pyrophosphate dependent phosphofructokinase and UDP-Glc pyrophosphorylase mRNAs required a hybridisation temperature of 37, 50 and 45°C respectively. The variation in temperature was attributed to the differences in probe length and GC content.

3.2. Introduction

Plants are complex structures that are composed of a number of different tissues that in turn comprise several different cell types (Walker *et al.*, 2001). The metabolic processes that occur within these different types of tissues and cells vary to a significant degree. Our understanding, however, of how metabolic processes are compartmentalized between the different tissue and cell types of such complex plant structures is very limited. An understanding of both metabolism and the role of individual enzymes within the different tissue and cell types is therefore urgently required. Molecular biology, cytogenetics and methods of *in situ* hybridisation (ISH) have already enhanced our understanding of the structure, function, organization and evolution of genes and the genome.

In situ hybridisation (ISH) has been reported to be a powerful tool in locating the expression of new genes and in detecting the temporal and spatial expression patterns of the gene transcripts (Drews, 1998). RNA ISH localizes mRNA to the cytoplasm of cells that are expressing the specific gene transcript sequences. Single stranded RNA (ssRNA) probes have been reported to have excellent hybridisation efficiencies (Drews, 1998). A problem somewhat peculiar to ISH analyses in plant tissues is the presence of large vacuoles and cells of differing size. These features result in very different amounts of cytoplasm per unit section area in different tissues (McFadden, 1995). The problems associated with this and other non-

radioactive hybridisation techniques include high background noise and the lack of consistency in signal specificity in the different tissue types (Drews, 1998). The parameters affecting the outcome of ISH results include: probe quality; plant tissue fixation; pre-hybridisation tissue treatment; hybridisation conditions and buffers, and stringency of post-hybridisation washes (Drews, 1998).

Single stranded RNA probes or riboprobes are commonly used in ISH studies as they offer several advantages over double stranded DNA (ds DNA) probes. Firstly, asymmetric probes cannot anneal during hybridisation. Secondly, RNA: RNA hybrids are considerable more stable than RNA: DNA hybrids. And thirdly, post-hybridisation RNase digestion dramatically reduces background caused by non-specific binding. Single-stranded RNA probes hybridize with an eight-fold greater efficiency than ds DNA (Cox *et al.*, 1984). Shorter probe fragments give higher signals for ISH, presumably because they penetrate the tissue better (Drews, 1998 and references therein). They are also able to find their target sequences free from molecules interfering with duplex formation (Hougaard *et al.*, 1997). Long probes possess more labeled molecules and form stable duplexes even if the target sequence is partially unavailable. However, tissue penetration by the long riboprobe becomes limited. The liability of probes (of any length) is that they can adhere to various cellular components thereby causing unspecific staining (Hougaard *et al.*, 1997). There is therefore a need to evaluate the stability of duplexes formed between the riboprobe and its target sequence. Hybrids that are very rich in G and C bases are much more stable than those that are rich in A and T/U bases (Morel and Cavalier, 2001). If too many G-C bases are present, non-specific combinations occur, whilst hybrids containing a large number of A-T/U bases are very easy to denature (Morel and Cavalier, 2001). A useful and commonly used approximation can be expressed as the temperature at which half the duplexes dissociate (Tm). This temperature can be calculated from the empirical formula formulated by Fitzpatrick-McElligott *et al.* (1988) and modified by Hougaard *et al.* (1997). The calculated Tm value is directly dependent on the sum of the GC content of the probe and the log value of the sodium content in the hybridisation buffer. Moreover, this temperature is inversely correlated with the difference between the probe length and percent formamide in the buffer.

Tissue fixation is one of the most crucial steps in ISH studies. It preserves the morphology of the tissue, which is essential for the accurate interpretation of ISH results. Fixation also cross-links RNA to proteins and other macromolecules, and is therefore critical for RNA retention during the hybridisation and wash steps. Tissue fixation must be rapid and efficient

in order to eliminate or minimize the action of endogenous RNases. In addition it must leave the mRNA in a condition where it is capable of forming stable duplexes with the riboprobe of choice (Hougaard *et al.*, 1997). Signal can either be reduced or eliminated if the tissue is either over- or under-fixed. If the tissue is over-fixed, the probe cannot penetrate the tissue. If the tissue is under-fixed, the RNA gets washed out of the tissue. Thus, to maximize signal, the determination of the appropriate duration of fixation is essential.

Nucleic acids located *in situ* are embedded in a cellular protein matrix following fixation. This must be permeabilized before efficient hybridisation can occur (Hougaard *et al.*, 1997). Three pre-treatment options are available: enzymatic (using proteases such as pronase, proteinase K or pepsin); chemical (using HCl or NaCl), and physical (using a detergent such as SDS or Triton X100 that cause partial deproteinization). Pronase is also used to partially digest the tissue so as to facilitate better probe penetration. Pronase-treated tissue gives better signal than untreated tissue. For *Arabidopsis* and grape berries, the best incubation time is 30 to 45 and 10 min, respectively (Drews, 1998 and Zwiegelaar, 2002). Depending on the tissue, a shorter pronase treatment may weaken the signal and a longer time may destroy the morphology of the tissue. The use of hydrochloric acid as part of the tissue pre-treatment regime is essential for RNA denaturation. The optimal duration of the HCl treatment is a fine balance between increased target accessibility versus the loss of mRNA and morphology (Kong and Simon, 1998; Hougaard *et al.*, 1997). Other pre-treatments also play an important role in reducing non-specific staining e.g. acetylation reduces the unspecific electrostatic binding of probes to tissue. The final step in the tissue pre-treatment procedure is dehydration, which is essential for the elimination of any aqueous medium from the section itself and to prevent dilution of the hybridisation buffer or probe.

To date, ISH protocols have been optimized and subsequently reported for many species of plants and animals (Duck 1994; Hougaard *et al.*, 1997; Sassa *et al.*, 2001). A procedure describing the localization of mRNA *in situ* for sugarcane tissue has yet to be reported. This study aimed to optimize the parameters for ISH studies on sugarcane culm tissue by varying the embedding medium; fixation time; tissue pre-treatment and hybridisation temperature.

3.3. Material and Methods

3.3.1. Plant material

Mature, non-flowering sugarcane stalks (*Saccharum* species hybrids, N19 variety), with approximately 18-20 internodes above ground, were randomly selected from the Schaffer

residence, Stellenbosch, South Africa. For the purpose of this study, a sample size of three was chosen.

Sections of sugarcane (N19 variety of *Saccharum* spp. hybrids) spanning half the core, mid-internodal and peripheral regions of different internodes were excised and sliced at a thickness of approximately 0.2 mm and a radius of 5.5 cm before being exposed to the different tissue pre-treatment procedures for embedding and subsequent sectioning.

3.3.2. Tissue preparation

3.3.2.1. Use of different embedding media

3.3.2.1.1. Wax

Sectioned tissue discs from internodes 3 and 7, were immediately placed into 2% (w/v) formaldehyde and 0.5 % (v/v) glutaraldehyde in 50 mM Na-Piperazine-N, N'-bis (2-ethanesulfonic acid) solution under vacuum and left overnight. Tissue sections were then treated according to the protocol outlined by Schwarzacher and Heslop-Harrison (2000). Sections were washed in phosphate-buffered saline solution (PBS), pH 7, dehydrated through a graded ethanol series, and left overnight in 100% (v/v) ethanol. Following this, the material was transferred to a solution containing 0.1 % (m/v) Eosin Y in 95% (v/v) ethanol. Thereafter the tissue segments were placed in a solution mixture containing equal parts of ethanol and xylene. Then the material was transferred into a solution of 1:1 xylene: molten paraffin wax (Paraplast X-tra, Sigma), and finally into molten wax. The molten wax was changed each day for 2 weeks and during this period twice subjected to vacuum infiltration at a pressure of 40 kPa for 4 hours. Thereafter, the samples were placed in Petri dishes filled with molten wax and placed in the refrigerator at 4°C to solidify. The embedded tissue was then cut into square blocks with a millimetre space of wax around the central tissue. These blocks of tissue were fixed onto wooden blocks, 2 x 2 x 2 cm and sectioned at a thickness of 10 µm using a microtome (Leitz Wetzlar, Germany). The sectioned material was placed onto a water droplet on a pre-cleaned glass slide (Superfrost Plus, Menzel-Glaser, Germany), and placed on a slide warmer at 40°C until the sections were dry. Thereafter the slides were placed in a container and treated accordingly for the purpose of *in situ* hybridisation.

3.3.2.1.2. Resin

Sugarcane culm tissue discs from internodes 3 and 11, were placed in 2% (v/v) glutaledehyde, 100mM sodium phosphate buffer (pH 6.9) and 0.2% (v/v) saturated aqueous picric acid and left overnight at 4°C (Schwarzacher and Heslop-Harrison, 2000). In brief, following fixation,

the tissue samples were washed for 5 min in the phosphate buffer. Thereafter the samples were dehydrated through a graded ethanol series for 10 min each in 10, 20, 30, 50, 70, 90% (v/v) EtOH in water. Then the samples were washed twice for 10 min in 100% EtOH. LR White Embedding Medium (Sigma) was introduced to the sample already containing ethanol in the following ratios 3:1 for 30 min, 1:1 for 30 min, 1:3 for 30 min and 0:1 for 48 hrs. Embedding moulds (Sigma) were filled with resin and the tissue was orientated in these moulds before they were polymerised for 15 h at 60°C. Tissue embedded in the resin was then sectioned using a Reichert Ultracut Microtome (LKB-Produkter AB, Sweden) at a thickness of 70nm.

3.3.2.1.3. Cryosectioning

Tissue sections from internodes 10 and 20 were treated according to the protocol described by Barthel and Raymond (1990). Sectioned sugarcane tissue was fixed with FAA (2% (v/v) formaldehyde, 63% (v/v) ethanol and 5% (v/v) acetic acid) with 50% (w/v) sucrose (for osmolarity protection) for 24 h. After rinsing the tissue with 20% (w/v) sucrose in 0.1 M sodium phosphate buffer in ratios of 1:2, 1:1 and 2:1 for 30 min each, the tissue was cryoprotected with 20 % sucrose in phosphate buffer at 4°C overnight. The tissue was then infiltrated with a mixture of 20% sucrose in phosphate buffer and O.C.T embedding medium (Tissue-Tek, USA) in the ratio 2:1 respectively for 30 min each at room temperature. The tissue was then transferred to an aluminium foil embedding mould filled with fresh infiltration medium before the foil mould was rapidly plunged into isopentane pre-cooled with liquid nitrogen. Fifteen micrometer sections were cut at –20°C using a Leica CM100 Cryostat.

3.3.2.1.4. Fresh sections

Sections from internodes 3 and 11 were cut with a hand microtome at a thickness of 0.5 mm and placed immediately into fixative (4% (w/v) paraformaldehyde with 0.3% (v/v) Triton X100) for 24 h. The tissue was then thoroughly washed with Milli-Q water before exposure to the *in situ* hybridisation protocol.

3.3.3. Tissue fixation and pre-treatment

Sugarcane culm tissue was bored out using a hand borer at a diameter of 11 cm. The lengths of tissue were then cut transversely at a thickness of 0.2 mm and placed immediately into fixative (4% (w/v) paraformaldehyde with 0.3 % (v/v) Triton X 100). Fixation times included 12, 24, 48 and 72 h. These times were linked to investigating the necessity of a tissue pre-treatment step involving tissue exposure to dilute HCl and pronase and acetylation. The

protocol used in this investigation was a modification of that reported by Simon (1998). The tissue was washed twice for 5 min each under running Milli-Q water. It was then subjected to a 10 min wash in 0.2 M HCl. Again, the tissue was washed for 5 min in Milli-Q water. The tissue was washed twice for 2 min in PBS. The tissue deproteinization treatment involved exposure of the tissue for 10 min in pronase containing 0.125mg.ml^{-1} in pronase buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA). This was followed by a 2 min wash in glycine (0.2% (v/v) in PBS). The tissue was again washed for 2 min in PBS. In order to improve the efficacy of the hybridisation, the tissue was post-fixed for 10 min in formaldehyde. This was followed by two 2 min washes in PBS. In order to inactivate the proteases and reduce the electrostatic binding of the probe to sections, the tissue section was washed for 10 min in acetic anhydride (1% (v/v) in 0.1M triethanolamine, pH 8). The tissue was finally washed for 2 min in PBS, before exposure to a dehydration series of 30, 50, 85 and 100 % (v/v) EtOH in MQ water.

3.3.4. Hybridisation temperature

The hybridisation temperatures for each of the three probes had to be optimised. It has been suggested that for RNA probes, annealing temperatures between 37 to 65 °C are able to better preserve tissue morphology and allow sensitive annealing of the probe to the target RNA (Lehmann and Tautz, 1994). Hence, the temperatures that were investigated included 37, 40, 45, 50, 55, 60 and 65°C using internode culm 7, which was chosen as a representative of the intermediate growth stage in sugarcane.

3.3.5. Microscopy

The sections were then studied with a Nikon Eclipse E400 Microscope.

3.4. Results

3.4.1. Varying embedding media

The embedding medium of paraffin wax facilitated the transformation of the semi-liquid biological material into a homogenous solid that could both preserve tissue structure and be sectioned with sufficient ease (Morel and Cavalier, 2001). Although RNA *in situ* hybridisation is most frequently performed using paraffin wax as an embedding medium, it was found in this study that wax as an embedding medium was rather viscous and was unable to adequately infiltrate the rigid sugarcane tissue. Hence sectioning of the material was rather

problematic especially with obtaining a strip of intact wax sections. Sections were not intact. The tissue also showed weak sensitivity to the riboprobe (Figures 3.1 a and b). To overcome such disadvantages, an alternate embedding medium, resin, was used. London resin is hydrophilic and does not require removal like wax. It was better able to penetrate the hard sugarcane tissue than wax (Figure 3.1 c and d). A disadvantage of this technique is the limited amount of endogenous RNA available in the ultra thin sections (70 nm) for interaction with the labelled probe (Li and Okita, 1995). Another drawback to the use of this medium is the tissue treatment step that precedes its infiltration into the tissue. Infiltration also influenced the cellular morphology. Cells adopted a shrivelled appearance, a result possibly influenced by both the severe pre-treatment and medium infiltration process. Such harsh pre-treatment conditions could potentially cause a redistribution of elements otherwise attached to the cellular water, including mRNA. Hence, the option of ice as an embedding medium was investigated. With the technique of cryosectioning, cooling slowed down all biological processes and hardened the tissue. Homogenous freezing has been claimed to be one of the best methods of preserving nucleic acids *in situ* (Morel and Cavalier, 2001). However in this study, fixation was not successfully achieved and hence there was loss of both cell turgor and morphology and tissue integrity (Figures 3.1 e and f).

Cellular morphology was most satisfactorily maintained by the embedding medium, wax. However, obtaining intact wax-embedded tissue sections was a challenge. Hence, the alternative was to use the technique that was selected to serve as a control to the standard wax embedding procedures undertaken in this study. This option involved the use of fresh hand-sectioned material that was fixed in paraformaldehyde only before exposure to the standard ISH protocol employed in this study (for methodology, refer to chapter 4). This technique was able to best preserve and maintain cell composition and tissue integrity (Figures 3.1 g-h). Cells of the sugarcane culm are large, 240 µm in length and 140 µm in diameter (Moore and Maretzki, 1996) and hence thicker sections would be best able to retain the cellular constituents.

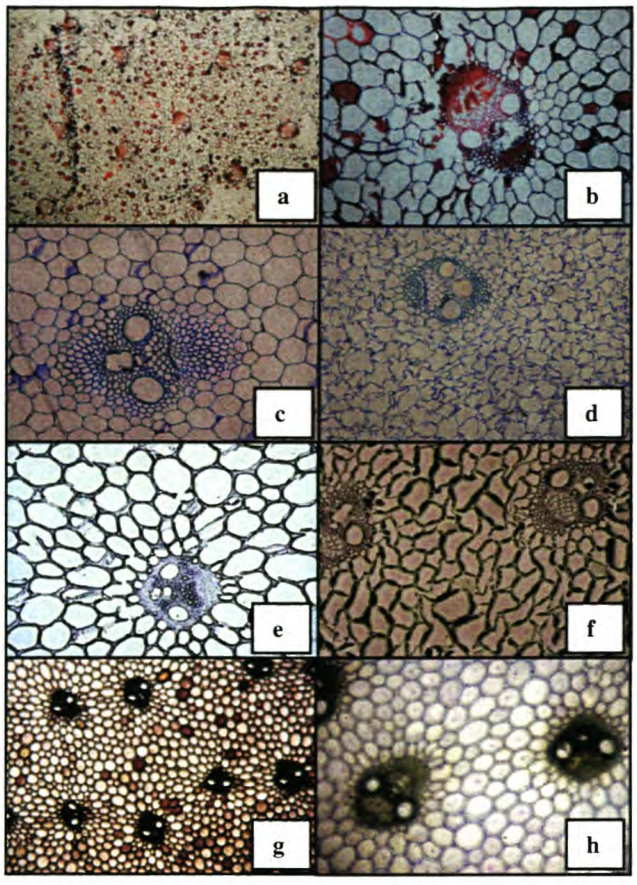

Figure 3.1: Sections of sugarcane tissue treated with different embedding media: wax sections of sugarcane internodes 3 (a, 40x) and 7 (b, 200x); resin sections of sugarcane internodes 3 (c, 200x) and 11 (d, 100x); cryosections of sugarcane internodes 10 (e, 200x) and 20 (f, 200x); and fresh sections of sugarcane internodes 3 (g, 100x) and 11 (h, 200x).

Such hand sectioning yielded the best results in that intact cells, cell wall and membrane morphology as well as integrity were well maintained. ISH studies were also successful and background signal was reduced.

3.4.2. Fixation time

Optimisation studies on the fresh hand sectioned material revealed that a fixation period of 24 h was adequate for the preservation of both cellular morphology and nucleic acid integrity in sugarcane. In previous studies, it was reported that paraformaldehyde almost always preserved tissue morphology and cell content, including mRNA, preservation and retention (Drews, 1998; Hougaard *et al.*, 1997). Paraformaldehyde as a fixative was able to better preserve the morphology of the large sugarcane internodal cells as compared to either glutaraldehyde or FAA. The latter two fixatives severely compromised both tissue integrity and cell turgor (Figures 3.1 a-f). However, other factors could have played a significant role.

These include the type of embedding medium used (e.g. wax, resin or ice) and the duration of both fixation and infiltration.

3.4.3. Pre-treatment of tissue

Tissue immediately exposed to ISH procedures without the pre-treatment step involving HCl, pronase, and acetylation showed an increase in background staining (Figure 3.2a). This resulted in a negligible difference between the sense and anti-sense probes that were used as control and treatment studies respectively (Figure 3.2b). The pronase treatment that was included in the ISH experiment significantly reduced background staining (Figure 3.2c) and improved detection of the cytoplasmic mRNA in tissue sections treated with the anti-sense probe (Figure 3.2d).

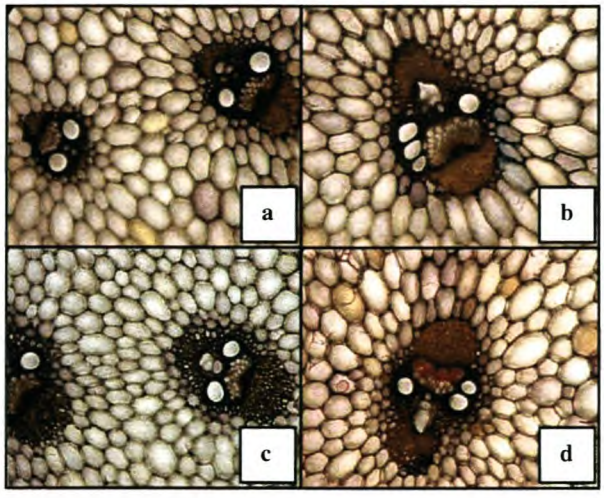

Figure 3.2: 24 h fixation of hand sectioned sugarcane internode 7. Exclusion of the tissue pre-treatment step revealed that there was no discernible difference between the sense (a, 200x) and anti-sense (b, 200x) probes. Inclusion of the tissue pre-treatment step showed no signal in the control (c, 200x) and the presence of the transcript, as determined by a purple-pink precipitate, following tissue hybridisation with the DIG-labeled anti-sense probe (d, 200x).

3.4.4. Hybridisation temperature

Each of the probes used in this experiment were of varying lengths and hence required different annealing temperatures for successful hybridisation with their target sequences. It was found that the different probes that were tested at a temperature range from 37 to 65°C, hybridised at different temperatures. UDP-Glc DH (561 bp) successfully hybridised at 37 °C, PFP (308 bp) at 50°C and UGPase (365 bp), 45 °C (Figure 3.3).

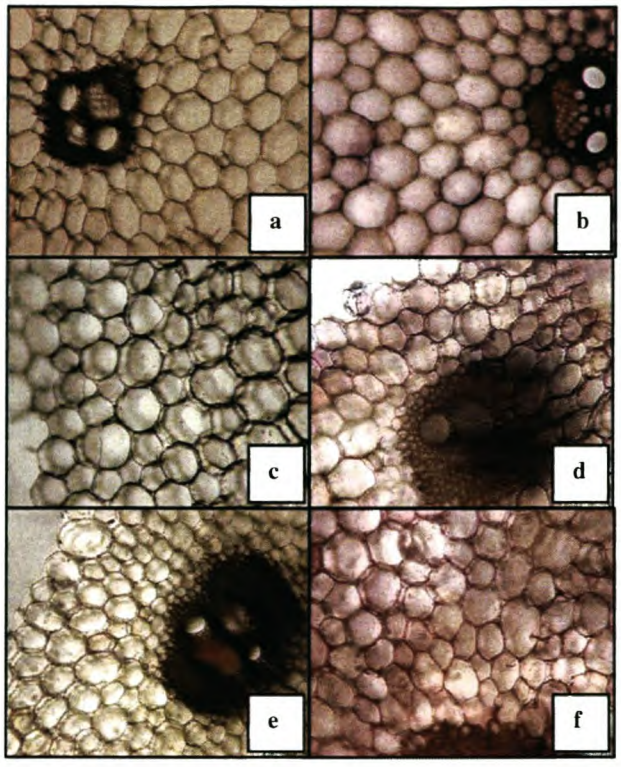

Figure 3.3: Staining of respective transcripts in internode 7 using ISH. The determined hybridisation temperatures were 37, 50 and 45 °C for the UDP-Glc DH (a, 200x, sense and b, 200x, antisense), PFP (c, 200x, sense and d, 200x antisense) and UGPase (e, 200x, sense and f, 200x, antisense) transcripts respectively.

3.5. Discussion:

This study has shown that *in situ* hybridisation studies can be successfully employed in conjunction with biochemical and physiological research to determine both the localization and metabolic function of enzymes in plant tissues.

Although the cellular constituents of sugarcane cells became fixed in position following paraformaldehyde treatment, pre-treatment and the subsequent infiltration of the embedding medium itself caused cell destruction and conformational changes to proteins, nucleic acids and structural polysaccharides (Sitte, 1996). Aggressive dehydration by organic solvents for both wax and resin embedding followed by infiltration by the respective media at either high (for paraplast wax) or low (for London Resin) temperatures resulted in membrane disruption and the redistribution of cellular elements.

Strong evidence suggests that ice, as an embedding medium could also be detrimental to the structure of the cell. If very rapid cooling is not achieved, the resultant ice crystals could incur injurious effects on the morphological, elemental distribution and in particular ISH and immunohistochemical analyses of cells (Sitte, 1996). Results from the present study supported this argument. Tissue slices exposed to fixation, pre-treatment and subsequent infiltration with an embedding medium (wax, resin or ice) all showed severe distortion of the cell membrane (Figure 3.1), and loss of cellular content which inadvertently implied the loss of cytoplasmic RNA which is critical for the success of any ISH investigation. ISH studies performed on tissue segments processed and preserved with an embedding medium would therefore result in the formation of artefacts that would compromise the validity of the results obtained. Hence, for the purposes of this investigation such tissue was avoided. Although cryofixation provided tissue sections that revealed almost intact cells with satisfactory morphological preservation, the drawback to this technique was the inadequate freezing of the tissue. This resulted in some cells exhibiting the phenomenon of "emptiness", or loss of cellular content, a feature possibly influenced by poor post-fixation. Due to the severe distortion of tissue morphology and cell membrane integrity following the chemical pre-treatments and exposure to infiltration by an embedding medium, the riboprobes under investigation were unable to adequately penetrate the tissue. These riboprobes were therefore incapable of forming stable duplexes with the endogenous mRNA that was either lost or redistributed intracellularly.

This study has further shown that chemical fixation with paraformaldehyde was able to best conserve tissue morphology without the added effects of using an embedding medium which either damaged or disrupted both cell membrane and content integrity. This could be attributed to either tissue rigidity, a feature peculiar to the mature sugarcane culm or poor fixation. One disadvantage to this technique is that the results can only be viewed under low magnification because of the tissue thickness. For a more comprehensive analysis, optimisation of a technique that allows for a more detailed analysis at the ultrastructural level is required.

The present study confirmed that tissue pre-treatment involving de-proteinization using pronase, post-fixation, acetylation, and dehydration were necessary for the successful implementation of ISH experimental protocols on sugarcane culm tissue. The pre-treatment process involving pronase and HCl helped modify proteins and their functional groups that usually become associated with nucleic acids in a dense matrix, following fixation. This

combined action reduced the possibility of the riboprobe becoming attached to the proteins surrounding the target nucleic acid sequences (Morel and Cavalier, 2001). Therefore the results obtained were easily discernible from the controls. It was therefore concluded that the tissue pre-treatment step was critical for the accurate detection of cytoplasmic mRNA.

Although, it has been reported that mRNA ISH studies are most successful at very high temperatures, 50 to 65°C (Hougaard *et al.*, 1997; Lehmann and Tautz, 1994), in the present study it was found that for sugarcane tissue, a moderate temperature range between 37 to 50°C was adequate for successful hybridisation. These temperatures were best able to facilitate riboprobe penetration through the tissue to form stable hybrids with the target sequences. Since the components of the hybridisation buffer were standardised, the only influencing factors were the probe length and their GC content. These two factors contributed significantly to the stability of the hybridisation between the endogenous mRNA and the probes.

This study revealed that the fixation and tissue pre-treatment were processes that were crucial to the success of *in situ* hybridisation experiments on sugarcane internodal tissues in order to preserve good morphology and retain cellular mRNA *in situ*.

CHAPTER 4

GENE EXPRESSION ANALYSIS OF UDP- GLUCOSE DEHYDROGENASE, PHOSPHATE-DEPENDENT PYROPHOSPHOFRUCTOKINASE AND UDP-GLUCOSE PYROPHOSPHORYLASE IN SUGARCANE

4.1. Abstract

This study aimed at investigating the cellular distribution within the sugarcane culm internode the transcripts encoding for UDP-Glc dehydrogenase (UDP-Glc DH), pyrophosphate-dependent phosphofructokinase (PFP) and UDP-Glc pyrophosphorylase (UGPase), involved in sugarcane sucrose metabolism. This was achieved by employing the technique of *in situ* hybridisation (ISH) along with northern and western blot analyses. Cellular gene expression analyses revealed that the UDP-Glc DH transcript was found in almost all the cell types. These included the xylem, phloem and parenchyma of the vascular complex and the storage parenchyma cells. The lignified schlerenchymous sheath surrounding the vascular bundles and the larger storage parenchyma cells present at increasing distances from the numerous vascular bundles, did not however show readily detectable levels of any of the three transcripts. The PFP transcript on the other hand was exclusively expressed in the parenchyma cells of the stem storage compartment and the vascular bundles, whilst UGPase was localised to the phloem and parenchyma of the vascular bundles and those comprising the storage compartment. The relative abundances of both the mRNAs coding for UDP-Glc DH, PFP and UGPase and proteins coding for UDP-Glc DH and PFP were higher in internode 3, indicative of the existence of divergent metabolic pathways in young, actively growing internodes. The observed steady-state mRNA levels coding for UDP-Glc DH, PFP and UGPase declined by internode 7 and the relative transcripts abundance thereafter remained constant throughout development. Hence it can be assumed that internode 7 represents a shift in the sink from utilisation to storage in the sugarcane plant.

4.2. Introduction

Sucrose, the major transport form of reduced carbon in higher plants, is either stored in the vacuole or exported to other parts of the plant via the phloem (Quick and Schaffer, 1996). Transported sucrose can either be retained in sink tissues or further metabolized to sustain cell maintenance and fuel growth or be converted to alternate storage compounds such as starch and lipids (Quick and Schaffer, 1996). The rapid movement of sucrose through the plant via the phloem is dependent on the sugar status in both the source and sink tissues (Farrar *et al.*, 2000). High sucrose levels in source leaves feed back to reduce the expression of genes

encoding photosynthetic proteins, whilst in sink tissues, a high sucrose content feeds forward to increase the expression of genes encoding enzymes associated with sucrose hydrolysis, respiration and growth (Farrar *et al.*, 2000). Furthermore, during the different developmental stages, the direction of organic carbon from one cell or tissue to another requires the metabolic co-ordination associated with the synthesis and degradation of sucrose by respective enzymes occurring at remote sites in the plant cell (Quick and Schaffer, 1996).

The present study targets three separate enzymes implicated in cytosolic sucrose metabolism, namely UDP-glucose dehydrogenase (UDP-Glc DH), pyrophosphate dependent phosphofructokinase (PFP) and UDP-glucose pyrophosphorylase (UGPase). These enzymes have previously been reported to regulate the flux of carbon towards cell wall biosynthesis, glycolysis and sucrose synthesis respectively, in the cytosol of almost all plant cells (Kruger, 1997) including those of the sugarcane culm (Lingle, 1999). Although biochemical studies have been performed on UDP-Glc DH (Turner and Botha, 2002) and PFP (Whittaker and Botha, 1999) derived from sugarcane, all three enzymes have been extensively studied in plants (reviewed by Kruger, 1997).

UDP-Glc DH catalyses the oxidation of UDP-Glc to UDP glucuronic acid (UDP-Glc UA), with the concomitant reduction of two molecules of NAD^+, (Stewart and Copeland, 1998). UDP-Glc UA is responsible for the synthesis of structural polysaccharides and nucleotide sugars in plants (Robertson *et al.*, 1995). Examination of the *Ugd* transcript and protein levels showed that highest activity of this enzyme was expressed in actively growing tissues such as developing roots and leaves in *Arabidopsis* (Seitz *et al.*, 2000) and in soybean (Tenhaken and Thulke, 1996). This indicates that UDP-Glc DH plays a role in providing the precursors for structural polysaccharide synthesis in roots and expanding leaves. Immunohistochemical studies localized this enzyme to the vascular tissue of the French bean (Botha and Small, 1987) and kinetic studies revealed an increase in the specific activity of UDP-Glc DH from cambial to differentiated xylem cells (Dalessandro and Northcote, 1977a). Such findings correlate with an increased demand for UDP-Glc UA and UDP xylose required for secondary thickening in angiosperms (Dalessandro and Northcote, 1977b).

The cytosolic enzyme, PFP, reversibly inter-converts fructose 6-phosphate and PPi to fructose 1,6-bisphosphate and Pi. Kinetic studies on PFP indicate that the fructose 2,6-bisphosphate regulates the activity of this enzyme (Mahajan and Singh, 1989). However, PFP catalyses a reaction close to equilibrium *in vivo*, and assigning a precise role to this enzyme is difficult

(Stitt, 1990; Botha and Botha, 1990; Kruger, 1997). It has been implicated in glycolysis, gluconeogenesis, PPi formation and Pi removal (Murley *et al.*, 1998). It was postulated further that PFP activity is a characteristic common to tissues undergoing extensive biosynthesis such as buds and developing roots (Krook *et al.*, 2000). Other studies revealed that changes in the total PFP activity occurred during developmental processes such as leaf development and maturation processes (Black *et al.*, 1987; Nielson, 1992). These studies provided evidence for the hypothesis that the presence of PFP in tissues is an indicator of sink tissue and sink strength (Black *et al.*, 1987; Black *et al.*, 1995). Recent work on sugarcane revealed that PFP activity is closely associated with sucrose content and its activity was found to decrease with increasing sucrose content in the maturing internodes of the sugarcane culm (Whittaker and Botha, 1999).

The enzyme, UGPase is responsible for the synthesis and pyrophosphorylysis of UDP-Glc, the key precursor for sucrose production, glycolysis and cell wall biosynthesis (Kruger, 1997). Although this enzyme exists in a predominantly soluble cytosolic protein form, a membrane bound UGPase has also been reported (Kleczowski, 1994). Immunohistochemical studies on developing rice endosperm tissue showed that 90 % of UGPase was found to be located in the cytosol whilst the rest was distributed in the amyloplasts and Golgi membranes (Kimura *et al.*, 1992). Little is known about the regulatory events that control UGPase activity and the expression of the corresponding gene(s). Even though the expression of UGPase activity has been shown to increase with phosphate-deficiency stress (Ciereszko *et al.*, 2001) and its regulation influenced by high sucrose concentrations (Spychalla *et al.*, 1994) and low temperatures (Zrenner *et al.*, 1993), as demonstrated in potato tubers, the UGPase expression pattern and its physiological significance remains unclear. Transgenic studies utilizing potato plants with a 50 % reduction in the UGPase activity, revealed that there was a corresponding decrease in the sucrose levels, thus emphasizing the role UGPase plays in sucrose synthesis (Borokov *et al.*, 1996). However, it might be possible that the direction of carbon flow through the UGPase step actually depends on the stage of tissue development (Kleczowski, 1994) or the metabolic status of the tissue itself (ap Rees, 1988).

Establishing the site(s) of localisation of the three enzymes, UDP-Glc DH, PFP and UGPase at the tissue and cellular levels is important for understanding the processes competing for carbon with sucrose storage in sugarcane culms. *In situ* hybridisation and or reporter gene expression technology can elucidate patterns of accumulation of transcripts following the induction of gene expression within tissues (Smith *et al.*, 1994). The use of techniques such

as northern and western blot analyses can also complement such investigations by providing quantitative information about the gene transcript and protein abundance in different tissue types (Sergeeva and Vreugdenhil, 2002). The present study used *in situ* hybridisation together with northern and western blot analyses to determine the tissue and cellular expression patterns of UDP-Glc DH, UGPase and PFP in the different internodal tissue of sugarcane.

4.3. Materials and Methods

4.3.1. Tissue preparation

For the purpose of this study, all experiments were replicated thrice. Internodes 3, 7, 10 and 13 were sampled as representatives of young, immature, mature and old tissues of sugarcane stalks (*Saccharum* spp. hybrid cv N19) comprising of 18-20 internodes. Transverse sections of the sugarcane internodes spanning half the core, mid-internodal and peripheral regions were excised and sliced at a thickness of approximately 0.2 mm and a radius of 5.5 cm. These tissue slices were placed into fixative containing 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) made with 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM $NaH_2PO_4.2H_2O$, pH 7, with 0.1 % (v/v) Triton X 100 and 0.1 % (v/v) Tween 20) overnight. In order for the tissue to be more accessible to the RNA probes, and to reduce any non-specific binding of the probe to the slide, the tissue was washed twice for 5 min each under running Milli-Q water then for 10 min in 0.2 M HCl followed by Milli-Q water for 5 min and twice for 2 min each in PBS. The tissue de-proteinization treatment involved exposure of the tissue for 10 min in 0.125 $mg.ml^{-1}$ pronase (Roche, Germany) in pronase buffer containing 50 mM Tris-HCl; pH 7.5, 5 mM EDTA. This was followed by a 2 min wash in glycine (0.2% (v/v) in PBS). The tissue was again washed for 2 min in PBS. Restabilization of the tissue is crucial before hybridisation and thus the tissue was post-fixed for 10 min in the fixative. This was followed by two 2 min washes in PBS. In order to inactivate the proteases and reduce the electrostatic binding of the probe to sections, the tissue sections were washed for 10 min 1% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8). The tissue was then washed for 2 min in PBS, before exposure to a dehydration series of 50 %, 85 % and 100 % (v/v) ethanol for a duration of 1 min in each.

4.3.2. Probe preparation and hybridisation

In situ hybridisation requires the use of short probes (Drews *et al.*, 1998). Plasmid vectors containing cDNA inserts encoding UDP-Glc DH, PFP and UGPase were obtained from in-house gene-banks. All restriction enzymes used in this study were obtained from Roche,

Germany. The inserts were sub-cloned into pBluescript (SK^+) using Pst I and Bam HI for UDP-Glc DH; Hind III for PFP, and Sma I, for UGPase, with T3 and T7 promoter sequences flanking the multiple cloning sites of each of the sub-cloned fragments. The DNA templates were linearised using Eco RI and Xba I for the UDP-Glc DH, Pst I and Xho I for the PFP and Xba I and Xho I for the UGPase gene sequences. The resulting fragments were purified using five volumes of 5mM $MgCl_2$ in 70% (v/v) EtOH according to the protocol outlined by Sambrook *et al.* (1989). One microgram of linearized template DNA was incubated for 2 h at 37°C in a 20 µl mixture containing 40 U of the appropriate RNA polymerase (T3 or T7), NTP labeling mixture containing 1 mM of each ATP, CTP and GTP, 0.65 mM UTP, 0.35 mM digoxigenin-labeled UTP, transcription buffer containing 400 mM Tris-HCL (pH 8), 60 mM $MgCl_2$, 100 mM dithiothreitol and 20 mM spermidine, and 20 U RNAse inhibitor. The reaction was stopped by the addition of 1 µl 0.5 M EDTA (pH 8) and the labeled RNA was precipitated with 2.5 µl 4 M LiCl and 75 µl EtOH at –20°C for 2 h. After pelleting and washing with cold 70% (v/v) EtOH, the transcripts were dissolved in 100 µl Milli-Q water. The riboprobes were then aliquoted in 10 µl volumes and stored at –80°C. Before storage, 5 µl of the transcript was run on a gel and thereafter a dilution series was set up in order to detect the riboprobe concentration using DIG quantification strips (Roche). The dilution of the probe was achieved as follows:

Generally, about 200ng ml^{-1} of the probe is required for a hybridisation reaction. The final hybridisation mixture consisted of the aforementioned probe concentration in hybridisation solution (Sigma, USA). Once the hybridisation mixture had been applied to the tissue (50 µl per tissue slice), the specimen was covered with RNAse-free hybrid-slips (Sigma). The slides were then placed onto tissue paper soaked in wash buffer, containing 2 x SSC and 50% (v/v) de-ionised formamide, in a plastic box and placed in an oven overnight at 37°C, 50°C and 45°C for UDP-Glc DH, PFP and UGPase riboprobe hybridisation respectively.

4.3.3. Washing

Hybridised tissue sections were placed into sterile 15 ml tubes and subjected to a series of stringency washes to remove the hybridisation mixture and unbound or weakly bound probes. The tissue was washed for 60 min in fresh wash buffer at 50°C. Subsequently the tissue was washed twice for 5 min each in NTE buffer containing 500 mM NaCl, 10 mM Tris-HCl (pH 8) and 1mM EDTA at 37°C. The samples were then incubated for 30 min in the same buffer with the addition of 25 µg ml^{-1} RNAse at 37°C and then washed twice for 5 min each in NTE

buffer at ambient temperature followed by wash buffer at 50°C for 1 h and PBS for 5 min at ambient temperature.

4.3.4. Detection

For the purpose of detecting RNA-RNA hybridisation sites in cells, a chromogenic, enzyme-mediated reporter system using the NBT/BCIP (Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl-phosphate) and the anti-DIG antibody, coupled to alkaline phosphatase was used. The tissue was washed for 5 min in a detection buffer comprising of 100 mM Tris-HCl (pH 7.5) and 150 mM (w/v) NaCl. Thereafter the tissue was placed in blocking solution (1% (w/v) Roche blocking reagent in the detection buffer) overnight at 4°C. The following day, the tissue was placed in fresh blocking solution containing the anti-DIG antibody coupled to alkaline phosphatase (Roche) at a concentration of 1:5000 for 90 min. The tissue was then washed twice for 40 min each in detection buffer with 0.3 % (v/v) Triton X 100 and then with the detection buffer for 5 min and with 100mM Tris-HCl (pH 9.5), 100mM NaCl and 50mM $MgCl_2$ for a further 5 min. The tissue was stained with a solution containing one NBT/BCIP tablet (Roche) per 10 ml of 10% (w/v) polyvinyl alcohol solution (Sigma) until significant colouration was observed.

4.3.5. Microscopy

The sections were studied with a Nikon Eclipse E400 Microscope.

4.3.6. Northern blot analysis

Total cellular RNA was prepared according to the method described by Bugos *et al* (1995). Aliquots of 10 to 20 µg of total RNA from internodes 3, 7, 10 and 13 were separated electrophoretically on a 1.2% agarose gel. The gel was cut to size and transferred to a positively charged Nylon membrane (Roche). The RNA was fixed to the membrane by UV cross-linking at 1 200 $mJ.cm^{-2}$ for 2.5 min. The probes used in the hybridisation included a 960 bp PCR product generated by employing the UDP-Glc DH specific primers, Rev3 and Fwd4, obtained courtesy of Mrs J van der Merwe. The gene specific DNA fragments for PFP and UGPase were purified following extraction from a 1.2% agarose gels. The PFP cDNA was cleaved using Pvu II and Pst I, thereby yielding a 1142 bp fragment whilst a fragment of 1092 bp was derived from the UGPase cDNA cleaved with Sal I and Pst I. These cDNA fragments and the PCR product were labeled with ^{32}P using the Prime-it II random primer labeling kit (Stratagene, USA). The blot was pre-hybridized for 6 h at 65°C in Rapid-hyb buffer (Amersham, UK). Hybridisation was performed in the same buffer overnight at 65°C

using the labeled probes and the blots washed according to manufacturer's instructions, developed and viewed using a Cyclone Storage Phosphor Screen, (Packard Instrument Company Inc, USA). In order to quantify the levels of mRNA expression during sugarcane internode development, the relative pixel unit of the highest units determined for each of the transcripts were used as the reference point and given a percentage value of 100. By comparing the highest value with the respective experimental samples, a series of percentages were obtained. These percentages were then used to normalise the experimental data (in relative pixel units).

4.3.7. Protein expression analysis

Total soluble protein was extracted from the same tissues as RNA following the protocol of Rose and Botha (2000). Protein concentrations were determined spectrophotometrically using the method described by Bradford (1976), with bovine serum albumin (BSA) as a standard. Twenty micrograms of protein representing the different internodes 3, 7, 10 and 13 were separated using SDS-PAGE electrophoresis. The samples were resolved on a discontinuous 12 % (v/v) gel followed by a 4 % (v/v) stacking gel (Laemmli, 1970). A premixed protein molecular weight marker (Roche) was used as a standard. The gel was equilibrated in transfer buffer (48 mM Tris, 39 mM (v/v) glycine, 20 % (v/v) methanol and 0.0375 % (v/v) SDS) for 20 min at 4°C. The protein was transferred onto a Hybond-C (Amersham) membrane using a Transfer blot, Semi-dry transfer cell (BioRad) at 10 to 15 V for an hour. The membrane was stained with Ponceau-S solution (0.2 % (w/v) in 3 % (v/v) trichloroacetic acid), destained with TBST buffer (20 mM Tris (pH 7.6), 137 mM NaCl and 0.1 % (v/v) Tween-20). Blocking was performed overnight with 4% (w/v) BSA in TBST buffer. Primary antibodies against sugarcane UDP-Glc DH and potato PFP were diluted 1: 2000 and 1: 500 respectively and inoculated into the blocking buffer and incubated for 6 h at room temperature. The membrane was then washed thrice with TBST buffer for 15 min each. The commercial secondary antibody, Anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase (Roche) was diluted 1: 2000 in 3 % (w/v) fat free milk in TBST and added to the membrane for an hour at room temperature. Thereafter the membrane was washed thoroughly in TBST, and twice for 5 min each in TBST containing 10 % (w/v) SDS. The membrane was washed in TBST again and finally developed using a detection buffer (1 NBT/BCIP tablet per 10 ml Milli-Q water).

4.4. Results

4.4.1. *In situ* hybridisation

Cellular localization of the UDP-Glc DH, PFP and UGPase transcripts was investigated using *in situ* hybridisation with DIG-labeled sense and anti-sense RNA probes. The controls (sense probes) showed no blue staining (Figures 4.1 to 4.3). The various tissue and cell types that comprise the sugarcane stem were described in chapter 2.

It was observed that the vascular tissue as well as the sucrose storing parenchyma cells showed similar blue staining pattern that were restricted to the cytoplasm of the cell. The blue staining was restricted to certain parts of the cell i.e. the cytoplasm. It is evident that the UDP-Glc DH transcript accumulated in the storage parenchyma cells and the phloem, vascular and parenchyma cells of the vascular complex (Figure 4.1). The transcript of the PFP gene on the other hand, was preferentially localised in the parenchyma cells of the vascular bundles and stem storage compartment (Figure 4.2), whilst the UGPase transcript accumulated in the storage parenchyma cells, the parenchyma associated with the vascular bundle and phloem (Figure 4.3).

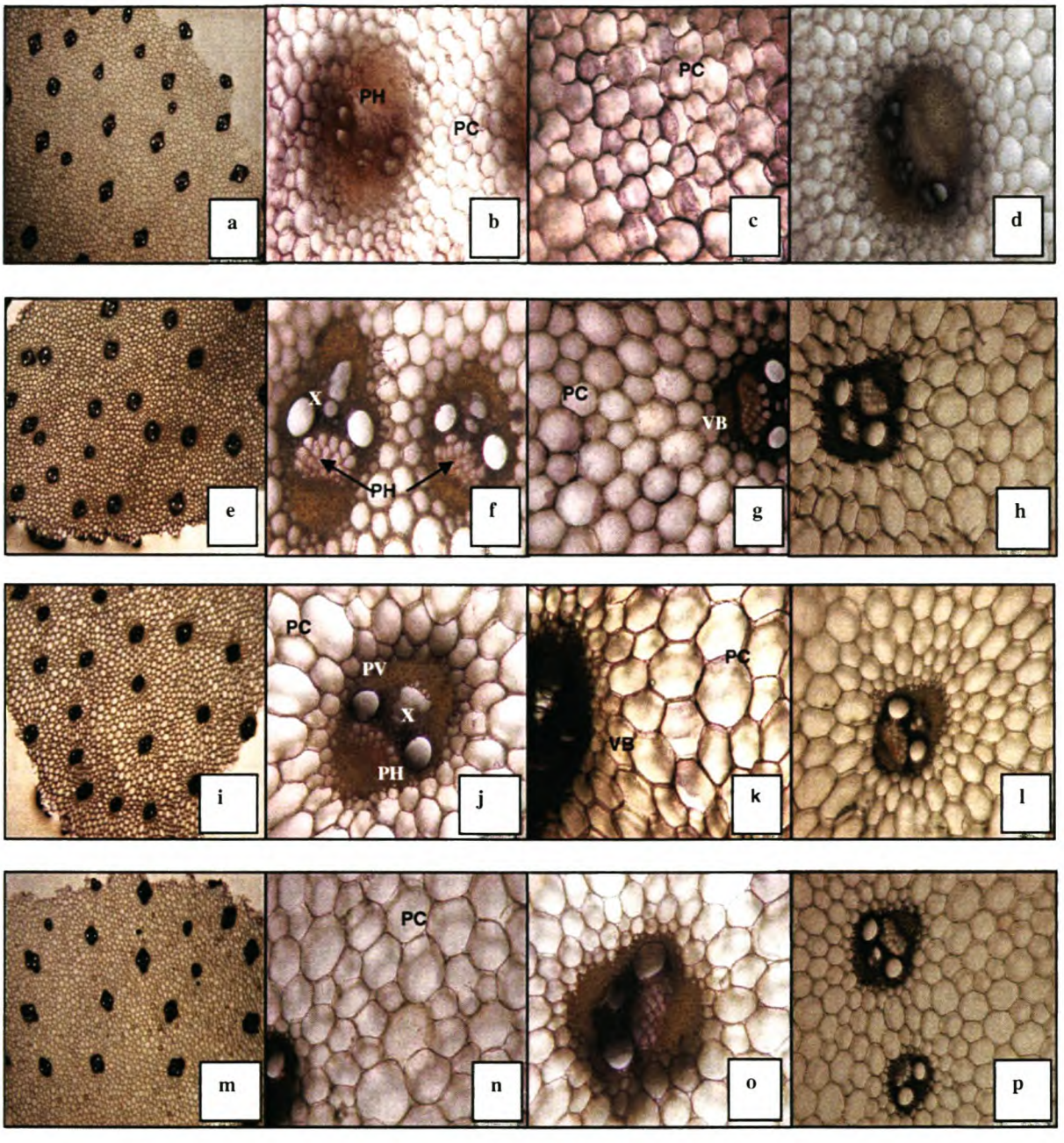

Figure 4.1: Expression pattern of a DIG labelled UDP-Glc DH transcript in sugarcane internodes 3 (a, 40x, b, 100x, c, 200x: antisense probe; d, 100x: sense probe), 7 (e, 40x, f, 200x, g, 200x: antisense probe; h, 100x: sense probe), 10 (i, 40x, j, 200x, k, 400x: antisense probe; l, 100x: sense probe) and 13 (m, 40x, n, 200x, o, 200x: antisense probe; p, 100x: sense probe) using *in situ* hybridisation. PH, phloem; X, xylem; PC, parenchyma contiguous to the vascular bundle; PV, parenchyma associated with the vascular bundle; VB, vascular bundle.

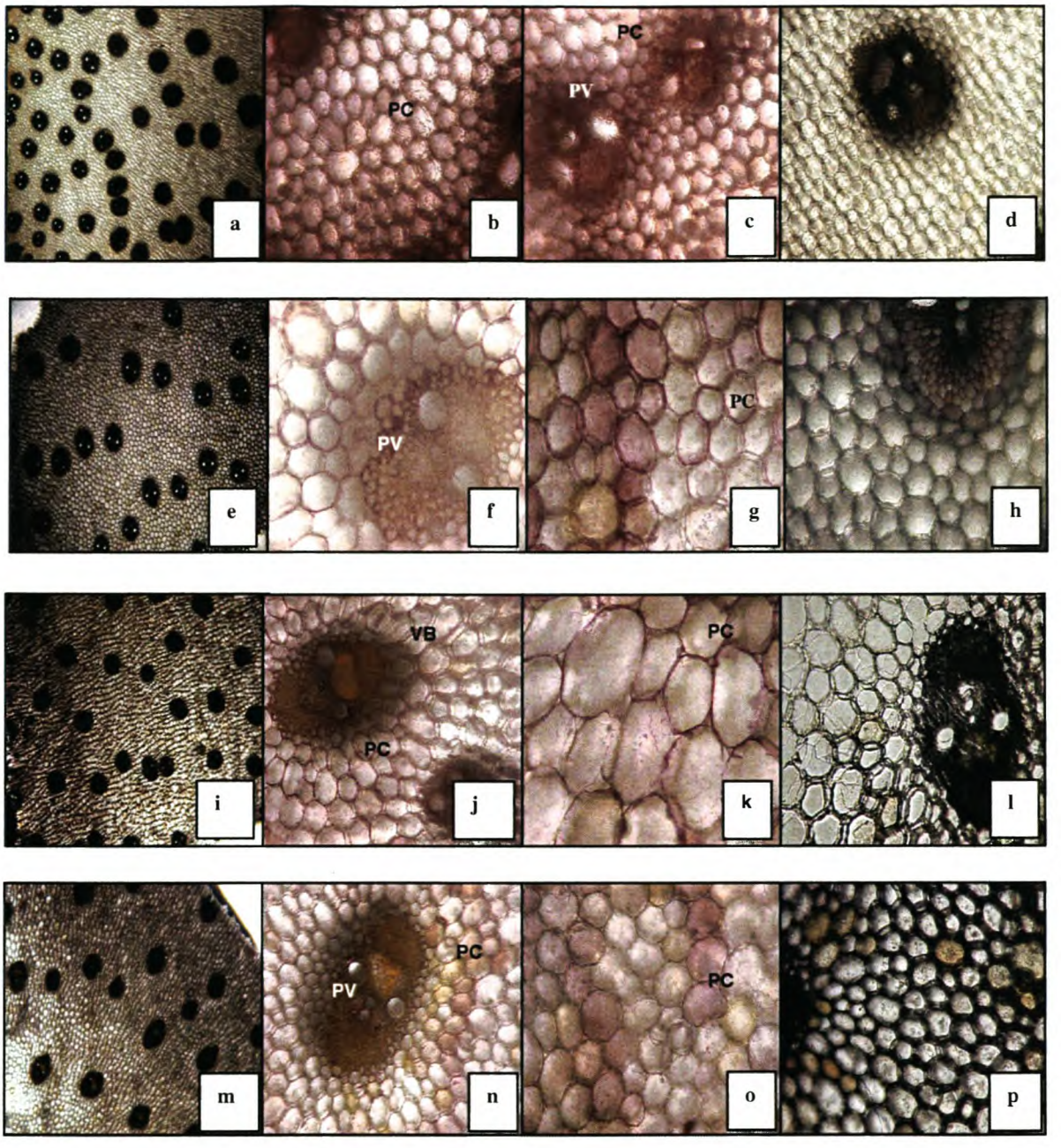

Figure 4.2: Localisation of a non-radioactive DIG labelled PFP transcript in the cells of sugarcane internodes 3 (a, 40x, b, 100x, c, 100x: antisense probe; d, 100x: sense probe), 7 (e, 40x, f, 200x, g, 200x: antisense probe; h, 100x: sense probe), 10 (i, 40x, j, 100x, k, 400x: antisense probe; l, 100x: sense probe) and 13 (m, 40x, n, 100x, o, 200x: antisense probe; p, 100x: sense probe) by *in situ* hybridisation. PC, parenchyma contiguous to the vascular bundle; PV, parenchyma associated with the vascular bundle; VB, vascular bundle.

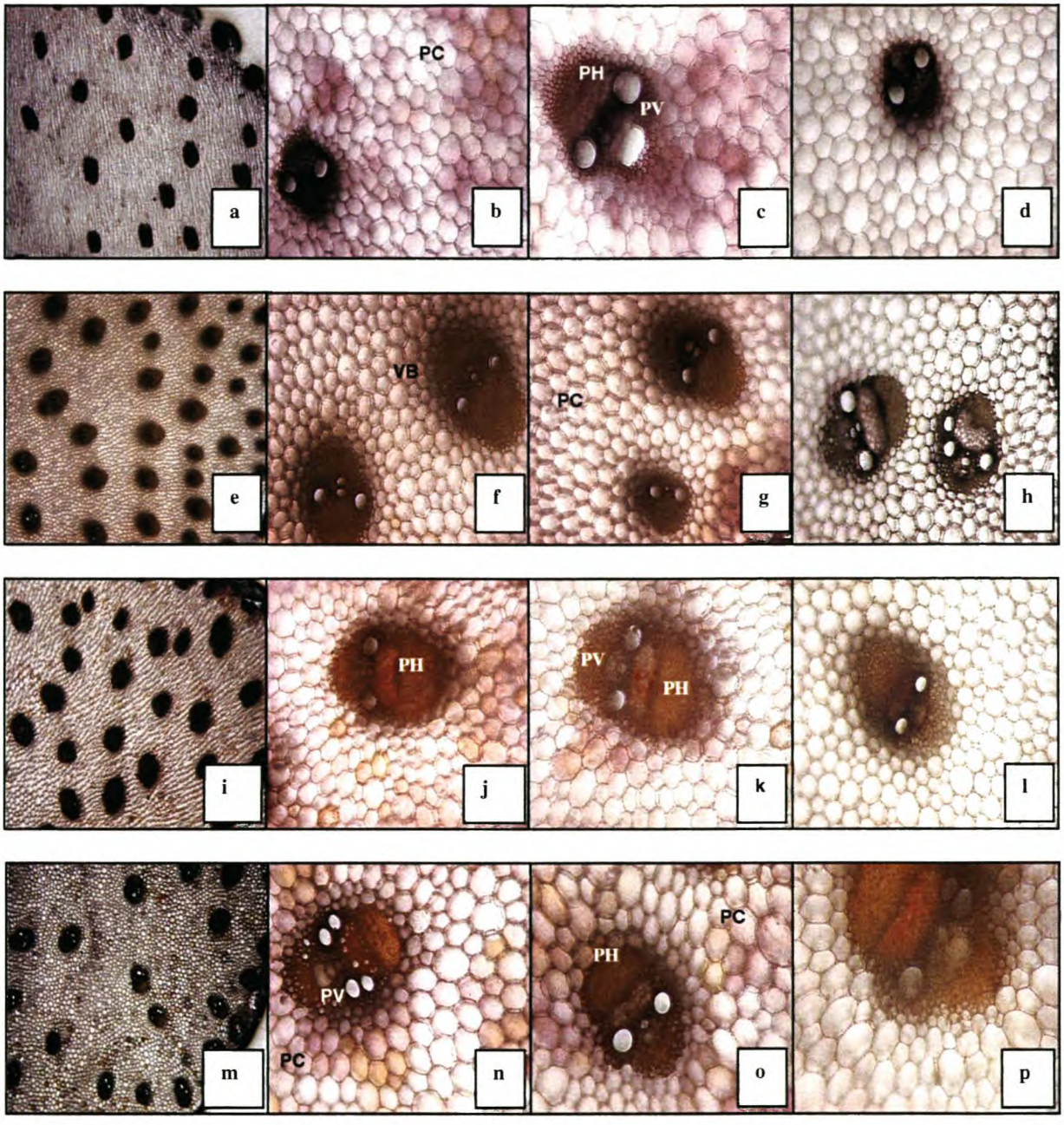

Figure 4.3: Detection of a non-radioactive DIG labelled UGPase transcript in sugarcane internodes 3 (a, 40x, b, 100x, c, 200x: antisense probe; d, 100x: sense probe), 7 (e, 40x, f, 100x, g, 100x: antisense probe; h, 100x: sense probe), 10 (i, 40x, j, 100x, k, 100x: antisense probe; l, 100x: sense probe) and 13 (m, 40x, n, 100x, o, 100x: antisense probe; p, 200x: sense probe) by employing the technique of *in situ* hybridisation. PH, phloem; PC, parenchyma contiguous to the vascular bundle; PV, parenchyma associated with the vascular bundle; VB, vascular bundle.

SUGARCANE STEM CELLS					
ENZYME	Pith Parenchyma	Vascular Bundle			
		Schleren-chyma	Paren-chyma	Xylem	Phloem
UDP-Glc DH	✓	×	✓	✓	✓
PFP	✓	×	✓	×	×
UGPase	✓	×	✓	×	✓

Table 4.1: Summary of the sites of UDP-Glc DH, PFP and UGPase expression in the different cell types of the sugarcane stem

4.4.2. Gene expression analysis

To determine the patterns of UDP-Glc DH, UGPase and PFP expression, the relative abundance of each of the transcripts was investigated in the different sugarcane internodes at various stages of development, including young, immature, mature and old. RNA gel blot analysis indicated that the relative transcript abundances for UDP-Glc DH (Figure 4.4), PFP (Figure 4.5) and UGPase (Figure 4.6) were higher in the young internodes. Whilst, the relative abundance of the mRNA coding for UDP-Glc DH declined in immature (internode 7), mature (internode 10) and old internodal tissues (internode 13), (Figure 4.4), the PFP transcript was only slightly detectable at internode 13 (Figure 4.5) and the UGPase transcript declined marginally at internode 7 and thereafter remained constant throughout development (Figure 4.6).

Single polypeptides with molecular masses of approximately 52 and 60 kDa cross-reacted with the UDP-Glc DH and PFP anti-sera (Figure 4.7). The immunoblots revealed that both the UDP-Glc DH and PFP proteins are highly expressed in internode 3 (Figure 4.7). Whilst the expression of UDP-Glc DH decreased along the length of the culm (Figure 4.7a), the expression of PFP was reduced to a non-detectable level in internodes 7, and remained the same throughout the rest of development (Figure 4.7b). A second weaker band on the UDP-Glc DH immunoblot could possibly reflect the non-specific binding of the antibody or an isozyme of the UDP-Glc DH enzyme, as was reported for poplar (Johansson *et al.*, 2002).

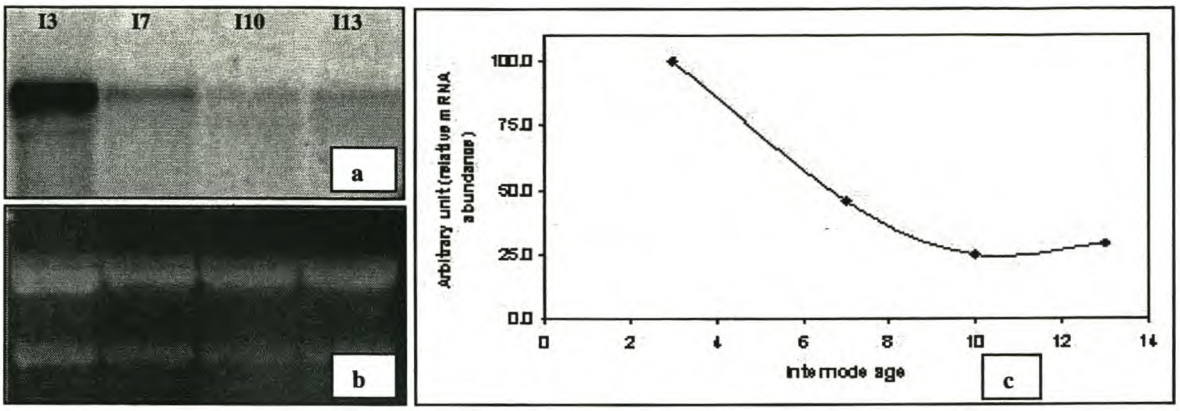

Figure 4.4: Analysis of UDP-Glc DH expression in sugarcane internodes 3 (I3), 7 (I7), 10 (I10) and 13 (I13) (a), with the corresponding gel of the total sugarcane RNA loaded (b). Signal intensity quantified with a phosphoimager and expressed as an arbitrary unit on an RNA and internode basis (c).

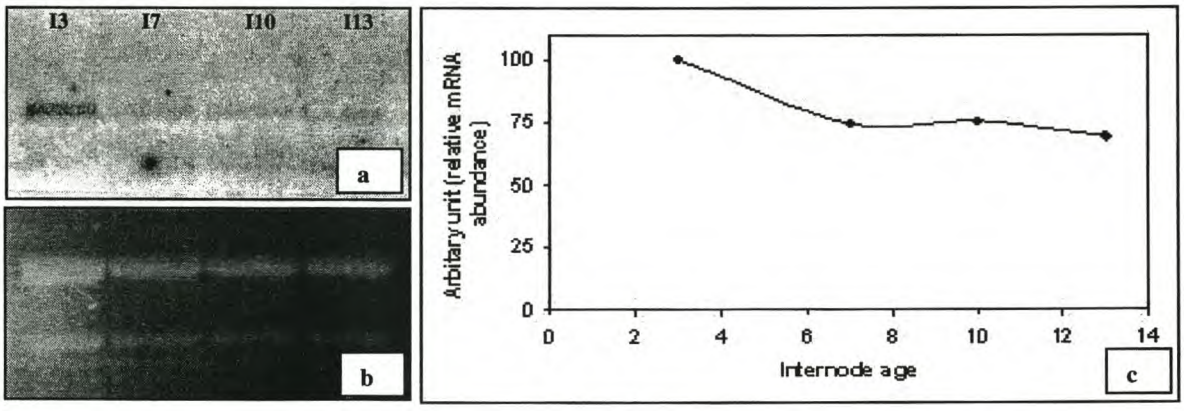

Figure 4.5: PFP gene expression sugarcane internodes 3 (I3), 7 (I7), 10 (I10) and 13 (I13) (a) and the total RNA loaded on an agarose gel (b). Signal intensity quantified and expressed as an arbitrary unit of relative RNA in the different internodes (c).

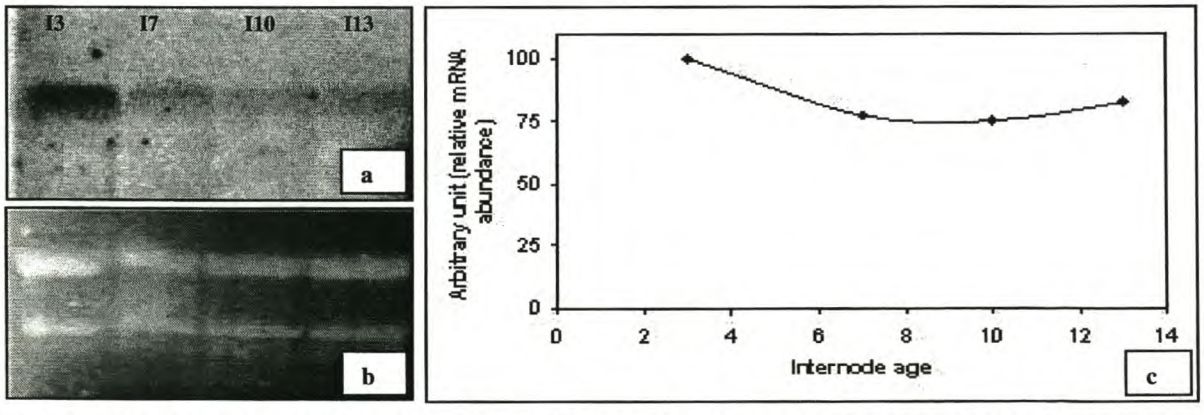

Figure 4.6: Gene expression analysis of UGPase in sugarcane internodes 3 (I3), 7 (I7), 10 (I10) and 13 (I13) (a) and the corresponding RNA gel blot (b). Signal intensity quantified and expressed as an arbitrary unit on both an RNA and internodal age basis (c).

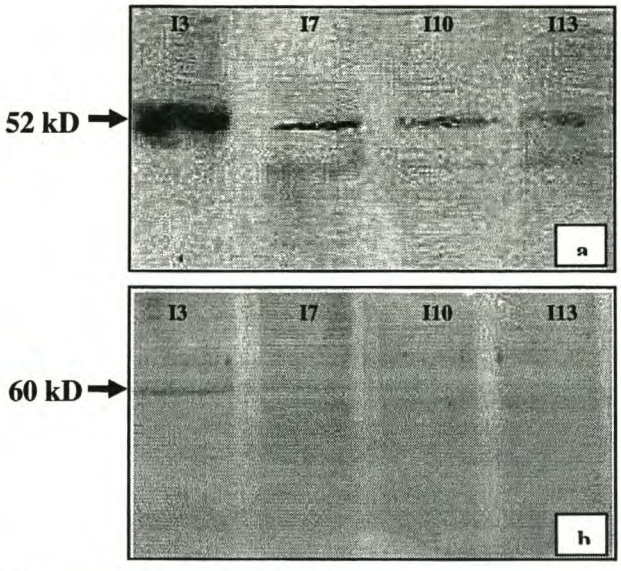

Figure 4.7: Western blots with protein extracts from internodes 3 (I3), 7 (I7), 10 (I10) and 13 (I13) separated on SDS-Page. A sugarcane anti-UDP-Glc DH antibody was used at a 1: 2000 dilution (a), whilst a potato anti-PFP antibody was diluted 1:500 (b)

4.5. Discussion

Phloem and xylem undergo elongation, expansion and secondary wall thickening before reaching a mature, differentiated state (Dalessandro and Northcote, 1977a). Once mature, these cells can withstand a substantial amount of tension and changes in turgor. Tension is usually a product of transpiration and gravitational forces acting simultaneously, whilst fluctuations in turgor is associated with the movement of organic solutes including sucrose between the symplast and apoplast, and water in the phloem and xylem elements, respectively (Thorpe and Minchin, 1996; Moore, 1995). Such activity creates a stress on the walls of xylem and phloem cells. The presence of UDP-Glc DH in the cells of phloem, xylem and parenchyma of the vascular complex and the storage parenchyma cells of the sugarcane culm (Figure 4.1; Table 4.1) was therefore a probable consequence of the requirement for cell wall polysaccharides in order to maintain both cell structure and function. A similar tissue specific expression pattern was demonstrated for *Arabidopsis* where the UDP-Glc DH gene was localised in the vascular system (Seitz *et al.*, 2000). However, in poplar, UDP-Glc DH was exclusively expressed in the leaves and developing xylem (Johansson *et al.*, 2002). Very little or no expression was observed in the phloem.

Earlier findings demonstrated that the demand for hemicellulose precursors was highest in actively dividing and rapidly expanding cells (Dalessandro and Northcote, 1977b). This was

confirmed in the present study. Higher levels of expression of both the UDP-Glc DH mRNA (Figure 4.4) and protein (Figure 4.7a) were detected in the young internodes of the sugarcane culm. The expression of both the transcript and protein decreased along the length of the culm to undetectable levels in older internodes. A similar trend was observed in *Arabidopsis* (Seitz *et al.*, 2000), soybean, wheat and canola (Stewart and Copeland, 1998) where UDP-Glc DH was mainly involved in young growing tissues but not in the mature tissues. This might be correlated with the lack of demand for UDP-GlcUA derived sugars in differentiated cells (Tenhaken and Thulke, 1996). Otherwise, the inositol oxidation pathway may take over the production of UDP-Glc UA for cell wall matrix synthesis (Seitz *et al.*, 2000; Johansson *et al.*, 2002) in the older tissues. The latter argument, however, requires further evaluation.

Investigations aimed at localising the sites of PFP expression on the cellular level revealed that the PFP transcript was found to occur mostly in the parenchyma cells associated with the vascular bundles, and those comprising the ground tissue (Figure 4.2; Table1). Expression of PFP in these cell types may be reflect a strategy employed by the cell for the maintenance of the proper balance of PPi/Pi and fructose-6-phosphate/fructose-2,6-bisphosphate especially during cellular processes such as phloem loading and unloading and assimilate transport from source to sink tissues (Moore, 1995). The observed trend of PFP activity in potato (Stitt, 1990) was similar to the findings in the present study. In sugarcane, the relative PFP mRNA (Figure 4.5) and protein (Figure 4.7b) levels were higher in the sucrose importing tissues of the young internodes whereas the levels decreased in the older sucrose-exporting internodes. The presence of PFP has been demonstrated to represent tissues undergoing extensive biosynthesis (Black *et al.*, 1995) such as buds, developing roots and other sink tissues (Xu *et al.*, 1989; Krook *et al.*, 2000). Therefore, in young, actively dividing sugarcane culm tissues, such as internode 3, imported sucrose could be utilized for the biosyntheses of cellular intermediates such as organic acids, amino acids and structural polysaccharides, as has been demonstrated for other plant species (ap Rees, 1985; Hatzfeld *et al.*, 1990). Furthermore, increased PFP activity may be associated with an increased rate of respiration and a decreased ability to accumulate sucrose. The undetectable levels of both transcript and protein in mature and older internodes (Figures 4.5 and 4.7b) provided further definitive evidence for the decrease in PFP activity with increasing sucrose content down the culm (Whittaker and Botha, 1997). This is highly probable considering that older internodes continue to accumulate sucrose by either importing this dissacharide or increasing the rates of sucrose synthesis. The latter is associated with a decrease of fructose-2,6-bisphosphate (Stitt *et al.*, 1987) which stimulates fructose 1,6-bisphosphatase but inhibits PFP activity (Stitt, 1990).

The UGPase transcript was found to accumulate in the phloem, parenchyma associated with the vascular complex and the storage parenchyma cells (Figure 4.3; Table 4.1). UGPase activity in the phloem and the associated parenchyma may reflect a requirement for either sucrose and/or hexoses during phloem loading and unloading and photoassimilate transport in source and sink tissues as described by Moore (1995). Parenchyma cells, at maturity acquire significant amounts of lignin and suberin on their cell wall surfaces and they become less dependent on the active transport system for maintaining a high internal sucrose concentration (Jacobsen *et al.*, 1992; Moore, 1995). However, these cells do continue accumulating sucrose (Whittaker and Botha, 1999; Rose and Botha, 2000). Hence the expression of the UGPase transcript may represent UGPase activity directed towards UDP-Glc formation, which in turn is aimed at sucrose synthesis (Kleczowski, 1994) in sugarcane storage parenchyma cells. UGPase may therefore regulate carbon flow according to the metabolic status and requirements of the tissue, which is dependent on the developmental stage of the plant. In sugarcane tissue, it was further demonstrated that UGPase mRNA is abundant at all stages of development, even though a marginal decrease in relative mRNA was observed at internode 7 (Figure 4.6). In young internodes of sugarcane, UGPase may operate in the direction of UDP-Glc synthesis, as was reported for actively growing tubers (Kleczowski, 1994) directed towards the synthesis of sucrose or cell wall polymers or both. In the young sugarcane internodes, imported sucrose is probably degraded by Susy to form fructose and UDP-Glc, which is then utilised to form glucose-1-phosphate, the substrate for glycolysis. In older internodes, UGPase may act in the direction of UDP-Glc formation, which is then used in the reaction catalysed by SPS to form sucrose (Kleczowski, 1994). However, this theory requires further kinetic analysis in sugarcane internodes.

Internode 7 represents a shift in the sink from utilisation to storage in sugarcane. This is depicted by the decline in mRNA and protein contents of enzymes implicated in sugarcane sucrose metabolism (Figures 4.4-4.7). However, the signal responsible for this shift is at present unknown. In young tissues, the high expression levels of UDP-Glc DH may reflect its correlative relationship with plant growth processes (Seitz *et al.*, 2000), especially primary cell wall synthesis (Kleczowski, 1994). PFP on the other hand, may be responsible for the gluconeogenic synthesis of PPi required for the conversion of sucrose to sugar phosphates via Susy and UGPase (Black *et al.*, 1987) in the growing sugarcane internodes. However, there also exists the possibility that PFP may operate in the glycolytic direction towards the synthesis of cellular precursors to remove the PPi produced in the reaction catalysed by UGPase where the resultant UDP-Glc is directed towards sucrose synthesis (Kruger, 1997).

This may be correlated with the Susy mediated sucrose cycling observed in *Chenopodium rubrum* cells, potato tubers, maize endosperm (Hatzfeld and Stitt, 1991) and cultured sycamore cells (Huber and Akazawa, 1986). High levels of both PFP and UGPase have been suggested to represent a strategy that plants adopt to adjust the equilibrium between the flow of carbon to glycolysis, the pathway leading to the synthesis of sucrose by SPS and structural cell components mediated by the activity of UDP-Glc DH, when the metabolic demands change (Krook *et al.*, 2000).

4.6. Conclusion

Since growing tissues are simultaneously respiring, expanding and synthesising cell walls, it could be expected that even with independent dedicated pathways, such separate pathways could co-exist in young tissues (Quick and Schaffer, 1996), including young sugarcane internodal tissues. Along with a decline in the total metabolic activity along the length of the sugarcane culm (Bindon and Botha, 2002; Botha *et al.*, 1996), the abundance of enzymes involved in strengthening cell walls, shuffling metabolites between glycolysis and gluconeogenesis, thereby conserving intracellular PPi levels and/or synthesising and degrading sucrose, also decrease (Figures 4.4-4.7). Hence whilst younger tissues are actively involved in sucrose metabolism, older tissues seem to accumulate increasing amounts of sucrose in the vacuole to such an extent that the vacuole occupies almost 90 % of the total cell volume (Komor, 1994).

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

The aims of this study were two-fold: firstly, the development of an *in situ* hybridisation (ISH) technique to investigate cellular gene expression in sugarcane and secondly, to identify the cellular localisation of the cytosolic enzymes, UDP-Glc DH, PFP and UGPase, that have been implicated in sucrose metabolism.

Currently the most effective and commonly utilised techniques to directly determine gene expression in sugarcane include Northern and Western blot analyses. These techniques provide a general, quantitative assessment of tissue-specific gene expression patterns (Drews *et al.*, 1998). Another technique that is able to provide a more detailed assessment of gene expression at a cellular level is that of *in situ* hybridisation (Hougaard *et al.*, 1997). *In situ* hybridisation, although routinely applied to determine the spatial patterns of gene expression at the cytological level in both plant and animals systems (Schwarzacher and Heslop-Harrison, 2000), has only recently been employed to investigate gene expression at the cellular level in sugarcane (Casu *et al.*, 2003). The gene expression technique described in this study was able to detect endogenous RNA sequences in younger internodes only. This could be a consequence of difficulties with embedding the characteristically hard and fibrous internodal tissues of the sugarcane stem with a medium such as wax. Hence, knowledge about gene expression in mature and old internodes of the sugarcane plant is rather limited. The development of an ISH technique that is able to permit the investigation of cellular gene expression at all stages of internode development was therefore required to enable identification of the site(s) of gene expression of separate enzymes involved in sucrose metabolism at a tissue-level.

The ISH technique developed and reported on in this study is a quick (4 day procedure), non-radioactive method that facilitates the examination of cytological gene expression in sugarcane internodal tissues at all stages of development. This technique excludes the use of a harsh embedding medium and the associated chemical and physical tissue processing procedure(s), thereby minimising cellular redistribution and preserving the *in situ* localisation of RNA. Furthermore, thicker sections (0.5 mm) were used in this study than in other standard ISH protocols and thus the relatively large cells of the sugarcane stem were maintained relatively intact with minimal loss of cytoplasmic content. The loss of

cytoplasmic content is a disadvantage that is commonly associated with the use of thin or ultra-thin sections of embedded sugarcane tissues.

This study demonstrated that fresh sections of sugarcane internodal tissues fixed for a period of 24 h and pre-treated according to the protocol described in chapter 3 enabled successful detection of the endogenous RNA sequences in the internodal tissues at various stages of development. However, the technique described in this study did present a few drawbacks. The hybridisation temperature for each riboprobe had to be determined in order to reduce and/or eliminate non-specific riboprobe binding. Furthermore, following fixation, tissue sections had to be immediately processed for ISH analyses and thus could not be preserved for further analysis. Such problems have not as yet been successfully addressed and therefore the ISH technique presented in this study requires further development.

Despite shortcomings, the technique was able to successfully establish the cellular site(s) of localised UDP-Glc DH, PFP and UGPase expression in sugarcane internodes at different stages of development (chapter 4). Previous studies on these enzymes demonstrated that the expression of UDP-Glc DH (Dalessandro and Northcote, 1977b), PFP (Kruger, 1997) and UGPase (Kleczowski, 1994) were associated with young actively dividing tissues, as determined by Northern and Western blot techniques. However, these earlier studies were unable to resolve the tissue site(s) of gene expression.

The current study revealed that the transcripts of all three enzymes were constitutively expressed in sugarcane internodes. UDP-Glc DH was expressed not only in xylem and parenchyma cells but also phloem cells, indicating that these cells types require cell wall matrix polysaccharides (Robertson *et al.*, 1995) for the maintenance of cell structure and function associated with assimilate transport. The expression patterns associated with PFP and UGPase suggest that they too may either directly or indirectly influence assimilate transport processes. PFP may regulate the PPi/Pi and fructose-6-phosphate/fructose-2,6-bisphosphate levels (Ashihara and Sato, 1993) during phloem loading and unloading in the parenchyma cells of the vascular bundles and in the stem storage compartment. UGPase, on the other hand, may be responsible for directing metabolites either towards sucrose synthesis or its hydrolysis (Kruger, 1997) in phloem and the parenchyma cells. The observed UDP-Glc DH, PFP and UGPase expression patterns may also be dependent on the requirements of the sink tissues for either photoassimilate mobilisation or its storage. Such findings have complemented the existing pool of knowledge about the involvement of UDP-Glc DH, PFP

and UGPase and further suggest that these enzymes play a crucial role in maintaining a stable carbon content in the sugarcane culm throughout its development (chapter 4). A priority in future studies will be to determine the sub-cellular localisation of UDP-Glc DH, PFP and UGPase in the different cell types of the sugarcane stem. It is proposed that electron microscope based ISH and immunohistochemistry be used for the simultaneous visualisation of the compartmentation of both gene expression and protein distribution within the same cell and tissue types. These methods would be able to detect differences in distribution patterns in the parenchyma cells that have been demonstrated in the present study to be the site of shared localisation for all three transcripts.

Although the expression of UDP-Glc DH, PFP and UGPase were highly cell type-specific, all three were localised in the parenchyma cells associated with the vascular bundles and stem storage compartment (chapter 4; Table 4.1). This finding implies that the sucrose storing parenchyma cells of the sugarcane stem either directly or indirectly influence fundamental processes such as phloem loading and unloading and assimilate transport.

Complementary northern and western blot analyses revealed that in the older, higher sucrose storing internodes, relative mRNA and protein abundance declined (chapter 4), whereas in younger tissues, these gene products were expressed at relatively high abundances. This is an important finding, as it demonstrates that there is a co-existence of divergent metabolic pathways in young sugarcane internodes and that the rate of metabolic turnover in older tissues decreases. Such findings have led to the development of the hypothesis that the increasing sucrose content in the parenchyma cells in older internodes is able to repress the expression of genes involved in sucrose mobilisation and synthesis and induce those required for its metabolism and storage (Whitakker and Botha, 1999; Pego *et al.*, 2000).

This study therefore revealed that cellular localisation studies together with nucleic acid blotting and probing methods are useful in obtaining a comprehensive view of plant gene expression. It further demonstrated an increase in the resolution with which tissue specific expression patterns of enzymes associated with sucrose metabolism can be determined on a cellular level using ISH, instead of classic gene expression methods. Furthermore, the identification of such patterns can be utilised for the purposes of genetic engineering and for the isolation of promoters to potentially enhance sucrose accumulation in the sugarcane stem.

Future directions in the use of the ISH technique developed in this study, could involve the study of specific genes involved in sucrose accumulation, its transport, biosynthesis and degradation. In addition, multiple ISH analyses and saturated hybridisation kinetics could be performed to evaluate the relative abundance of different mRNAs and the exact sub-cellular site(s) at which these transcripts differ. This would be used in conjunction with immunohistochemistry, the measurements of the activities of these enzymes and the determination of the distribution and concentration of metabolites *in situ*. The co-ordinated use of these techniques would allow for the elucidation of the regulation of the metabolic processes involved in sugarcane development and sucrose accumulation.

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