DEVELOPMENT OF *IN SITU* HYBRIDISATION TO EXAMINE TISSUE-SPECIFIC EXPRESSION PATTERNS OF THE INVERTASE GENES IN SUGARCANE CULM

by

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DECLARATION

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

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Gabrielle Turner Date

ABSTRACT

The goals of this project were firstly to develop the tissue preparation and *in situ* hybridisation protocols for sugarcane culm tissue, and secondly to use the developed techniques to examine the expression patterns of three invertase isoforms in sugarcane internodes of various developmental stages. Sugarcane invertases have been the focus of intense research for many years, yet almost nothing is known of their tissue-specific distribution. It was thought that by characterising their expression patterns using *in situ* hybridisation, more knowledge of their functions and involvement in sucrose accumulation would be gained.

Although *in situ* hybridisation is now regularly used to study gene expression in plants, there is to date only a single publication describing its use on immature sugarcane tissue. Therefore this technique needed further development, and this was achieved by comparing different tissue preparation methods, as well as by systematically testing the various parameters pertaining to each method. The *in situ* hybridization technique was also developed by testing and comparing a number of key parameters. It was found that fixing whole mount tissue for 48 h preserved sugarcane tissue adequately. High hybridization temperatures and probe concentrations provided the best signal, and including pre-treatment with HCl and Pronase was essential in sensitizing the tissue to the probe. A less viscous detection buffer reduced both osmotic effects and time required for signal detection.

In the second part of this study, the developed method was used to examine the expression patterns of the three invertase isoforms in young, maturing and mature internodes of sugarcane, and the results were complemented with Northern blot analysis. Transcript of all three isoforms was found to be present in the storage parenchyma and in the phloem tissue. Transcript levels of all three isoforms declined in maturing tissue, with soluble acid invertase declining sharply and dropping below detection in maturing and mature tissue. Transcript levels of cell wall invertase and neutral invertase declined only gradually, and appreciable levels of both were still present in mature tissue. Acid

invertase is suggested to be mainly involved in internode elongation, while cell wall invertase would appear to play important roles in phloem unloading and turgor control. Neutral invertase is suggested to be involved in either sucrose cycling or maintenance of hexose pools, however the function of this enzyme remains unclear.

This study has demonstrated the value of *in situ* hybridization, yet at the same time has shown its limitations, especially when more traditional biochemical techniques are not employed to complement the results. Although the precise functions of the invertase isoforms in sugarcane remain inconclusive, this study has opened up the way for tissue-specific promoter design and future *in situ* studies of sugarcane invertases.

OPSOMMING

Die doel van hierdie projek was tweeledig: eerstens om weefselvoorbereiding en *in situ*-hibridisasie-protokolle vir die stingelweefsel van suikerriet te ontwikkel; en tweedens om die ontwikkelde tegnieke te gebruik om die uitdrukkingspatrone van drie invertase-isovorme in die suikerriet-internodes van verskeie ontwikkelingstadia te ondersoek. Suikerriet-invertases is al vir jare lank die fokus van intense navorsing, maar baie min is bekend oor hulle weefselspesifieke verspreiding. Die idee was om meer kennis oor suikerriet-invertases se funksies en betrokkenheid by sukrose-akkumulasie te verkry deur *in situ*-hibridisasie te gebruik om hulle uitdrukkingspatrone te karakteriseer.

Alhoewel *in situ*-hibridisasie deesdae gereeld gebruik word om geenuitdrukking in plante te bestudeer, is daar tot op datum slegs een publikasie wat die gebruik daarvan in onvolwasse suikerrietweefsel beskryf. Hierdie tegniek moes dus verder ontwikkel word, en dit is gedoen deur verskillende weefselvoorbereidingsmetodes te vergelyk en sistematies die verskillende parameters wat op elke metode van toepassing is te toets. Die *in situ*-hibridisasie-tegniek is ook ontwikkel deur die toetsing en vergelyking van 'n aantal sleutelparameters. Daar is gevind dat suikerrietweefsel voldoende gepreserveer word deur die intakte gemonteerde weefsel vir 48 uur te fikseer. Hoë hibridisasie-temperature en hoë peilerkonsentrasies het die beste sein gegee; die insluiting van voor-behandeling met HCl en Pronase was noodsaaklik om die weefsel meer gevoelig vir die peiler te maak. Osmotiese invloede en die tyd nodig vir seindeteksie is verminder deur die viskositeit van die buffer te verminder.

In die tweede deel van die studie is die ontwikkelde metode gebruik om die uitdrukkingspatrone van die drie invertase-isovorme in jong, ontwikkelende en volwasse internodes te ondersoek en die resultate is deur 'n noordelike oordraganalise gekomplementeer. Transkripte van al drie isovorme is in die stoorparenchiem en floëemweefsel gevind. Transkripvlakke van al drie isovorme het afgeneem in ontwikkelende weefsel, met oplosbare suurinvertase wat skerp afgeneem en tot onder die

deteksie-limiet gedaal het in ontwikkelende en volwasse weefsel. Transkripvlakke van selwandinvertase en neutrale invertase het slegs geleidelik afgeneem en merkbare vlakke van albei was teenwoording in ontwikkelende en volwasse weefsel. Daar word voorgestel dat suurinvertase hoofsaaklik betrokke is by internodeverlenging, terwyl selwandinvertase skynbaar 'n belangrike rol in floëem-ontlading en turgor-beheer speel. Daar word voorgestel dat neutrale invertase betrokke is óf by die sukrose-sirkulering óf by die onderhoud van heksose-poele; die funksie van hierdie ensiem is egter steeds nie duidelik nie.

Hierdie studie het die waarde van *in situ*-hibridisasie gedemonstreer maar terselfdetyd ook die beperkinge daarvan uitgewys, veral as meer tradisionele biochemiese tegnieke nie gebruik word om die resultate aan te vul nie. Alhoewel daar onsekerheid is oor die presiese funksies van die invertase-isovorme in suikerriet, het die studie die weg gebaan vir weefselspesifieke promotorontwerp en toekomstige *in situ*-studies van suikerrietinvertases.

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LIST OF ABBREVIATIONS AND ACRONYMS

°C	degrees centigrade
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate toluidine salt
bp	nucleic acid base pair
cDNA	complementary deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
g	gram
h	hour
L	litre
m	metre
M	Molar
NAD	nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium chloride
RNA	ribonucleic acid
RnaseA	ribonuclease A
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
Susy	sucrose synthase
UDP	uridine 5'-diphosphate
UV	ultraviolet

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CHAPTER 1

General introduction

Approximately one million people are estimated to be dependent on the sugar industry in South Africa, with direct and indirect employment estimated at 240 000 people (South African Sugar Association (SASA) Directory, 2000). This industry represents one of the world's leading competitors in high quality sugar production, and was estimated to contribute R1.7 billion to the country's foreign exchange earnings in 2000 (SASA Directory, 2000). Thus apart from producing an important foodstuff, the sugar industry generates a wealth of employment and foreign income. Maximising the yield from such an industry is obviously desirable, and this ultimately requires an understanding of the biology of the plant concerned.

Sugarcane produces large amounts of biomass, and stores high concentrations of sucrose (up to 60% of total dry mass) in the stem (Lingle, 1999). Improving the sucrose yield by producing a sugarcane variety with an increased stalk weight would inevitably result in higher harvesting, transport and milling costs (Lingle, 1999). Improving sucrose yield by increasing the sucrose concentration or the juice purity (ratio of sucrose to total sugar) represents a far more viable and cost-effective option. Yet the improvement of the commercial sugarcane plant by traditional breeding methods appears to have reached a threshold, and now technologies utilizing genetic manipulation offer a greater chance of improving sucrose content of the crop. In order to be effective, such manipulation must target the metabolic pathways ultimately responsible for sucrose accumulation. Sucrose accumulation has been studied more in sugarcane than any other plant (Hawker, 1985), since very high concentrations of sucrose are attained, yet despite such numerous studies the biochemical basis of sucrose accumulation in sugarcane is still poorly understood (Moore, 1995). Invertase is a major sucrolytic enzyme involved in the regulation of carbohydrate partitioning and mobilisation in many plant organs (Avigad, 1982), and occurs in multiple isoforms. Although the various isoforms present in sugarcane have been characterised, very little is known of their specific distribution within the stem or their particular functions in the metabolism of the plant. In addition, what little is known

of their distribution has been inconsistently reported in different studies. Much of the past research has involved extraction studies, with virtually no consideration of the enzymes *in situ*. Knowledge of the tissue-specific distribution of these isoforms would greatly aid the elucidation of their functions, and this in turn would enable the manipulation of these enzymes in order to increase sucrose accumulation in the sugarcane stem.

In order to resolve the variance seen in the distribution of the invertase enzymes, both within and between internodes of different developmental stages, the tissue-specific expression patterns of the three isoforms were studied using *in situ* hybridization. This technique first required development (*chapter 3*), since its use on sugarcane tissue is to date still experimental. Once optimized, the *in situ* method was used together with RNA blot analysis to form a clearer picture of the expression patterns of the invertase genes in sugarcane culm (*chapter 4*).

CHAPTER 2

Invertase expression and sucrose

Although their precise role in the control of carbohydrate metabolism is not always clear, the invertase isoforms are nonetheless inextricably involved in sucrose metabolism. Hence it is necessary to first understand sucrose biochemistry, accumulation and storage, so that this knowledge can be integrated with that of invertase expression patterns to develop a hypothesis for invertase function.

2.1 Sucrose biochemistry

Sucrose is not only a leading commercial commodity important in human nutrition, but also plays a central and vital role in plant life (Avigad, 1982). According to Kruger (1997), sucrose has three fundamental roles in plants: firstly, it is the major product of photosynthesis, and accounts for the majority of the CO_2 absorbed by a plant during photosynthesis. Secondly, sucrose is the main form in which carbon is translocated in plants, including translocation from both photosynthetic and non-photosynthetic tissues. Thirdly, sucrose is one of the main storage sugars in plants, occurring not only in specialised storage organs such as tap roots, but also in tissues such as stems and leaves.

2.1.1 Sucrose synthesis and breakdown

Sucrose is metabolized in a number of ways, as illustrated by Figure 2.1. The ability to synthesize sucrose is a widespread characteristic of higher plant cells. Sucrose is derived from the pool of hexose phosphates present in plant cells, and synthesis occurs exclusively in the cytosolic compartment of the cell (Kruger, 1997). Early studies indicated that two reactions can lead to the synthesis of sucrose (Leloir and Cardini, 1953, 1955). The first of these reactions involves production of sucrose by a transglucosylation reaction from UDP-glucose to fructose as the acceptor, catalysed by sucrose synthase (E.C. 2.4.1.13). The second reaction involves production of sucrose 6-phosphate by a transglucosylation from UDP-glucose to fructose-6-phosphate as the acceptor, and is catalysed by sucrose phosphate synthase (E.C. 2.4.1.14). Sucrose

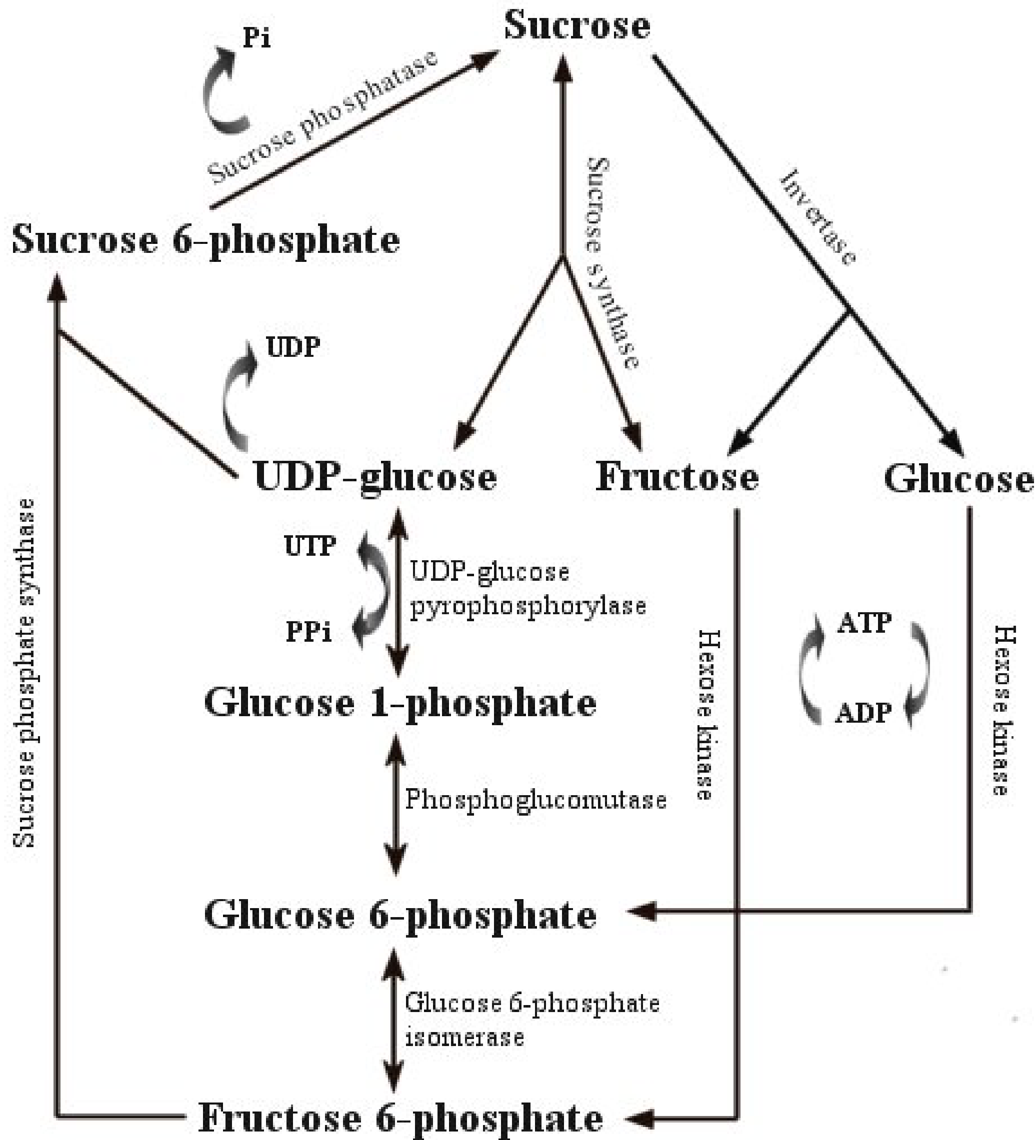


Figure 2.1 Pathways of sucrose synthesis and breakdown

phosphatase (E.C. 3.1.3.24) then hydrolyses the sucrose 6-phosphate, releasing free sucrose. Although both reactions can result in the synthesis of sucrose, more recently it has been established that the latter of the two reactions is mostly responsible for producing sucrose in plants (Kruger, 1997). Both sucrose phosphate synthase and sucrose synthase contribute to sucrose synthesis in immature internodes of sugarcane, however, in mature internodes sucrose phosphate synthase activity has been shown to exceed that of sucrose synthase by more than three-fold (Botha and Black, 2000).

Sucrose can be degraded in the apoplastic space, the cytosol or the vacuole. There are two types of enzymes capable of breaking down sucrose in plants, namely sucrose synthase and invertase (E.C. 3.2.1.26). The first of these catalyses a readily reversible reaction in the cytosol, involving the hydrolysis of sucrose to UDP-glucose and fructose (Avigad, 1982). Although the kinetics of sucrose synthase favours its synthetic activity, in most tissues it has been shown to act in the direction of sucrose cleavage (Kruger, 1997). This could be due to high sucrose concentrations tipping the equilibrium towards cleavage. The second enzyme reaction catalyses the irreversible hydrolysis of sucrose into glucose and fructose, and due to the different invertase isoforms, this reaction can take place in the vacuole, the cytosol or the apoplastic space (Avigad, 1982).

2.1.2 Sucrose accumulation and storage in sugarcane

The maturation of sugarcane is characterised by the accumulation of sucrose in developing internodes (Moore, 1995), and coincides with a redirection of carbon from water-insoluble components and respiration to produce this sucrose (Botha and Whittaker, 1995). Early research on sucrose accumulation in sugarcane began with experiments on tissue slices suspended in radiolabelled sugar solutions, and resulted in the following model (Glasziou and Gayler, 1972): sucrose from the phloem diffuses into the apoplastic space, where it is hydrolysed into glucose and fructose by the action of an extracellular invertase. Glucose and fructose are then taken up into the storage cells by membrane localised carriers or hexose transporters, where they are phosphorylated and synthesised into sucrose-phosphate. The sucrose moiety of sucrose-phosphate is then transported across the tonoplast and stored in the vacuole (Gayler and Glasziou, 1972).

Although this model was widely accepted, an increased understanding of phloem unloading has shown that the majority of sink tissues employ a symplastic unloading mechanism (Patrick, 1997). In addition, Lingle (1989) repeated the uptake studies over shorter time periods to minimise contamination, and found that less than 15 % of sucrose is hydrolysed before uptake into sugarcane internodes. According to Moore (1995), there are at least three possible pathways of post-phloem transport of sucrose: firstly, transport

may occur through the plasmodesmata between the sieve elements and the surrounding cell layer, and then through free space to the storage parenchyma (Hawker, 1985). A second possibility is that sucrose is unloaded from the sieve elements into the storage parenchyma cells via the plasmodesmata, and then sugars are leaked into the apoplastic space (Oparka and Prior, 1988). The third possibility involves simultaneous symplastic and apoplastic transport through the storage parenchyma, so that the sugars in the apoplast are a mixture of those from leakage and phloem unloading (Moore, 1995).

The theories involving either symplastic transport or both symplastic and apoplastic transport would seem more plausible when considering the anatomy of the sugarcane stem: Walsh *et al*. (1996) found that vascular bundles in the central region of the stem were mostly responsible for sucrose transport, and that these bundles were surrounded by a fibre sheath with lignified and/or suberised cell walls. Such cell wall reinforcements would isolate the phloem apoplast from that of both the xylem and storage parenchyma, theoretically making it impossible for sucrose to follow a strictly apoplastic path between the phloem complex and the parenchyma (Walsh *et al*., 1996). Sucrose could rather follow a symplastic route through the plasmodesmata that have been observed to connect the vascular bundles with the storage parenchyma (Welbaum *et al*., 1992). In this case, the presence of sucrose and an invertase in the apoplastic space could be due to the sink cell regulating its turgor, and hence the pressure gradient driving phloem import, by unloading sucrose into the apoplast (Patrick, 1990). Alternatively, the mechanism could switch with development: younger internodes could employ an apoplastic unloading mechanism, while an increase in lignification would require a predominantly symplastic mechanism in older internodes.

In addition to uncertainty about the transport of sucrose from the phloem to storage tissue, enzymatic activities in sugarcane add more confusion to the understanding of sucrose accumulation. Contrary to earlier findings (Hatch and Glasziou, 1963), all the invertase isoforms have been shown to be present in significant amounts throughout the sugarcane stem, including tissue containing high amounts of sucrose (Vorster and Botha, 1999). It is unclear how sucrose accumulation is maintained in an environment where

sucrolytic activity is so high, although this may support the idea that high rates of cycling occur between sucrose and hexose-phosphate pools (Botha *et al*., 1996). Such a system of constant recycling and turnover would allow rapid switching between net storage or mobilisation of sucrose, with only small changes in enzymes and metabolites (Wendler *et al*., 1990). Alternatively, endogenous invertase activity may be inhibited by some or other factor, although this requires further investigation (Vorster and Botha, 1999).

Sucrose accumulation in sugarcane is a complex process, and is probably the result of a number of different processes and enzymes. Whether or not the invertase isoforms play an important role in this accumulation is unsure, however a better knowledge of their specific expression patterns within sugarcane tissue will hopefully identify their involvement. Previous research has failed to define such expression patterns due to the use of whole tissue assays, which do not differentiate between tissue types e.g. vascular and ground tissue.

2.2 Characteristics of invertase

Invertases are probably very important sucrolytic enzymes involved in the regulation of carbohydrate partitioning and mobilisation in many plant organs (Avigad, 1982). Because sugars in plants are not only nutrients but also important regulators of gene expression (Koch, 1996), invertases may also be indirectly involved in the control of cell differentiation and plant development (Sturm, 1999). All plant invertases are ß-D-fructofuranosidases (Avigad, 1982). Invertases are present in most plant organs in multiple isoforms, and may be classified according to their pH optima and solubility properties. Three types of invertase have been purified from a number of plant species and characterised at the biochemical level. They are either classified as soluble acid invertase, insoluble acid invertase or soluble neutral invertase. Each type of invertase is probably specified by several genes (Tymowska-Lalanne and Kreis, 1998). For example, three different genes code for insoluble acid invertases in carrot (Unger *et al*., 1994). The physiological advantage of having multiple isoenzymes of invertases might be a greater flexibility in the control of sucrose metabolism, translocation or storage under different

internal and external conditions and at various developmental stages of a tissue, organ or plant (Tymowska-Lalanne and Kreis, 1998).

Plant invertase gene expression and enzyme activity are both known to be influenced by a variety of intracellular and extracellular factors, with the level of invertase activity being regulated by both the end products and the substrate itself (Burch *et al*., 1992; Roitsch *et al*., 1995). There is evidence that the synthesis and/or activity of invertase might be regulated by hormones produced or accumulated in sink tissues (Weil and Rausch, 1990; Silva and Ricardo, 1992). Plant invertases have also been shown to be regulated by wounding or pathogen infection (Sturm and Chrispeels, 1990; Benhamou *et al*., 1991), temperature (Zhou *et al*., 1994), light (Krishnan *et al*., 1985) and gravity (Wu *et al*., 1993a,b). These factors modulate invertase activity either by activation or by repression, acting at the level of gene expression and/or at the level of protein activity (Tymowska-Lalanne and Kreis, 1998).

The physiological roles of the invertase isoforms appear to be diverse, and studies suggest that their functions vary depending on the organ, tissue or cells in which they are expressed (Kim *et al*., 2000). Thus localisation of these enzymes to specific tissues and organs is essential for identifying the role played by each isoform, and furthermore for understanding the metabolism and overall physiology of specific plant species. Although the exact role of the invertases may vary between plant species, there are nonetheless functional similarities. This makes it worthwhile considering the functions of other plant invertases, since they may provide valuable clues to those of the sugarcane invertases.

2.2.1 Soluble acid invertases

2.2.1.1 Biochemistry and regulation

Soluble acid invertases or vacuolar invertases (VI) have acid pH optima ranging from pH 3.5 to 5.5 in different species (Fahrendorf and Beck, 1990; Walker and Pollock, 1993). Vacuolar invertase enzymes are glycoproteins localised in the vacuole (Isla *et al*., 1998; Sturm and Chrispeels, 1990), and in most cases possess monomeric catalytic units of approximately 40 to 80 kDa (Fahrendorf and Beck, 1990; Miller and Ranwala, 1994;

Walker and Pollock, 1993). These enzymes have a K_m for sucrose in the low millimolar range, with K_m values of 2 to 13mM reported in the literature (Avigad, 1982). Sugarcane acid invertase has been reported to have a K_m of 2.8 mM, while the V_{max} of the purified protein has been reported to be 2.7 µmol sucrose hydrolysed $h^{-1}mg^{-1}$protein (Rosario and Santisopasri, 1977).

Vacuolar invertase activity is competitively inhibited by fructose and non-competitively by glucose in a number of species, including *Saccharum officinarum* and *Solanum tuberosum* (Sampietro *et al*., 1980; Burch *et al*., 1992). However in *Avena sativa*, these sugars actually stimulate vacuolar invertase activity (Kaufman *et al*., 1974). Both glucose and sucrose inhibit vacuolar *Ivr1* activity in *Zea mays*, however in the case of vacuolar *Ivr2* in *Zea mays* the situation is reversed, with enzyme activity being activated by these sugars (Xu *et al*., 1996). Such a different effect on two similar genes seems to emphasize the specificity of sugar signalling in gene expression. Acid invertase genes have also been found to be regulated by wounding and stress. Activity of vacuolar acid invertase was shown to markedly increase in aging slices of sweet potato tuber (Matsushita and Uritani, 1974), while subjecting maize plants to a water stress was found to cause a dramatic increase in acid invertase activity in the leaves of the plants (Pelleschi *et al*., 1999).

2.2.1.2 Distribution patterns and functions of soluble acid invertases

Vacuolar invertase activity is generally high in immature, actively growing tissues, and declines in activity with increasing maturity of organs, which store sucrose (Hatch *et al*., 1963; Moore, 1995). Organ or tissue specificity is not necessarily a permanent feature, since the dominant sucrolytic activity in an organ or tissue often depends on the developmental stage of the plant. For example, in snap bean pods, vacuolar invertase is the dominant sucrolytic enzyme during early pod elongation phases, whereas sucrose synthase becomes dominant during seed dry matter accumulation (Sung *et al*., 1994).

Vacuolar invertase is specifically expressed in certain tissues in grape berries, and this distribution appears to be developmentally regulated. Immunohistochemistry showed VI to be present in both the pericarp and seeds of grape berries 10 d after anthesis (Famiani

et al., 2000). Vacuolar invertase was non-uniformly localised in clumps of parenchyma cells, and was also present in the vasculature of the berry, the cells lining the locular sac, and within the seed it was it was associated with the developing seed coat (Famiani *et al*., 2000). The presence of the enzyme in the vasculature suggests some function in the unloading of assimilate into the developing berry. A similar function could be suggested for the vacuolar invertase localised in the cells lining the locular sac and that associated with the developing seed coat, since these tissue layers would appear to be the point of contact between the young seed and the rest of the berry. The role of vacuolar invertase in the loading or unloading of sucrose has been suggested for other tissue types, such as in cucumber petioles where it is localised in the extrafasicular phloem (Kingston-Smith *et al*., 1999). Tissue prints of pea and barley leaves have also revealed the acid invertase protein to be predominantly located in the vascular regions (Kingston-Smith and Pollock, 1996).

At a much later stage of development in grape berries (50 d after anthesis), vacuolar invertase was shown to be present throughout the parenchyma tissue of the pericarp, but was most concentrated in the layer of cells directly underlying the epidermis (Famiani *et al*., 2000). In grapes the major forms of stored carbohydrates are glucose and fructose, which are mainly derived from imported sucrose (Kanellis and Roubelakis-Angelikis, 1993). After the start of ripening, at approximately 50 d after anthesis, these hexoses begin to accumulate (Famiani *et al*., 2000). Hence the function of vacuolar invertase throughout the parenchyma of the grape berry, at this stage of development, would appear to be to continually hydrolyze imported sucrose and maintain high levels of stored glucose and fructose. The function of invertase in the cells underlying the epidermis could be to fuel the synthesis of secondary metabolites, and similarly the PEPCK found to be abundant in these cells was suggested to generate the PEP used in the synthesis of such metabolites (Famiani *et al*., 2000). This suggestion is supported by the observation that these enzymes are also abundant in the glandular cells of certain trichomes in tobacco and cucumber, which are known to produce a variety of antimicrobial secondary metabolites (Leegood *et al*., 1999; Kingston-Smith *et al*., 1999). Hence vacuolar invertase would appear to play more than one role in the development of grape berries,

due not only to its consistently high activity throughout development, but also due to its heterogeneous distribution throughout the berry tissues (Famiani *et al*., 2000).

The role of acid invertase in response to wounding has recently been demonstrated in sugar beet taproot tissue. Wounding induced both a cell wall invertase isoform and a vacuolar isoform, however, while cell wall invertase mRNA was strongly induced within 10h of wounding, vacuolar invertase mRNA levels increased only 24h after wounding (Rosenkranz *et al*., 2001). A further 7-fold increase was observed over the following 4d, and this coincided with a strong and persistent increase in hexose concentrations. So while cell wall invertase may be important in the initial wound response, vacuolar invertase induction is required for mobilization of the large vacuolar sucrose pool, which would presumably provide substrate for the wound-activated cellular metabolism (Rosenkranz *et al*., 2001).

Soluble acid invertase would also appear to play some role in pollen development. In wheat anthers, vacuolar invertase activity was shown to increase steadily throughout development, peaking sharply at anthesis and showing up to 12-fold more activity than sucrose synthase at comparable stages (Dorion *et al*., 1996). Furthermore, the most dramatic effect of an imposed water stress, which has been shown to cause pollen sterility, was on acid invertase: activity was suppressed 4-fold during the stress, and failed to return to normal levels following the stress (Dorion *et al*., 1996). High invertase activities have also been reported in pollen of mature maize and *Camellia*, where once again activity was much higher than that of sucrose synthase (Bryce and Nelson, 1979; Nakamura *et al*., 1980). Such high activity relative to that of sucrose synthase seems to imply that sucrose imported into anthers is primarily cleaved by acid invertase, before being further processed for starch synthesis (Dorion *et al*., 1996).

To date there is no literature available regarding the tissue-specific distribution of acid invertase in sugarcane, although distribution patterns on a broader scale have been described. As mentioned previously, vacuolar invertase is present in significant amounts throughout the sugarcane stem (Vorster and Botha, 1999), but generally decreases in

activity with increasing maturity. It has been shown to reach a peak during elongation of an internode (Lingle and Smith, 1991), and the elongation rate has been correlated with acid invertase activity (Lingle, 1999). Following elongation, acid invertase drops to a low level, and hence it would seem that vacuolar invertase plays a major role in internode elongation. This role in sugarcane could involve the provision of energy for metabolism and growth, or perhaps the supply of hexoses for channeling into cell wall growth.

Despite the suggestion by recent work that acid invertase plays an insignificant role in sucrose accumulation (Botha and Birch, 2001), there is evidence that it actually does play some form of indirect role during sucrose accumulation in sugarcane. Zhu *et al*. (1997) found that a significant non-linear negative relationship existed between soluble acid invertase activity and sucrose accumulation in individual internodes. The hyperbolic function they found to exist between the two means that high acid invertase activity always results in low sucrose content, but that low acid invertase activity could result in either high or low sucrose content (Zhu *et al*., 1997). They also showed that while no relationship existed between SPS activity and sucrose concentration, the difference between SPS activity and soluble acid invertase activity was strongly and positively correlated with sucrose concentration, both in individual internodes and on a whole-stalk basis (Zhu *et al*., 1997). So it would appear that acid invertase may limit sucrose accumulation but not necessarily control it, and when combined with the activity of SPS it would seem to be a fairly important determinant of sucrose accumulation. Hence determining the tissue-specific expression patterns of this invertase isoform in sugarcane is necessary so that future work can effectively target this tissue for down-regulation or modification of the activity of this enzyme.

2.2.2 Insoluble acid invertases

2.2.2.1 Biochemistry and regulation

Insoluble acid invertases have many similar properties to the VI isoform: they share the same pH range, are similar in size, and are active as monomeric glycoproteins (Fahrendorf and Beck, 1990; Weil and Rausch, 1990; Lauriere *et al*., 1988). However these isoforms are insoluble in aqueous solution, instead being associated with the plant

cell wall. They occur extracellularly (Lauriere *et al*., 1988), and are thus referred to as apoplastic or cell wall bound invertases (CWI). Sugarcane CWI has a K_m of 8 x 10^{-3} M for sucrose (Moore and Maretzki, 1996). An apoplastic invertase from *Chenopodium rubrum* has been shown to be activated by glucose (Roitsch *et al*., 1995), while cell wall invertase levels in *Avena sativa* stem tissues have also been shown to be stimulated by glucose, fructose and sucrose (Kaufman *et al*., 1973).

2.2.2.2 Distribution patterns and functions of insoluble acid invertases

Cell wall invertase activity is high in rapidly growing tissues such as meristems and elongating internodes, and activity declines as growth slows (Roitsch and Tanner, 1996; Sturm and Chrispeels, 1990). Cell wall invertase has been suggested to be involved in various processes including early development, environmental sensing, osmoregulation, phloem unloading, and reloading of sucrose leaked into the apoplast (Tymowska-Lalanne and Kreis, 1998).

There are two well-characterised cell wall acid invertase genes in maize, namely *Incw1* and *Incw2* (Taliercio *et al*., 1999). Expression of these genes has been shown to be organ-specific: RNA-blot analysis of *Incw1* shows the highest levels of expression in cell-suspension cultures, etiolated shoots, roots, and low levels in developing kernels (Carlson and Chourey, 1999). *Incw2* mRNA is predominant in developing kernels but is also present in etiolated shoots (Taliercio *et al*., 1999). In maize kernels, cell wall invertase is the predominant form, contributing almost 90 % of the total invertase activity (Cheng *et al*., 1996). Immunohistological analysis has revealed cell wall invertase to be restricted to the endosperm transfer cells of the kernel, represented by one or two cell layers in the basal portion of the endosperm, as well as along the upper parts of the vascular bundles of the pedicel (Cheng *et al*., 1996; Cheng and Chourey, 1999). A number of roles have been proposed for *Incw2* in this location, mostly based on analyses on the *mn1* (*miniature 1*) seed mutation. Several lines of evidence have demonstrated that the *Mn1* locus encodes the endosperm-specific cell wall invertase *Incw2* (Cheng *et al*., 1996), hence any characteristics of the *mn1* mutation can in some way be attributed to an invertase deficiency and thus used to propose functions for this isoform. One suggestion is that

Incw2 plays a critical role in cell division in the endosperm (Cheng and Chourey, 1999). This is not only drawn from the fact that the *mn1* mutant is characterised by a severely reduced endosperm, but also that the highest level of invertase activity in normal maize kernels coincides with the cell division phase in the endosperm (Cheng *et al*., 1996). This is further supported by studies on cotyledonary cells of *Vicia faba*, where greater cell wall invertase activity and high hexose levels were correlated with extended mitotic activity (Weber *et al*., 1996). Cell wall invertase in the maize basal endosperm cells may also exert a regulatory force in the pull of the photosynthate column from the pedicel and into the endosperm (Miller and Chourey, 1992). Another possible function of invertase here may be to release hexoses essential for normal regulation of downstream genes engaged in carbon assimilation, based on the observation that *mn1* seed mutants failed to revert to the normal phenotype during culture on hexose media (Cheng and Chourey, 1992).

The role of cell wall invertases in the proliferation of endosperm cells has been observed in other cereal species. During grain filling in rice (*Oryza sativa* L.), transcripts for a cloned cell wall invertase were detected only in the very early stage of caryopsis development, viz. 1-4 d after flowering (Hirose *et al*., 2002). Expression of this clone, designated *OsCIN1*, correlated with the highest level of cell wall invertase activity, as well as with a rapid increase in caryopsis length. At this early stage of development there was little dry weight increase, however the caryopsis length was rapidly increasing, which is known to reflect the rapid increase in the number of endosperm cells in rice (Hoshikawa, 1993). This suggests that *OsCIN1* is involved in the proliferation of endosperm cells rather than starch accumulation (Hirose *et al*., 2002). *In situ* localization on caryopses 1 DAF found *OsCIN1* mRNA to be concentrated in the vascular parenchyma of the dorsal vein, the importing vein in the caryopsis, and the integument and its surrounding cells (Hirose *et al*., 2002). Its function here could be similar to that of *Incw2* in the maize basal endosperm cells, viz. to exert a regulatory force in the pull of assimilate from the pedicel and into the rapidly dividing endosperm.

In seeds of the fava bean (*Vicia faba*), the cell wall invertase isoform *VfCWINV1* was found to be exclusively expressed in the chalazal vein and the inner rows of cells of the thin-walled parenchyma of the seed coat (Weber *et al*., 1995). These cells represent the end of the sieve element system, and the inner rows of cells of the thin-walled parenchyma are proposed to be the site of photosynthate exchange to the apoplastic space (Offler *et al*., 1989). The specific expression of *VfCWINV1* in this unloading area could help establish sink strength by increasing the concentration gradient of sucrose between cells of the thin-walled parenchyma and the apoplastic space, and by lowering the water potential in the apoplast due to accumulation of hexoses (Weber *et al*., 1995). Sucrose hydrolysis by extracellular invertases also appears to be part of the import mechanism in sorghum (Wolswinkel, 1992).

Analysis of mRNA levels of three extracellular invertases of tomato revealed another sink tissue-specific expression pattern. While mRNA for *Lin5* cell wall invertase was found to be most abundant in fruits, *Lin6* mRNA was localized to seedling roots, small flower buds and tumors induced by *Agrobacterium tumefaciens* (Godt and Roitsch, 1997). However, the mRNA for *Lin7* showed the most specific expression pattern, since it was only detected in the anthers of large flower buds and flowers. This localization correlated with a high extracellular invertase activity in this flower organ (Godt and Roitsch, 1997). *In situ* hybridization studies found *Lin7* mRNA to be specifically expressed in the tapetum and in the pollen grains, a location which suggests some function in assimilate unloading or pollen development (Godt and Roitsch, 1997). Alternatively, the purpose of cell wall invertase here could be similar to that of *Incw2* in the maize endosperm, viz. an involvement in cell division. The purpose of the larger flowers expressing a different isoform to the smaller flowers is unclear, although it is possible that the cell wall invertase encoded by *Lin7* unloads sucrose more efficiently and can hence sustain the metabolic requirements of the larger flowers.

In situ hybridisation studies on stem and leaf tissues of wounded pea plants revealed an accumulation of invertase transcript around the phloem tissue (Zhang *et al*., 1996). Some cell wall invertase mRNA was also detected in epidermal cells and the xylem, although

the authors suggest the latter represents a non-specific binding of the probe in some of the vessel walls. Zhang and co-workers (1996) suggest invertase in the phloem tissue either maintains a steep sucrose concentration gradient between the source and sink regions of the plant, or alternatively provides hexose and hence energy for metabolism of the companion cells.

The cell wall isoform of acid invertase is very poorly characterised in sugarcane, as it has received relatively little attention when compared with the soluble isoform (Albertson *et al*., 2001). In addition, there is considerable difficulty in distinguishing the two isoforms due to their very similar properties (Tymowska-Lalanne and Kreis, 1998). Hence there is to date no literature available on the tissue-specific localisation of cell wall invertase in sugarcane stem tissue. Recent work has, however, found that this isoform is highly active in young leaves and decreases considerably with increasing leaf maturity (Albertson *et al*., 2001). The same article describes an improved method for measuring cell wall invertase activity, and this should hopefully result in more conclusive work being performed on activity profiles in the sugarcane stem. Furthermore it is clear from the limited information available that determining the tissue-specific expression patterns of sugarcane cell wall invertase, both within and between internodes, would contribute a great deal to our understanding of this isoform.

2.2.3 Soluble neutral invertases

2.2.3.1 Biochemistry and regulation

Alkaline or neutral invertases (NI) have pH optima ranging between pH 6.8 and 8, and are mostly active as multimers (Lee and Sturm, 1996; Van den Ende and Van Laere, 1995). Since these enzymes are extremely labile and activity is rapidly lost after tissue homogenization, their purification is difficult (Sturm, 1999), hence neutral invertase research has lagged behind that of the acid invertases. Enzymes of the NI group are non-glycosylated and are thus probably localised in the cytoplasm (Lee and Sturm, 1996). The major inhibitors of neutral invertase activity would appear to be Tris and fructose (Lee and Sturm, 1996; Ross *et al*., 1996; Morell and Copeland, 1984). In addition, carrot neutral invertase is inhibited by $CuSO_4$ (Lee and Sturm, 1996), and several neutral

invertase enzymes purified to date show inhibition by Hg^{2+} ions (Van den Ende and Van Laere, 1995; Vorster and Botha, 1998). Sugarcane neutral invertase displays typical hyperbolic saturation kinetics with sucrose as substrate, with a K_m and V_{max} of 9.8 mM and 7.32 nkat.mg^{-1} respectively (Vorster and Botha, 1998).

2.2.3.2 Distribution patterns and functions of soluble neutral invertases

Neutral invertases are not as universal in occurrence as the acid invertases, and tomato plants for example lack any detectable activity (Quick and Schaffer, 1996). A study on the sucrose-cleaving enzymes in *Lilium longiflorum* floral organs also found no detectable activity in flower buds (Ranwala and Miller, 1998). Neutral invertase activity tends to be highest in mature storage tissues such as taproots (Lee and Sturm, 1996), and also in other sinks such as broad bean cotyledons (Ross *et al*., 1996). The functions of the neutral invertase isoforms have only recently come under scrutiny, however, several roles have been suggested including provision of energy for cellular maintenance, regulation of sucrose storage, and regulation of hexose levels (Quick and Schaffer, 1996). It has also been suggested that neutral invertase compensates for low acid invertase activity in certain tissues (Ricardo and ap Rees, 1970). It is also conceivable that the two enzymes play different roles in development, and hence display different activity profiles at different stages. In *Cucumis melo* ovaries 3 days prior to anthesis, there was little acid invertase activity when compared to alkaline invertase (Gao *et al*., 1999). At anthesis acid invertase began to increase, until it was significantly more active than alkaline invertase at 10 DAA. Thus it would appear that alkaline invertase plays an important role in the early stages of ovary development, while acid invertase plays a dominant role during the period of fruit set and growth (Gao *et al*., 1999).

Sugarcane neutral invertase has, in the past, been reported as having low activity in meristematic tissues and increasing activity in tissues that store sucrose (Hatch and Glasziou, 1963). This prompted the theory that this enzyme controls sucrose movement from vascular to storage tissue in mature internodes (Hatch *et al*., 1963). More recent work has in fact shown the opposite to be true: the highest neutral invertase activities are found in the youngest internodes (Zhu *et al*., 1997; Vorster and Botha, 1999; Rose and

Botha, 2000). Such differences are probably due to sampling techniques: past research has relied on data collected from entire internodal tissue. However the sugarcane internode comprises differentiated cell types, and there are striking differences, not only between the top and bottom of an internode, but also between the core and periphery (Rose and Botha, 2000). Biomass production occurs at the bottom of the internode, while vascular bundles are more densely packed around the periphery than in the core of the internode (Jacobsen *et al*., 1992). Hence it is conceivable that the metabolism in these different parts will be adapted to specific functions (Rose and Botha, 2000), and also that the distribution and activity of neutral invertase will vary between the different regions.

To our knowledge there is no existing literature describing the tissue-specific distribution, at the *in situ* level, of any neutral invertase isoforms. However Rose and Botha (2000) conducted a study in which sugars and neutral invertase were extracted from anatomically different regions of internodes at different developmental stages. They found striking differences between and within internodes: at the bottom of the internode, activity of NI decreased by 43 % between internodes 3 and 9, largely due to the sharp drop occurring between internodes 6 and 9. In the peripheral region, approximately a 30 % drop occurred, largely due to the sharp drop between internodes 3 and 6. Within the internodes, neutral invertase activity was higher in the periphery bottom than the core bottom for all three internodes. This is interesting since biomass production occurs at the bottom of an internode, and the density of vascular bundles increases from the core to the periphery of an internode (Jacobsen *et al*., 1992). This could suggest that neutral invertase is somehow involved in production of new vascular tissue, whether it be by the release of energy for respiration or by providing carbon for the manufacture of cell walls. At the top of the internode, neutral invertase activity decreases from the core to the periphery, and so here it could be more involved in maintaining hexose pools in the storage parenchyma. However this is entirely speculative, and requires further investigation by means of precisely localising neutral invertase to specific tissues within each internode.

CHAPTER 3

Development of tissue preparation and *in situ* hybridization protocols for sugarcane tissue

Abstract

Although *in situ* hybridization is now regularly used to study gene expression in plants, there is to date only a single publication describing its use on sugarcane tissue. Therefore this technique needs to be optimized to suit the characteristics of sugarcane tissue. This study aimed to develop both the tissue preparation and *in situ* hybridization techniques to the point where they were quick and easily reproducible for sugarcane. This was achieved by comparing different tissue preparation methods viz. wax-embedding, cryofixation and whole mounts, and systematically testing parameters such as fixative type, fixation period and duration of embedding. The *in situ* hybridization technique was developed by testing and comparing parameters such as temperature of hybridization, necessity of pre-treatment, probe concentration, and type of detection buffer. It was established that sugarcane is most amenable to whole mount tissue preparation, and that 48 h fixation preserves tissue adequately. High hybridization temperatures and probe concentrations provided the best signal, and including pre-treatment with HCl and Pronase was essential in sensitizing the tissue to the probe. A less viscous detection buffer reduced both osmotic effects and time required for signal detection. These results are significant as they have paved the way for future *in situ* hybridization studies on sugarcane tissue, and will contribute to a wider knowledge of gene expression patterns in this species.

3.1 Introduction

In situ hybridization allows direct visualization of the spatial location of specific sequences that is crucial for elucidation of the organization and function of genes (Wilkinson, 1998). The basic principle of this technique is that a labelled, single-stranded nucleic acid probe will bind to a complementary strand of cellular DNA or RNA and, under the appropriate conditions, will form a stable hybrid in the tissue (Maliga *et al*., 1995). This hybrid can then be detected and viewed under a microscope. The advantage of *in situ* hybridization over more conventional methods for the detection of nucleic acids (i.e. northern and Southern blots) is that it allows determination of the precise spatial distribution of a nucleic acid sequence in a heterogeneous cell population (Maliga *et al*., 1995). While it is used for a variety of applications, this technique is most often used to localize specific RNA sequences in cells (Angerer *et al*., 1984; Dixon *et al*., 1991). Ideally, an *in situ* hybridization procedure should fulfil a number of criteria such as good tissue preservation, high resolution and high sensitivity, and should be quick, relatively inexpensive and non-hazardous (Engler *et al*., 1998). Since no existing protocol meets all these requirements, the researcher must choose a protocol and adapt it to his or her needs. The basic steps that need to be considered are tissue preparation, probe synthesis, pre-hybridization treatment, hybridization, and post-hybridization washing and detection.

3.1.1 Tissue preparation

Tissue preparation prior to the actual hybridization is a particularly important aspect of methodology development, since tissue with poorly preserved target sequences and anatomy will hinder accurate interpretation of expression patterns. Typical tissue preparation methods include fixation, followed by dehydration and embedding in a medium such as wax or resin. Most fixatives are based on protein cross-linking agents, such as paraformaldehyde or glutaraldehyde, since these give a greater accessibility and retention of cellular RNA than precipitating fixatives (Wilkinson, 1998). Paraformaldehyde is used most often, since it penetrates faster than glutaraldehyde (Hayat, 1989). The conditions for fixation are a compromise: stronger fixation yields a better preservation of cellular morphology, but the increased cross-linking lowers the accessibility of the target (Wilkinson, 1998). Fixation is usually carried out at 4°C to

inhibit endogenous ribonucleases, and to minimise the extraction of cellular constituents (Wilkinson, 1998). The tissue is then thoroughly dehydrated with a graded ethanol or methanol series, which is important since any remaining water in the tissue will hinder infiltration of the embedding medium and consequently make sectioning difficult (Hayat, 1989). Paraffin wax is the most popular embedding medium, since thin sections can be cut and morphology is usually well-preserved (Schwarzacher and Heslop-Harrison, 2000). Some researchers have obtained good results using cryostat sections for *in situ* hybridization (Meyerowitz, 1987; Nasrallah *et al*., 1988), however the morphological preservation after using this method is often poor, samples are difficult to handle, and some tissues are simply not amenable to cryosectioning (Schwarzacher and Heslop-Harrison, 2000). Another method involves the use of whole mounts, i.e. whole organs or thick tissue pieces. The major advantages of this method compared with using tissue sections are that it allows very rapid analysis, and that a large number of samples can easily be prepared and hybridised at once (Xu and Wilkinson, 1998). Choice of method depends on whether the researcher is interested in gene expression at the cellular, tissue or organ level, and also to some extent on how amenable the tissue is to a particular method of preparation.

3.1.2 Probe synthesis

A number of different types of nucleic acid probes can be prepared for *in situ* hybridization, including double-stranded DNA probes, oligonucleotides, and single-stranded RNA probes (Wilkinson, 1998). Double-stranded DNA probes have the advantage of not requiring sub-cloning into transcription vectors, however they tend to reanneal during hybridization (Burgess, 1992). Oligonucleotide probes can readily be designed from published sequences and allow excellent penetration into the tissue due to their small size, but are less sensitive than longer nucleic acid probes (Wilkinson, 1998). Single-stranded RNA probes are most commonly used, especially when the target sequence of interest is an RNA molecule (Schwarzacher and Heslop-Harrison, 2000). RNA:RNA hybrids are more stable than DNA:RNA hybrids, and in addition post-hybridization treatment with RNase A can remove unhybridized single-stranded RNA and thus reduce non-specific background (Leitch *et al*., 1994). The major drawback to

using RNA probes or riboprobes is that they are extremely labile and so must be treated with extra precaution (Leitch *et al*., 1994), for example by avoiding repeated freezing and thawing of probe stocks.

There is some ambiguity regarding the optimal length of a probe to be used for *in situ* hybridization. Longer probes usually give stronger signals since it is possible to incorporate more labelled nucleotides into them, however very long probes can reduce signal since they penetrate the cross-linked tissue less efficiently (Wilkinson, 1998). Some authors maintain that it is best to synthesize as long a probe as possible and then reduce it to smaller fragments by alkali hydrolysis (Xu and Wilkinson, 1998). Other literature states that the best probe length for *in situ* hybridization is between 100 and 300 bases (Leitch *et al*., 1994). While this is entirely up to the researcher, it would seem logical to synthesize a longer probe if the target sequence is suspected to be of low abundance, so that even very low levels can be detected due to a stronger signal.

3.1.3 Pre-hybridization treatment

Before hybridization, tissue is pre-treated to reduce non-specific hybridization to non-target nucleic acids and to reduce non-specific interactions with proteins or other components that may bind the probe (Leitch *et al*., 1994). Pretreatment also reportedly functions to increase sensitivity of the tissue to the probe (Oliver *et al*., 1997). It usually involves proteolytic digestion of material with proteases, for example proteinase K or pre-digested pronase E, and is used to remove proteins that increase background and interfere with probe access (Schwarzacher and Heslop-Harrison, 2000). Pre-treating tissue by heating in a microwave has been reported to have several advantages over conventional protease pre-treatment, including reducing the required hybridization time and markedly minimising background (Lan *et al*., 1996). However in the study concerned the pre-treatment was applied to animal tissue, and preliminary experiments in our own laboratory suggested this technique to be unsuitable for plant tissue (Zwiegelaar, personal communication).

Another part of the pre-treatment regime involves acetylation. This neutralizes positively charged molecules, such as basic proteins, and prevents non-specific binding of probe to the tissue (Leitch *et al*., 1994). Some protocols, especially those using tissue sections on slides, include a dehydration step prior to hybridization to ensure that the probe is not diluted by any residual buffer solution (Wilkinson, 1998). However when whole mounts are being used, this step can often destroy the tissue and even generate background, and should be omitted (Schwarzacher and Heslop-Harrison, 2000). In addition, whole mounts should be pre-hybridized by incubating in the hybridization buffer for 1-2 h at the hybridization temperature, which ensures penetration into the tissue and blocking of non-specific binding sites (Schwarzacher and Heslop-Harrison, 2000).

3.1.4 Hybridization

For the actual hybridization process, the labelled probe is made up in a hybridization buffer which typically contains formamide, salts and dextran sulphate, with optional incorporation of SDS and bovine serum albumin (Leitch *et al*., 1994). Alternatively the probe may be added to a commercially prepared buffer that has been specifically formulated for *in situ* hybridization. It is necessary to consider both the temperature and duration of hybridization. Hybridization time depends on the amount and length of the probe, as well as on the amount of the target sequence present. Hybridising for 4-6 h is usually adequate for highly abundant sequences, but for rare target sequences it is sometimes advantageous to hybridise for at least 72 h (Schwarzacher and Heslop-Harrison, 2000). Temperature of hybridization is perhaps more important, since this is one of the factors that controls the stringency of the experiment. Hybridization typically takes place at 37°C for DNA:DNA hybrids and 50-55°C for RNA:RNA hybrids, which is usually 20-25°C below the melting temperature or T_m (Leitch *et al*., 1994). The T_m is the temperature at which 50% of the hybrids are dissociated, and can be calculated if the base composition and length of the probe is known (Wilkinson, 1998). Increasing the temperature increases the dissociation of hybrids that are non-perfectly matched and vice versa (Schwarzacher and Heslop-Harrison, 2000), and so can be adjusted according to the desired stringency.

3.1.5 Washing and detection

Following hybridization it is necessary to remove unbound probe by washing. More importantly, post-hybridization washing removes probe that has bound to sequences related to the intended target or non-specifically to other cellular components (Wilkinson, 1998). Many protocols include washing in buffer containing RNase A to digest unbound probe, however this may cause a reduction in signal (Jowett *et al*., 1996). This is most likely due to the possibility of steric hindrance preventing the probe from hybridising along its full length to homologous targets, resulting in the single-stranded regions being digested (Wilkinson, 1998). Thus it may be wise to determine whether this step is absolutely necessary in a particular experiment, especially if poor signal is achieved.

The final step in an *in situ* hybridization experiment is the detection of the probe in the tissue. If the probe has been radioactively labelled, this is achieved by exposure to X-ray film. The most sensitive method for detection of hapten-labelled probes involves amplification of the signal by using an enzyme-conjugated antibody, for which chromogenic substrates are available that yield an insoluble coloured product (Wilkinson, 1998). The latter method is safer and is most commonly used, however there is the drawback that the hybridization signal cannot be quantified.

Despite years of research on sugarcane physiology, metabolism and molecular biology, there is to date only a single publication describing the use of *in situ* hybridization to identify mRNA expression patterns in sugarcane tissue (Casu *et al*., 2003). However the researchers only succeeded in applying their technique to young and maturing tissue, and a method also applicable to mature tissue would obviously be of great use. This study reports for the first time the development of a tissue preparation and *in situ* hybridization protocol which is specifically suited to sugarcane culm tissue of all developmental stages.

3.2 Materials and methods

3.2.1 Plant material

Mature, non-flowering stalks of a *Saccharum* species hybrid, variety N19, were used. Stalks were cut in the early morning and taken to the laboratory. Internode 1 was defined as that which subtended the first visible dewlap (Moore, 1987). Cylinders of culm tissue were bored out of the respective internodes with a cork borer, and then discs of tissue were cut as described below.

3.2.2 Development of tissue preparation method

Three different methods of tissue preparation were tested, including conventional wax embedding, cryosectioning, and hand-cut sections.

3.2.2.1 Chemical fixation and wax embedding

Tissue discs were cut from internodes 3, 5, 7, 9 and 11, and were fixed and embedded in wax according to a method modified from Schwarzacher and Heslop-Harrison (2000). Since specimen size affects quality of fixation, a variety of tissue dimensions were tested. These included discs 2 mm thick and 10 mm in diameter, discs 1 mm thick and 10 mm in diameter, and discs 0.5 mm thick and 5 mm in diameter. Three different fixatives were tested, viz. 4% paraformaldehyde in phosphate buffer (pH 7.2), 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (pH 7.5), and FAA (10% formaldehyde, 5% glacial acetic acid and 50% absolute ethanol). Tissue was initially fixed under vacuum for 2 h at room temperature, then at 4°C for a variety of fixation periods. These included fixation for 24h, 48h, 72h, 5d and 7d. Tissue was then rinsed in saline solution (145 mM NaCl in water) for 30 min on ice, followed by dehydration in a graded ethanol series for 1 h each. Dehydration was continued in 100% ethanol overnight at 4°C. The following day tissue was dehydrated for a further 1 h in fresh 100% ethanol, and then transferred to a dilute safranin solution (0.05% in 100% ethanol) for 5 min. Once stained the tissue was rinsed three times for 2 min in 100% ethanol, and then placed in 1 part 100% ethanol to 1 part xylene for 1 h. Tissue was then transferred to pure xylene for 3 h, with the xylene being refreshed hourly. Tissue was left in fresh xylene and the same volume of solid paraffin wax pellets overnight at 60°C. Tissue was then transferred to pure molten wax,

which was changed twice a day. Five different embedding periods were tested, viz. 3d, 7d, 10d, 2w and 3w. Both Paraplast Plus wax (Sigma) and Paraffin wax (BDH Laboratory Supplies) were tested as embedding media. Tissue was polymerized by placing into flexible plastic molds containing molten wax and allowing to cool. Sectioning was attempted with both stainless steel microtome blades and disposable blades, and sectioning at a thickness of 5µm, 10µm, 20µm and 40µm was attempted.

3.2.2.2 Cryosectioning

Culm tissue was treated according to a protocol modified from Barthel and Raymond (1990). Tissue discs 1mm thick and 10mm in diameter were cut and immediately dropped into FAA (10% formaldehyde, 5% glacial acetic acid and 50% absolute ethanol). Tissue was fixed for 2h under vacuum, then passed through a series of solutions of 20% (w/v) sucrose solution and 0.1 M sodium phosphate buffer, in ratios of 1:2, 1:1 and 2:1 for 30 min each. The tissue was soaked overnight in a solution of 20 % sucrose in 0.1 M sodium phosphate buffer, and the following day was placed into a 1:1 mixture of 20 % sucrose solution and OCT cryoprotectant solution (Tissue-Tek, USA) for 30 min. Tissue discs were then plunged into liquid nitrogen-cooled isopentane, and 20µm sections were cut with a Leica CM100 Cryostat at -20°C. Sections were lifted into cooled slides, then allowed to thaw at room temperature and immediately examined under a microscope.

3.2.2.3 Thick hand-cut sections

Tissue discs were cut from internodes 3, 6 and 13, and immediately dropped into 4 % paraformaldehyde in phosphate buffer (pH 7.2). Again, a variety of tissue sizes was tested. These included the following dimensions: discs 1 mm thick and 10 mm in diameter; 1 mm thick and 5 mm in diameter; 0.5 mm thick and 10 mm in diameter; 0.5 mm thick and 5 mm in diameter. Different fixation periods were tested, viz. 4 h, 12 h, 24 h, 48 h and 72 h. All of these were performed at 4°C. Following fixation tissue was briefly rinsed in water, and then treated for hybridization as described below.

3.2.3 Development of *in situ* hybridization method

The basic *in situ* hybridization method was adapted from Schwarzacher and Heslop-Harrison (2000). Fixed tissue slices were rinsed in distilled water, and then placed into 0.2 M HCl for 10 min. After rinsing in distilled water and PBS, the tissue was incubated in Pronase (0.125 $mg.ml^{-1}$ predigested Pronase in Pronase buffer) for 15 min. Pronase activity was stopped with 0.2 % glycine, and sections were then re-fixed in 4 % paraformaldehyde for 10 min. The fixative was rinsed off with PBS, and the tissue was then acetylated (1 % acetic anhydride in triethanolamine, pH 8.0). Following a final rinse in PBS, sections were placed onto marked slides that had been washed in Rnase-Away solution (Molecular BioProducts). Each section was covered with 50 µL hybridisation mix (200$ng.ml^{-1}$ probe in hybridisation buffer (Sigma)) and incubated for 16 h at 37°C. Sections were then washed in wash buffer (2x SSC, 50 % formamide) for 60 min at 50°C, followed by 2 washes of 5 min each in NTE at 37°C. The tissue was then incubated in NTE with 20$\mu g.ml^{-1}$ RNaseA at 37°C for 30 min, followed by 2 washes of 5 min each in NTE at room temperature. Sections were washed again in wash buffer for 60 min at 50°C, followed with a brief rinse in PBS and 5 min equilibration in 100 mM Tris, pH 7.5, containing 150 mM NaCl (Buffer 1). Sections were blocked in 1 % blocking reagent (Roche) in Buffer 1 at 4°C overnight, and then incubated with anti-digoxygenin-alkaline-phosphatase conjugate diluted 1:3000 in the blocking solution at room temperature for 90 min. Sections were washed four times for 20 min in Buffer 1 containing 0.3 % Triton X-100, then once for 5 min in Buffer 1 and equilibrated for 5 min in Buffer 4 (100 mM Tris, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$). Detection took place in 1.5 $\mu l.ml^{-1}$ BCIP and 1.5 $\mu l.ml^{-1}$ NBT in Buffer 4 containing 10 % polyvinyl alcohol.

Since this basic method did not yield satisfactory results (e.g. low or absent signal, excessive background signal, etc.), a number of experimental parameters were systematically changed and tested in order to determine the most optimal method. The parameters that were tested included temperature of hybridization, necessity of pre-treatment, probe concentration, necessity of pre-hybridisation, and composition of the detection buffer.

3.2.3.1 Temperature of hybridization

The most optimal temperature for hybridization was determined by performing the basic procedure detailed above and attempting hybridization at 37°C, 50°C and 55°C. This had to be performed for each of the three RNA probes, since probes of different lengths and base composition often form stable hybrids with tissue at different temperatures (Schwarzacher and Heslop-Harrison, 2000).

3.2.3.2 Necessity of pre-treatment

This was determined by repeating the basic method, and running a parallel experiment in which the digestion with 0.2 M HCl, incubation in Pronase, and re-fixation in paraformaldehyde was precluded.

3.2.3.3 Probe concentration

The most optimal probe concentration was determined by running 3 parallel experiments in which probe concentrations of 200 $ng.ml^{-1}$, 500 $ng.ml^{-1}$ and 1000 $ng.ml^{-1}$ were tested and compared. Although this was done for each of the three RNA probes, the chosen concentration had to be the same for each probe in order for the resulting signal intensities to be comparable.

3.2.3.4 Necessity of pre-hybridisation

In order to assess whether pre-hybridisation was necessary to block non-specific binding sites, tissue was pre-hybridised in the hybridisation buffer for 2h before hybridisation with the actual probe, and then compared with tissue that had not been pre-hybridised.

3.2.3.5 Composition of detection buffer

Since the detection buffer used in the basic protocol was very viscous, buffers containing different amounts of polyvinyl alcohol were compared. Buffers with 10% and 5% polyvinyl alcohol were tested, as well as a buffer that contained no polyvinyl alcohol. An additional detection buffer consisting of BCIP/NBT tablets dissolved in Buffer 4 was also tested and compared with the other buffers.

3.3 Results

3.3.1 Chemical fixation followed by wax embedding

Although this method of tissue preparation is routine for many *in situ* hybridization studies, major difficulties were encountered when applying the method to sugarcane stem tissue. Fixing tissue discs overnight, as recommended by Schwarzacher and Heslop-Harrison (2000), resulted in large white patches in the tissue, which caused the discs to float in the molten wax. The cause of these white patches appeared to be inadequate fixation, since increasing the fixation time reduced both their number and size. However even fixation periods as long as 7d failed to completely eradicate all unfixed patches in the older internodes, where the patches were most prevalent. Sectioning of such non-uniformly fixed tissue was difficult and resulted in highly damaged cell morphology (Figure 3.1, a and b), even at a thickness of 40µm. Extended embedding (3 weeks) in wax and application of a gentle vacuum during infiltration did little to improve sectioning properties. There was little appreciable difference in fixation quality between the fixatives tested, and using different waxes had no effect on the quality of the sections.

3.3.2 Chemical pre-fixation followed by cryofixation

Cutting frozen sections was somewhat easier than sectioning wax-embedded tissue, however the reproducibility of this method was extremely low. Sections repeatedly broke up, both while being cut and while being transferred onto slides. In addition, preservation was poor, since broken cell walls were frequently observed and some cells had a haphazard shape (Figure 3.1, c and d). Such damage was most probably due to the formation of large ice crystals during the freezing process, which would puncture the plasmalemma and cell walls.

3.3.3 *In situ* hybridization on thick hand-cut sections

This method proved to be the easiest and quickest to perform, and not only allowed good preservation of morphological detail (Figure 3.1, e and f), but also minimized the use of chemical embedding media which can affect the distribution of cellular constituents (Hayat, 1989). The major drawbacks were that tissue was difficult to store i.e. once cut,

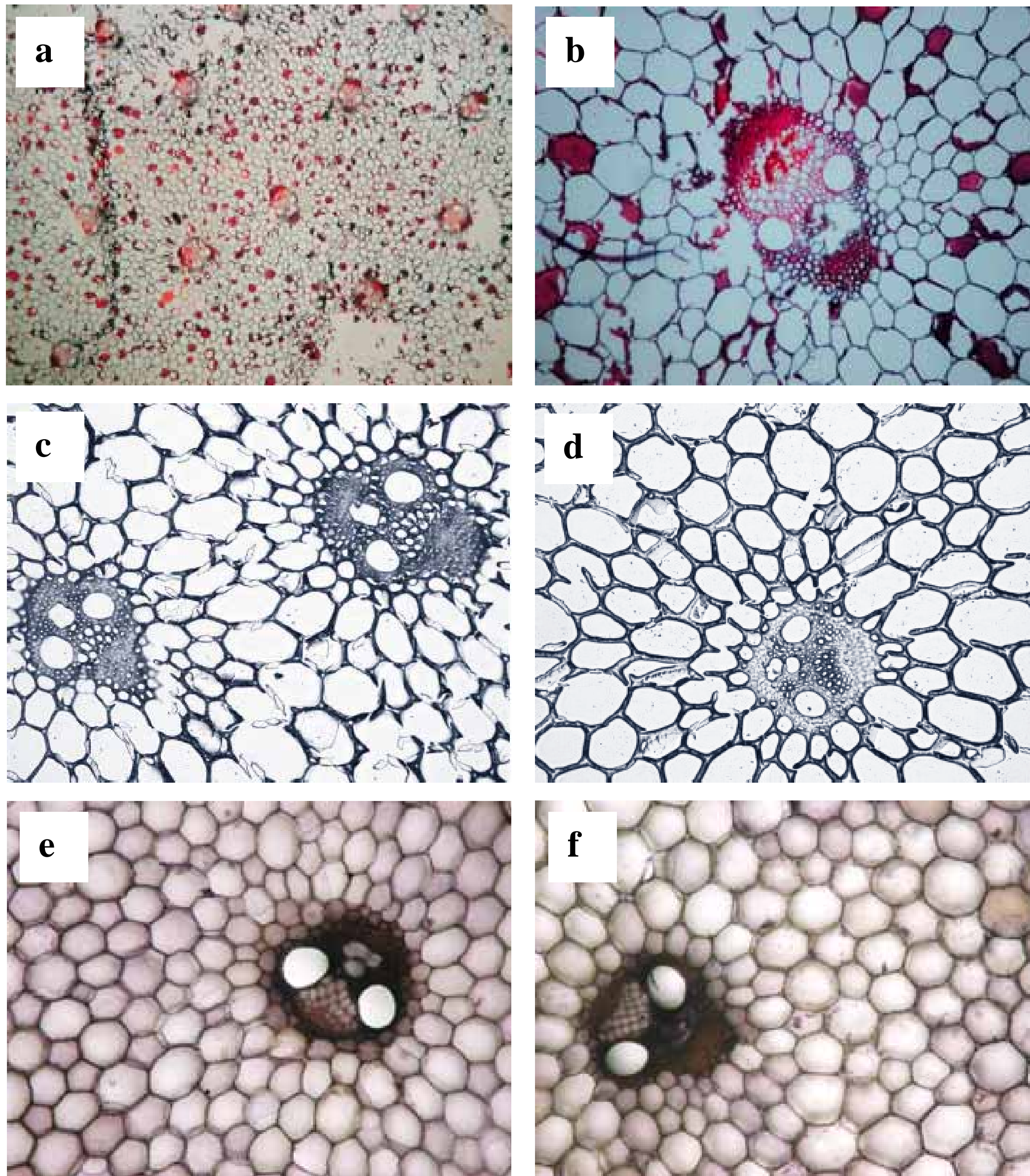


Figure 3.1 Comparison of different tissue preparation methods for sugarcane culm: sections of internodes 3 (a) and 7 (b) that have been embedded in wax; sections of internodes 10 (c) and 20 (d) that have been frozen and cryosectioned; fresh sections of internodes 3 (e) and 13 (f) that have been cut by hand.

tissue needed to be fixed and hybridized immediately, and in addition resolution was frequently jeopardized due to the thickness of the sections. In terms of fixation time, 48 h fixation seemed to preserve morphology satisfactorily without over-fixing (Figure 3.2 d). After 4h fixation the cellular morphology was severely deteriorated, which was evident by the highly compressed, irregular shape of most of the parenchyma cells (Figure 3.2 a). This effect, which is most likely due to cell shrinkage, was most prevalent in the young internodes. Fixing for 12 h did little to improve this (Figure 3.2 b), and even after 24 h fixation there was evidence of cell shrinkage around the vascular bundles (Figure 3.2 c). Fixing tissue for 72 h did not appear to greatly reduce the hybridization signal (not shown), however shorter fixation times are naturally more desirable. Changing the dimensions of the tissue pieces did not have any apparent effect on fixation quality, although sections 10 mm in diameter were easier to handle than those 5 mm in diameter. Sections with a 10 mm diameter also allowed both core and peripheral culm tissue to be sampled at the same time. Thinner sections i.e. 0.5 mm thick, provided better resolution than sections 1 mm thick.

3.3.3.1 Temperature of hybridization

While many *in situ* hybridization protocols suggest a hybridization temperature of 37°C, in reality the temperature required to produce satisfactory signal is often somewhat higher. Hybridization of all probes to tissue at 37°C resulted in a diffuse pink-coloured staining (Figure 3.3 a, g and m), which was quite obviously the result of non-specific binding since the same staining was apparent in tissue hybridized with the sense probes (Figure 3.3 d, j and p). Increasing the temperature to 50°C allowed antisense tissue to be easily distinguished from sense tissue for neutral invertase (Figure 3.3 n and q, respectively) and cell wall invertase (Figure 3.3 h and k, respectively). However in the case of acid invertase, hybridizing at 50°C resulted in no appreciable difference between anti-sense and sense tissue (Figure 3.3 b and e, respectively). Increasing the hybridization temperature to 55°C reduced non-specific binding sufficiently to allow antisense to be distinguished from sense tissue (Figure 3.3 c and f, respectively), however this temperature seemed too stringent for cell wall and neutral invertase probes judging by the

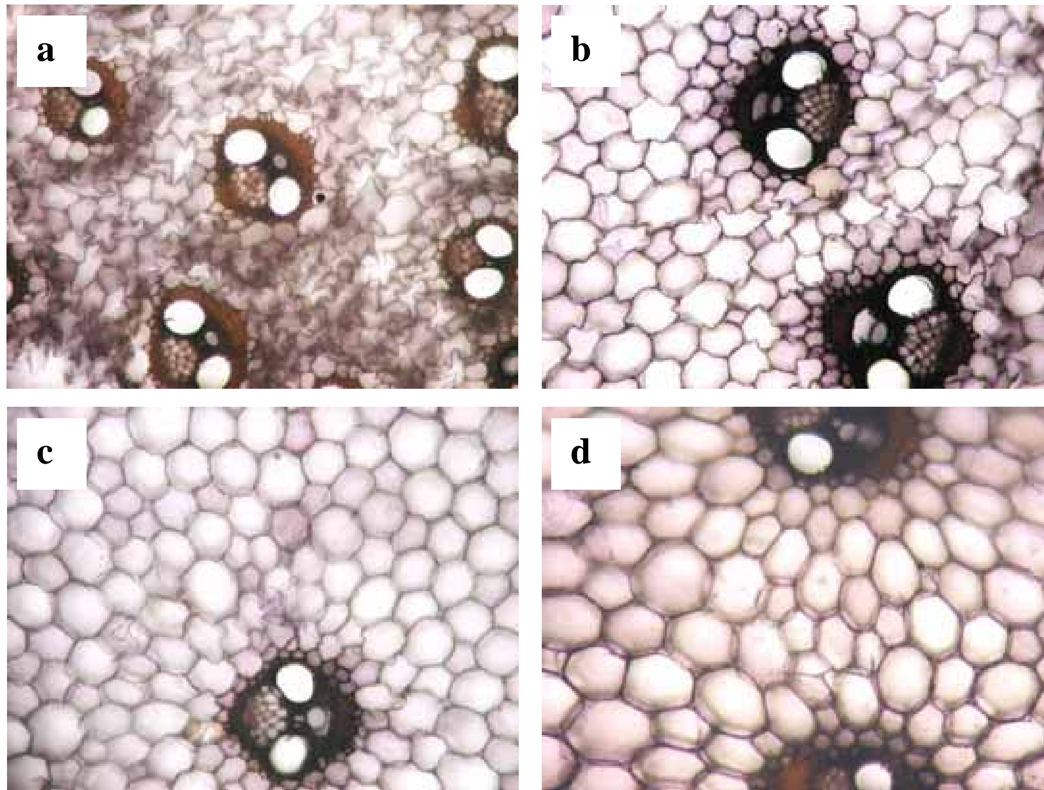
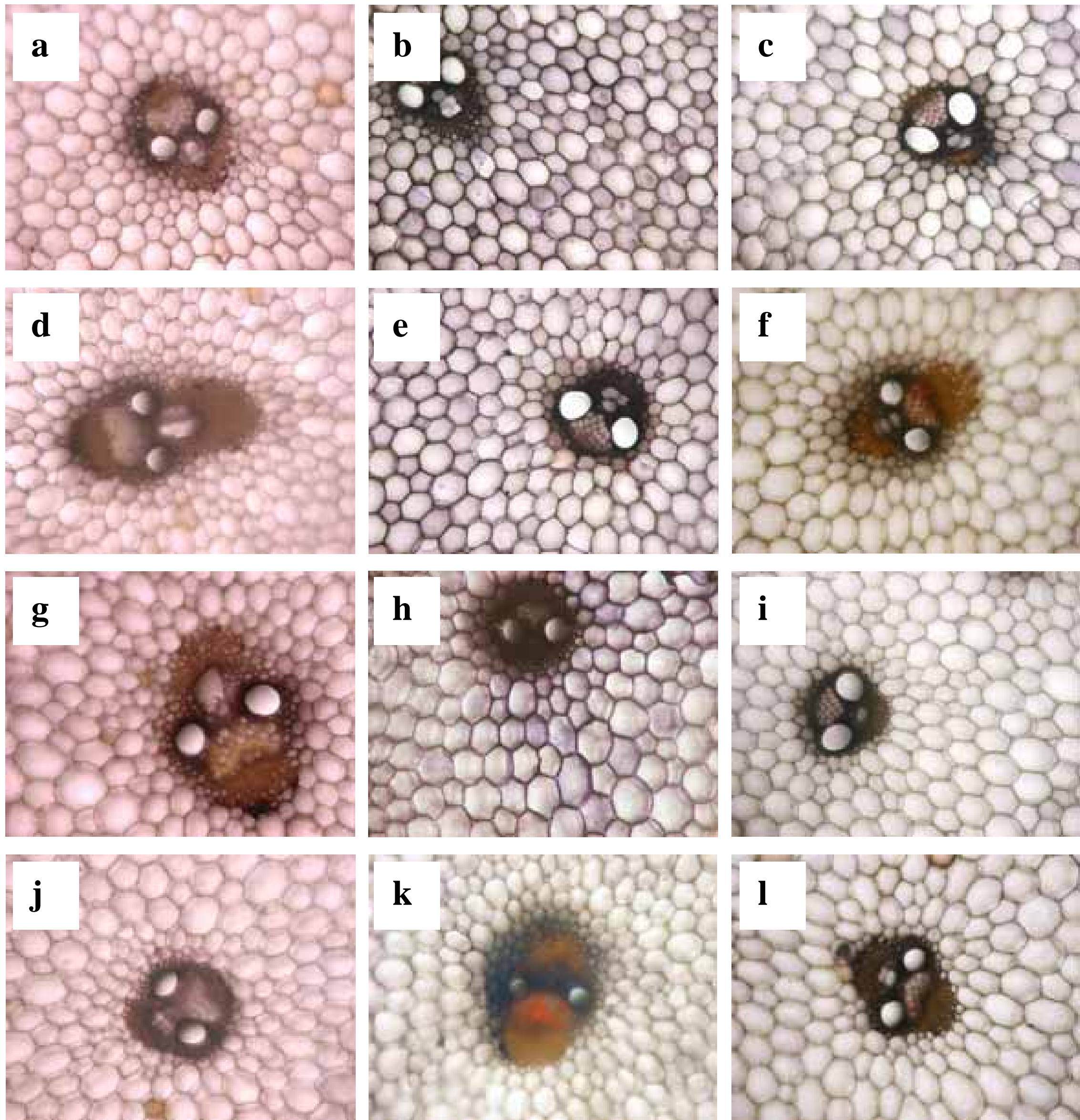


Figure 3.2 Comparison of different fixation periods for thick hand-cut sections of sugarcane culm. Sections from internode 3 were fixed in 4 % paraformaldehyde for 4h, 12h, 24h and 48h (a, b, c and d, respectively), at 4°C.

lack of signal in both antisense (Figure 3.3 i and o, respectively) and sense tissue (Figure 3.3 l and r, respectively).

3.3.3.2 Necessity of pretreatment

Excluding pretreatment with HCl and Pronase resulted in no appreciable signal in either antisense or sense tissue (Figure 3.4 c and d), in contrast to tissue treated with these solutions where hybridization signal was visible (Figure 3.4 a).



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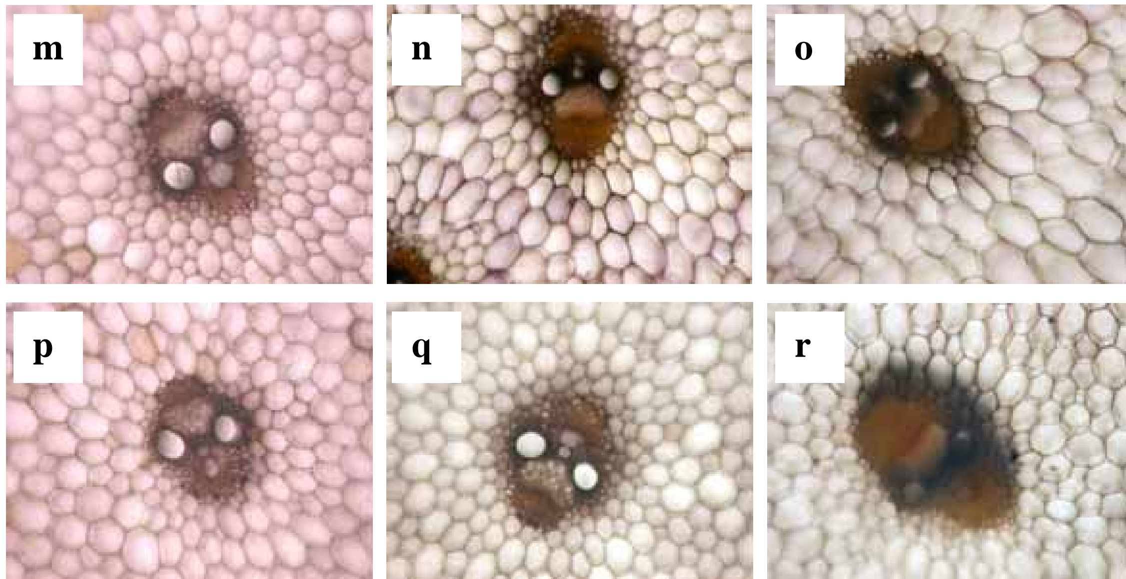

Figure 3.3 Comparison of hybridization temperatures for acid invertase mRNA (a-f), cell wall invertase mRNA (g-l), and neutral invertase mRNA (m-r). Hybridization was attempted at 37°C (left-hand column), 50°C (middle column), and 55°C (right-hand column) for all three isoforms. Tissue sections from internode 7 were used, and were fixed for 48h at 4°C. Tissue was hybridised with probes diluted to 200 $ng.ml^{-1}$ in each case. d-f, j-l, and p-r represent sense tissue for acid, cell wall and neutral invertase, respectively.

3.3.3.3 Probe concentration

Diluting probe to a concentration of 500 $ng.ml^{-1}$ seemed to give the best signal intensity for all three riboprobes (Figure 3.5 b, e and h). Increasing the probe concentration to 1000 $ng.ml^{-1}$ gave more intense colouring for cell wall and neutral invertase (Figure 3.5 f and i), however dramatically reduced the hybridisation signal in the case of acid invertase (Figure 3.5 c). This is probably since the concentration of the probe stock was very low, and achieving a concentration of 1000 $ng.ml^{-1}$ involved using almost no buffer.

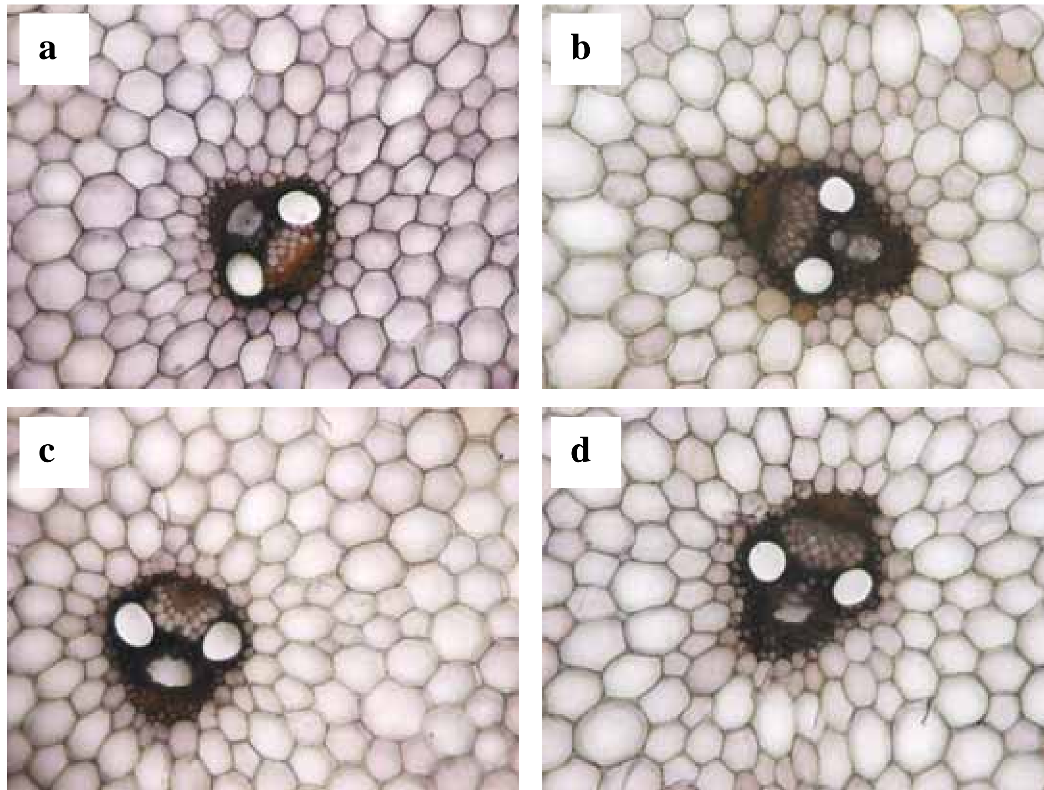

Figure 3.4 Comparison of pretreated (a and b) and non-pretreated (c and d) tissue. Pretreatment allows a clear distinction between anti-sense and sense tissue (a and b respectively). Omitting pretreatment seems to dramatically reduce signal, resulting in no visible difference between anti-sense and sense tissue (c and d respectively). Tissue sections from internode 7 were used, and were probed with neutral invertase probe (200 $ng.ml^{-1}$) at 50˚C for 16h.

3.3.3.4 Necessity of pre-hybridisation

Although it is recommended as a means of reducing non-specific binding and to allow adequate tissue penetration, pre-hybridization appears to dilute the probe, resulting in no visible difference between anti-sense and sense tissue (Figure 3.6 c and d, respectively). Omitting pre-hybridisation results in good differentiation between anti-sense and sense tissue (Figure 3.6 a and b, respectively), which in turn demonstrates that this step is not necessary for adequate penetration of the tissue by the probe.

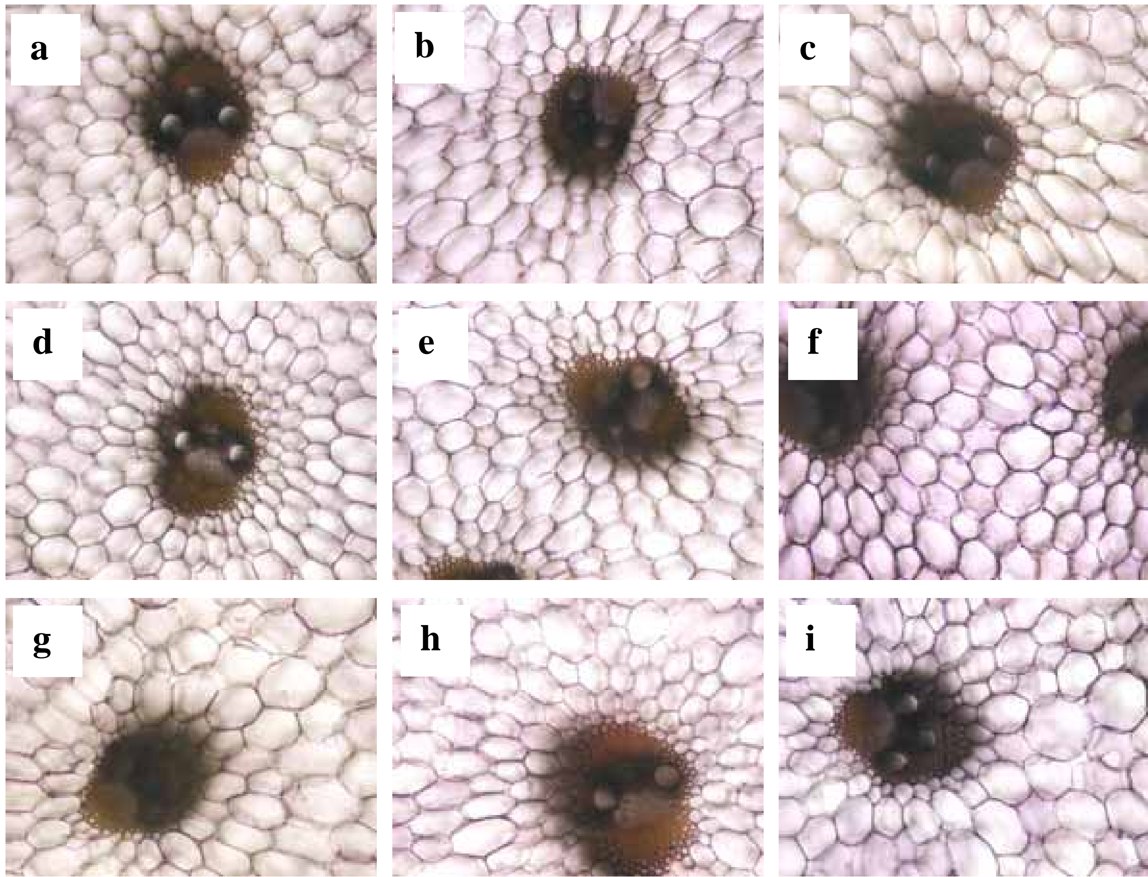

Figure 3.5 Comparison of different concentrations of probes for acid invertase mRNA (a-c), cell wall invertase mRNA (d-f), and neutral invertase mRNA (g-i). Probes were tested on tissue sections from internode 7, at concentrations of 200 $ng.ml^{-1}$ (a, d and g), 500 $ng.ml^{-1}$ (b, e and h) and 1000 $ng.ml^{-1}$ (c, f and i).

3.3.3.5 Composition of detection buffer

There was little noticeable difference in signal intensity or quality when either 10% or 5% PVA was included in the detection buffer (Figure 3.7 a and b, respectively). However development of the signal occurred about twice as quickly when buffer containing 5%

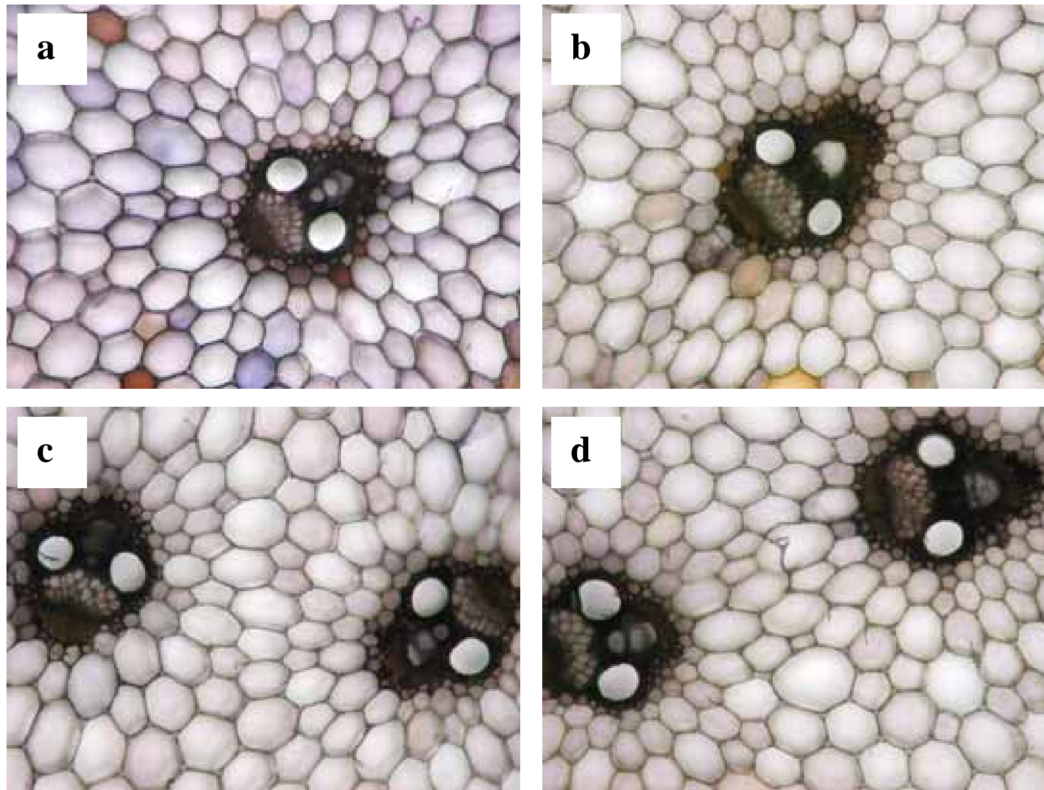

Figure 3.6 Comparison of tissue treated normally (a and b) and tissue prehybridized in hybridisation buffer (c and d). Omitting prehybridization allows a clear distinction between anti-sense and sense tissue (a and b respectively). Prehybridization appears to dilute the probe, resulting in no visible difference between anti-sense and sense tissue (c and d respectively). Tissue sections from internode 7 were used, and were probed with neutral invertase probe (500 $ng.ml^{-1}$) at 50˚C for 16h.

PVA was used. Omitting PVA from the detection buffer seemed to result in a less intense, diffuse pink colouration (Figure 3.7 c), while using pre-mixed NBT/BCIP tablets resulted in dark pink colouration and uncharacteristic brown staining (Figure 3.7 d). Development of hybridisation signal when using the latter two detection buffers occurred at roughly the same rate as when using buffer containing 5% PVA.

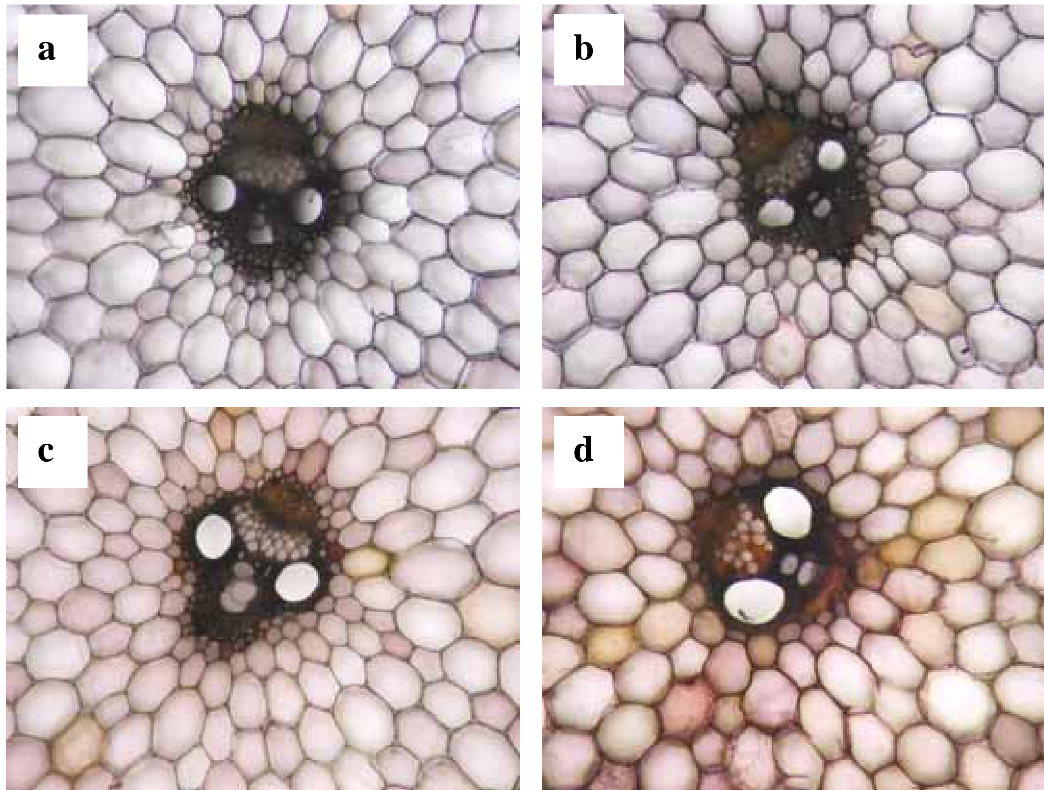

Figure 3.7 Comparison of detection buffers: tissue was incubated in buffer containing either 10% PVA (a), 5% PVA (b) or no PVA (c). An additional buffer, in which pre-mixed NBT/BCIP tablets were used instead of diluting from stock solutions, was tested (d). Tissue sections were cut from internode 7, fixed for 48h at 4˚C, and probed with neutral invertase riboprobe (500 $ng.ml^{-1}$).

3.4 Discussion

Wax embedding is now routinely used in the preparation of a wide variety of both plant and animal tissues for *in situ* hybridization, and so it is somewhat peculiar that sugarcane tissue is not amenable to this method. The difficulties encountered in applying this method to sugarcane could be attributed to the increased lignification and suberization that occurs as the internodes mature (Jacobsen *et al*., 1992). Lignin and suberin layers could possibly hinder the uniform diffusion of fixative through the tissue section, and more especially the infiltration of the embedding medium, which is considerably more viscous. Yet the wax-embedding technique has been successfully applied to woody stem tissues (Kwon *et al*., 2001), which presumably also have a very high degree of lignification. Casu and co-workers report using the wax embedding technique to prepare sugarcane for *in situ* hybridization, however they only succeeded in applying the method up to internode 5 (Casu *et al*., 2003). There is no mention of using the technique on older internodes, and so it is possible they encountered a similar problem to the one described here. Another factor which may inhibit proper fixation is the high concentration of sucrose in the older internodes. Formaldehyde is incapable of fixing carbohydrates (Hayat, 1989), and although much of the stored sucrose would probably leach out during fixation, it is possible that some would get trapped by the fixation of surrounding cells and result in the observed white patches. This explanation seems plausible in light of the high concentrations of stored sucrose in mature internodes, and the fact that the white patches mentioned occurred mostly in older internode pieces.

Another problem encountered with the wax sections, and indeed also with the frozen sections, was that they lacked any appreciable hybridization signal. This could be attributed to the fact that the actual hybridization protocol had not yet been optimised. However another possibility is that there was simply not enough cytoplasm within the cells to provide sufficient signal. Parenchyma cells of the sugarcane culm are large, being on average 140 µm in diameter and 240 µm in length (Moore and Maretzki, 1996), and hence 10 µm or even 40 µm sections would contain little, if any, cytoplasm. Thicker sections would include whole cells, and this would greatly enhance visualisation of the hybridisation signal.

Most *in situ* hybridization protocols suggest fixation times of between 30 min and 4 h, warning that longer fixation adversely affects the strength of the hybridization signal (Schwarzacher and Heslop-Harrison, 2000). However highly lignified or suberised tissue, such as sugarcane culm tissue, would hamper the penetration of the fixative, and so longer fixation periods are necessary. Furthermore fixing for 48h, as opposed to shorter fixation periods, does not seem to significantly reduce hybridisation signal. The results of this study in fact show that fixing for short periods such as 4h not only results in severely deteriorated cellular morphology, but also yields a less intense hybridisation signal. This demonstrates that fixation is not only important for preserving tissue morphology, but also for fixing target sequences so that they are prevented from leaching out of the tissue during subsequent pretreatments and high-temperature incubations.

From the results of this study, it is clear that inclusion of pre-treatment with HCl and Pronase is necessary to make the target sequences accessible to the probe. This increase in sensitivity with pre-treatment has been demonstrated before in both animal and plant tissues (Oliver *et al*., 1997; Busch *et al*., 1994), and is now usually a routine part of *in situ* hybridisation protocols. On the other hand pre-hybridisation, which is usually necessary for large pieces of tissue (Schwarzacher and Heslop-Harrison, 2000), seemed only to dilute the probe and reduce hybridisation signal completely in this study.

The use of polyvinyl alcohol in detection buffers was first reported by DeBlock and Debrouwer (1993). They report that its addition to the NBT/BCIP detection system enhances the alkaline phosphatase reaction and greatly reduces diffusion of the reaction intermediates, which results in a twenty-fold increase in sensitivity (DeBlock and Debrouwer, 1993). The results of this study support these findings: including PVA in the detection buffer results in a far more intense signal, which can be interpreted as an increase in sensitivity. Use of PVA also reportedly allows more precise determination of the site of hybridization (DeBlock and Debrouwer, 1993), and this was seen in some of the tissue where the cytoplasm was clearly discernible from the vacuole. A drawback to using the recommended concentration of 10% PVA was that occasionally tissue would

undergo massive shrinkage when placed in the detection buffer, an effect most probably caused by severe osmotic differences between the hydrated section and the hypertonic detection solution. Since this phenomenon occurred at random, even when all other experimental parameters were kept constant, it was tentatively concluded that the underlying cause was probably differences between the relative water contents of individuals. The problem was sidestepped by reducing the PVA concentration to 5%, which resulted in no shrinkage and gave comparably good hybridisation signal. In addition, development of the signal took place twice as quickly as when using 10% PVA.

Although most of the results presented are based on experiments using maturing tissue i.e. internode 7, the same experiments were performed on immature and mature tissue (results not shown). Although somewhat laborious, this ensured that the developed technique could be applied to all developmental stages. The results of this study demonstrate that *in situ* hybridisation can successfully be employed to examine gene expression in sugarcane stem tissue of all ages, and also reinforces the importance of adapting so-called “routine procedures” to suit the peculiarities of the tissue of interest. And while not perfect, the developed technique is quick, reproducible and easy, and will greatly aid future research in this field.

CHAPTER 4

Tissue-specific expression patterns of invertase genes in sugarcane internodal tissue

Abstract

Sugarcane invertases have previously been characterised from whole tissue homogenised samples, an approach which is useful but which gives no indication of the tissue-specificity of these enzymes. Knowledge of the tissue-specific expression patterns of the invertase isoforms is important for understanding their involvement in sucrose accumulation. This study aimed to describe the tissue-specific expression patterns of the three invertase isoforms in young, maturing and mature internodes of sugarcane. This was performed using non-radioactive *in situ* hybridization, the results of which were complemented with Northern blot analysis. Results of *in situ* hybridization revealed transcripts of all three isoforms to be present in the storage parenchyma and in the phloem tissue. Northern blot analysis showed that transcript levels of all three isoforms declined in maturing tissue, with soluble acid invertase declining sharply and dropping below detection in maturing and mature tissue. Transcript levels of cell wall invertase and neutral invertase declined only gradually, and appreciable amounts were still present in fully mature tissue. The results suggest that acid invertase is involved in internode elongation, and may play a small role in determining sucrose accumulation in this specific variety. Cell wall invertase would appear to be involved in turgor control and phloem unloading, the latter of which has important implications for future work. The function of neutral invertase is tentatively suggested to be maintenance of sugar levels, however ultimately the results leave the role of this enzyme unresolved.

4.1 Introduction

Most sugarcane varieties are capable of accumulating sucrose to more than 20% on a fresh weight basis in mature internodes (Glasziou and Gayler, 1972). This phenomenon is commercially useful, but despite years of research the biochemical processes controlling sugar accumulation are still poorly understood. This is largely due to the highly fragmented and inconsistent nature of current knowledge, caused by different sampling techniques and analytical methods used on different sugarcane varieties. Thus it is difficult to efficiently integrate the existing knowledge to target the appropriate metabolic pathways, and manipulate them so as to increase sucrose accumulation.

Understanding sucrose accumulation in sugarcane requires, among other things, an understanding of the enzymes involved in sucrose metabolism. Research in the past has largely focused on the sucrolytic enzymes, and particularly on the invertases (Hatch and Glasziou, 1963; Albert *et al*., 1996; Vorster and Botha, 1999; Rose and Botha 2000). A number of invertase isoforms exist, classified according to their pH optima, solubility and subcellular localization (Tymowska-Lalanne and Kreis, 1998), and these have all been identified and analysed to some extent in sugarcane. Sugarcane soluble acid invertase, with a pH optimum of 4.4 and K_m of 1.3 x 10^{-2} M for sucrose, has been shown to be highly active in immature tissue but almost absent in older tissue (Hawker and Hatch, 1965). It tends to reach a peak during elongation of an internode, and the elongation rate has been correlated with acid invertase activity (Lingle and Smith, 1991; Lingle, 1999). Its role in sucrose accumulation has been suggested to be insignificant (Botha and Birch, 2001), but there is evidence to the contrary. Zhu *et al*. (1997) found that a significant non-linear negative relationship existed between soluble acid invertase activity and sucrose accumulation in individual internodes. They also found a positive correlation between the difference between SPS activity and soluble acid invertase activity, and sucrose concentration (Zhu *et al*., 1997). Thus it appears that acid invertase might play a role in sucrose accumulation.

Sugarcane cell wall invertase has a pH optimum of 3.8 and a K_m of 8 x 10^{-3} M for sucrose (Hawker and Hatch, 1965), but otherwise has been poorly characterised (Albertson *et al*.,

2001). It is present in most types of tissues, but has been shown to decrease in activity with increasing tissue maturity (Vorster and Botha, 1999). Cell wall invertase has been shown to be involved in phloem unloading in maize kernels and wounded pea stem tissue (Cheng and Chourey, 1999; Zhang *et al*., 1996). Other putative functions in plants include environmental sensing, reloading of leaked sucrose and osmoregulation (Tymowska-Lalanne and Kreis, 1998). A function in phloem unloading and osmoregulation would seem likely in the sucrose-accumulating, potentially high-turgor environment of sugarcane sink tissues, but this is somewhat speculative and requires further investigation.

Sugarcane neutral invertase has a pH optimum 7.0 and a K_m of 9.8 x 10^{-3} M for sucrose (Vorster and Botha, 1998), and has attracted increased interest over the last decade. Initial work on this enzyme revealed increasing activity with increasing tissue age (Hatch and Glasziou, 1963), but more recently it has been shown that the highest neutral invertase activities are found in the youngest internodes (Zhu *et al*., 1997; Vorster and Botha, 1999; Rose and Botha, 2000). Neutral invertase has been suggested to be involved in providing energy for cellular maintenance, regulating sucrose storage, and regulation of hexose pools (Quick and Schaffer, 1996), as well as compensating for low acid invertase activity in certain tissues (Ricardo and ap Rees, 1970). In sugarcane specifically, it is proposed to be important in sucrose turnover at all stages of culm maturation (Vorster and Botha, 1999).

To date there is no literature on the tissue-specific distribution of invertase isoforms in sugarcane. Previous work on other plant species has illustrated how useful such knowledge can be in determining the function of a particular enzyme (Cheng *et al*., 1996; Hirose *et al*., 2002). The aim of this study was to determine the tissue-specific expression patterns of the sugarcane invertase isoforms at a range of developmental stages, and together with complementary Northern blot analysis, to use this information to explore their functions and involvement in sucrose accumulation.

4.2 Materials and methods

4.2.1 Plant material

Mature, non-flowering stalks of a *Saccharum* species hybrid, variety N19, were cut in early summer (November) in the morning at the Welgevallen experimental plot in Stellenbosch, and taken to the laboratory. Internode 1 was defined as that which subtended the first visible dewlap (Moore, 1987). Cylinders of culm tissue were bored out with a cork borer for *in situ* hybridization studies, while whole internodes were ground up for RNA extraction and Northern blot analysis.

4.2.2 RNA extraction

RNA was extracted from internodes 3, 6, 10 and 13, according to a modified method from Bugos *et al*. (1995). Tissue was ground into a fine powder in liquid nitrogen, and 5 g of this powder was transferred into a centrifuge tube containing 10 ml homogenization buffer (0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl and 1% SDS) and 10 ml of 25:24:1 phenol:chloroform:isoamyl alcohol. The mixture was vortexed at high speed, followed by addition of 0.7 ml 3 M sodium acetate (pH 5.2). The mixture was again vortexed and then stored on ice for 15 min. Following centrifugation at 12 000 g for 15 min at 4°C, the aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. RNA was precipitated overnight at -20°C, and then recovered by centrifugation at 10 000 g for 10 min. The pellet was washed with 70% ethanol, centrifuged at 10 000 g for 5 min and briefly dried. The RNA pellet was resuspended in 800 µl DEPC-treated water and insoluble material removed by centrifugation at 10 000 g for 5 min at 4°C. The supernatant was transferred to a microcentrifuge tube, and RNA precipitated by addition of LiCl to a final concentration of 2 M and incubating overnight at 4°C. RNA was recovered by centrifugation at 12 000 g for 20 min at 4°C. The pellet was washed with 70 % ethanol, centrifuged at 12 000 g for 5 min and then briefly dried. The pellet was resuspended in DEPC-treated water, and RNA quantified spectrophotometrically.

4.2.3 Northern blot analysis

For each sample 15 μg RNA, detected spectrophotometrically, was loaded on a 1.2 % (w/v) agarose gel and separated at 120 V until the dye front had run 8 cm. The gel was stained with ethidium bromide solution (approximately 0.4 μg ml^{-1}), viewed and photographed under UV light, and trimmed. The gel was equilibrated in 10x SSC for 30 min, and the nylon membrane (Roche) was wet in water and then equilibrated in 10x SSC while the transfer stack was set up. RNA was transferred onto the membrane using the downward transfer method (Chomczynski and Mackey, 1994) overnight at room temperature. After transfer the membrane was briefly dried and UV cross-linked for 2.5 min at 1200 mJ cm^{-2}.

Probes were prepared by random primer incorporation of ^{32}P-dCTP (Prime It II Random Labelling Kit, Stratagene). Unincorporated label was removed using Qiagen quick spin columns. The membrane was pre-hybridized in 10 ml RapidHyb buffer (Amersham, UK) for 3 h at 65°C, following which the labelled probe was added and the membrane hybridized at 65°C overnight. The membrane was washed twice for 5 min each in 0.2x SSC, 0.1 % sodium dodecyl sulphate (SDS), followed by washing twice for 15 min each in 0.1x SSC, 0.1 % SDS. All washes were done at 65°C. Hybridisation was visualised using a Cyclone Storage Phosphor Screen (Packard Instrument Company Inc., USA).

4.2.4 Preparation of RNA probes: *in vitro* transcription

All restriction enzymes used were obtained from Promega. A 240 bp *PstI* fragment from maize vacuolar invertase (*IVR2*, GenBank accession no. U31451) cDNA was subcloned into the corresponding sites of the *pSPT18* vector. A 303 bp *HindIII* fragment from sugarcane neutral invertase (*pESNI 510*, cloned from *Lolium temulentum* neutral invertase, Genbank accession no. AJ003114) cDNA was subcloned into the *pSPT19* vector. Lastly, a 400 bp *HindIII* fragment from sugarcane cell wall invertase (*Shcw1*, Genbank accession no. AY302084) cDNA was subcloned into the *pSPT19* vector. The plasmids were linearised, and then purified using phenol/chloroform extraction and ethanol precipitation. Digoxigenin (DIG)-labelled antisense and sense RNA probes were next synthesised by an *in vitro* transcription reaction using the DIG-RNA Labelling Kit

(Boehringer Mannheim): 1 μg of purified plasmid DNA was mixed with 2 μl 10x concentrated DIG RNA labelling mix, 2 μl 10x transcription buffer (Boehringer Mannheim), 2 μl T7 or SP6 RNA Polymerase (Boehringer Mannheim), and made up to a final volume of 20 μl. The mixture was incubated for 2 h at 37°C, followed by addition of 2 μl 0.2 M EDTA (pH 8.0) to stop the polymerase reaction. The labelled RNA transcript was precipitated for 2 h at -20°C using 4 M LiCl and ice-cold absolute ethanol. RNA was recovered by centrifugation at 13,000 g for 15 min at 4°C, followed by washing in chilled 70 % ethanol and centrifugation at 13,000 g for 5 min. The pellet was briefly dried and resuspended in DEPC-treated water, and stored in aliquots at -80°C. The size and integrity of the probes were checked electrophoretically on agarose gels, while the level of label incorporation into the probes was determined using DIG-Quantification Teststrips (Boehringer Mannheim) according to the manufacturer's instructions.

4.2.5 Tissue preparation and RNA *in situ* hybridization on stem tissues

Cylinders of tissue 10 mm in diameter were bored from internodes 3, 6 and 13. These internodes were chosen as representatives of young, maturing and mature stem tissue, respectively (Botha and Black, 2000). The tissue cylinders included both core and peripheral tissue. Sections, 0.5mm thick, were cut using a hand microtome and immediately dropped into 4% paraformaldehyde, and fixed for 48 h at 4°C. Following fixation sections were briefly rinsed in distilled water, and then placed into 0.2 M HCl for 10 min. After rinsing in distilled water and phosphate-buffered saline (PBS), the tissue was incubated in Pronase (0.125 mg ml^{-1} predigested Pronase in Pronase buffer) for 15 min. Pronase activity was stopped with 0.2 % glycine, and sections were then re-fixed in 4 % paraformaldehyde for 10 min. The fixative was rinsed off with PBS, and the tissue was then acetylated (1 % acetic anhydride in triethanolamine, pH 8.0). Following a final rinse in PBS, sections were placed onto marked slides that had been washed in Rnase-Away solution (Molecular BioProducts). Each section was covered with 50 μL hybridisation mix containing 500 ng ml^{-1} probe in hybridisation buffer (Sigma), and incubated for 16 h at the appropriate temperatures for neutral invertase and cell wall invertase probes (50°C), and 55°C for the acid invertase probe (see chapter 3). Sections

were then washed in wash buffer (2x SSC, 50 % formamide) for 60 min at 50°C, followed by 2 washes of 5 min each in NTE at 37°C. The tissue was then incubated in NTE with 20µg.ml^{-1} RNaseA at 37°C for 30 min, followed by 2 washes of 5 min each in NTE at room temperature. Sections were washed again in wash buffer for 60 min at 50°C, followed with a brief rinse in PBS and 5 min equilibration in 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (Buffer 1). Sections were blocked in 1 % (m/v) blocking reagent (Roche) in Buffer 1 at 4°C overnight, and then incubated with anti-digoxygenin-alkaline-phosphatase conjugate diluted 1:3000 in the blocking solution at room temperature for 90 min. Sections were washed four times for 20 min in Buffer 1 containing 0.3 % Triton X-100, then once for 5 min in Buffer 1 and equilibrated for 5 min in Buffer 4 (100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$). Detection took place in 1.5 µl.ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) and 1.5 µl.ml^{-1} nitro blue tetrazolium chloride (NBT) in Buffer 4 containing 5 % (m/v) polyvinyl alcohol, and progression of the colour reaction was terminated by washing the tissue in PBS containing 0.1 % Tween-20 and 20 mM EDTA. Alkaline phosphatase produces an NBT-formazan precipitate which stains the cells containing the mRNA of interest, and the precipitate is viewed as a blue/purple stain under bright field illumination. Sections were viewed and photodocumented using bright field microscopy at 100x magnification on a Nikon Eclipse E400 microscope.

4.3 Results

4.3.1 *In situ* hybridisation

Presence of transcript in tissue hybridised with anti-sense probe was defined by presence of a blue-purple precipitate, visible as a blue-purple staining under the light microscope. Tissue hybridised with sense probe showed no such colouration, and acted as a control. Due to the large vacuoles present in storage parenchyma, many of the cells appear "empty", since the cytoplasm is pressed against the cell wall.

Results of *in situ* hybridisation revealed similar distribution patterns for the three invertase isoforms. Transcript for acid, cell wall and neutral invertase was present in the storage parenchyma and the parenchyma contiguous to the vascular bundle, as well as in the phloem tissue (Figures 4.1 to 4.3). There appeared to be transcript present in the vascular parenchyma, although the thickness of the sections made this difficult to discern properly. Some blue-purple colouration was evident in the xylem vessels, however this is due to non-specific binding of the probe. The observed expression pattern was consistent for all culm developmental stages, as well as for both core and peripheral tissues. Abundance of transcript for all three isoforms appeared to decrease as tissue matured due to weaker colour intensity, however, due to the non-radioactive nature of the signal this could not be quantitatively verified. No acid invertase transcript was detectable in maturing or mature tissue (Figure 4.1 i-l).

4.3.2 Northern blot analysis

Developmental expression patterns of the three invertase isoforms were determined using Northern blot analysis. A weak signal for acid invertase in young tissue suggested low transcript levels in this tissue (Figure 4.4 a). Acid invertase transcript levels decreased sharply between internodes 3 and 6 (Figure 4.4 c) and thereafter remained undetectable. Transcript levels for cell wall invertase gradually decreased with increasing tissue maturity (Figure 4.5), with the largest drop occurring between internodes 3 and 6. A sharp drop in transcript between internodes 3 and 6 was also observed for neutral invertase, however transcript levels appeared to increase again between internodes 10 and 13 (Figure 4.6 c).

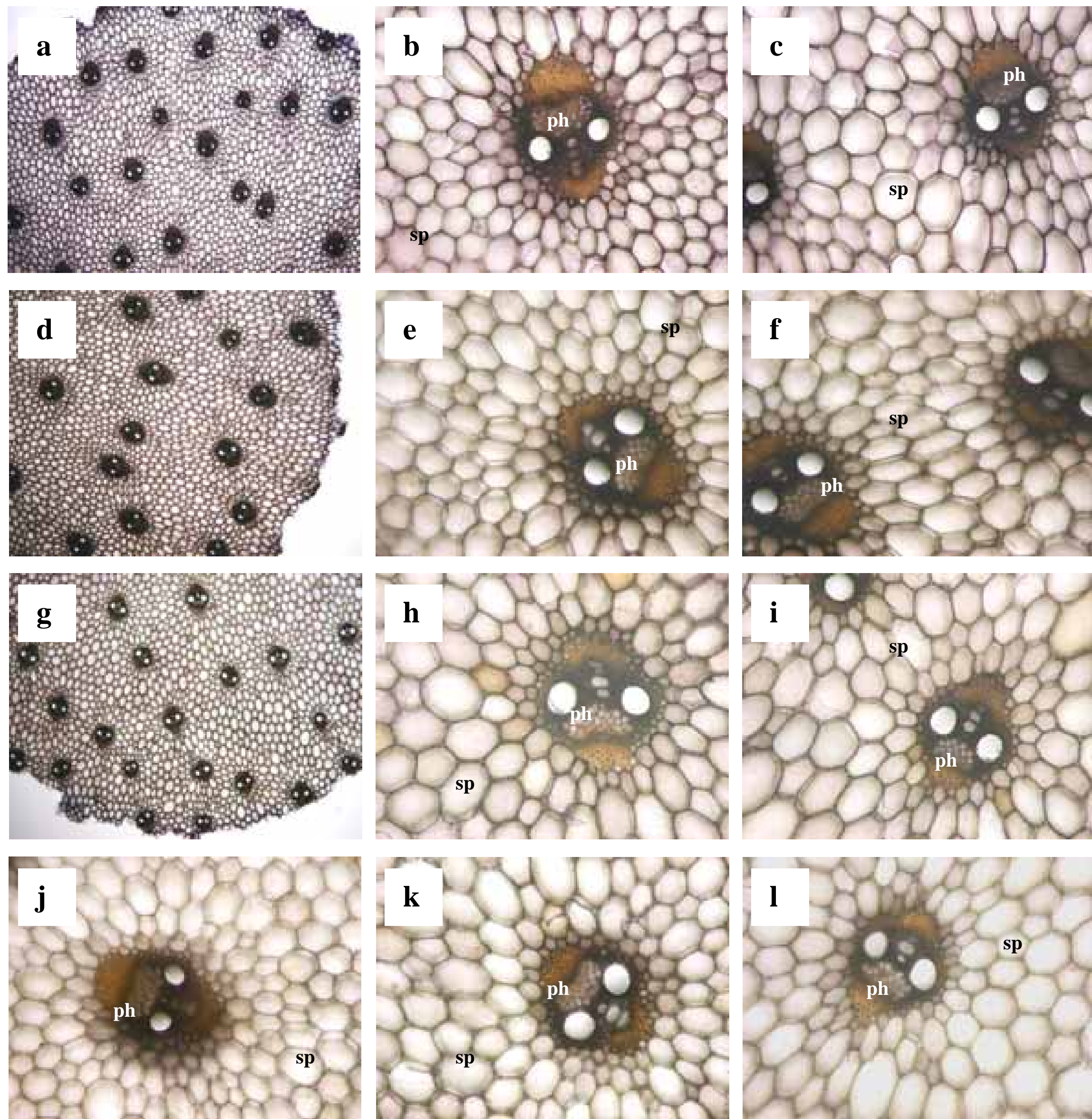

Figure 4.1 Distribution of acid invertase mRNA in young (internode 3, a-c), maturing (internode 6, d-f) and mature (internode 13, g-i) sugarcane culm. Young, maturing and mature tissue hybridised with sense probe showed no blue-purple precipitate (j, k and l respectively); b, e and h represent core tissue; c, f and i represent peripheral tissue; ph, phloem; sp, storage parenchyma; a, d and g, 40x; b, c, e, f, h -l, 100x.

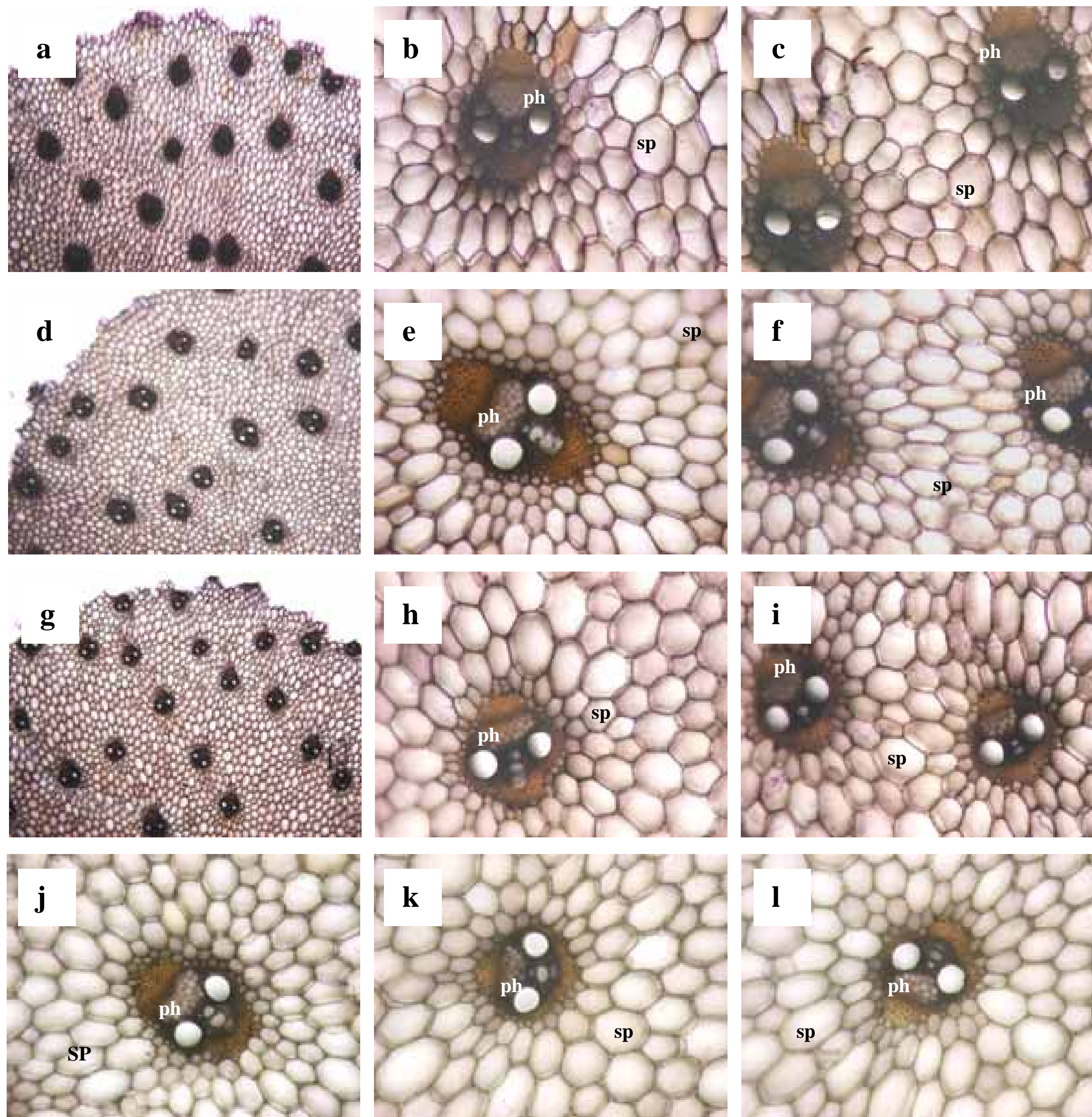

Figure 4.2 Distribution of cell wall invertase mRNA in young (internode 3, a-c), maturing (internode 6, d-f) and mature (internode 13, g-i) sugarcane culm. Young, maturing and mature tissue hybridised with sense probe showed no blue-purple precipitate (j, k and l respectively); b, e and h represent core tissue; c, f and i represent peripheral tissue; ph, phloem; sp, storage parenchyma; a, d and g, 40x; b, c, e, f, h -l, 100x.

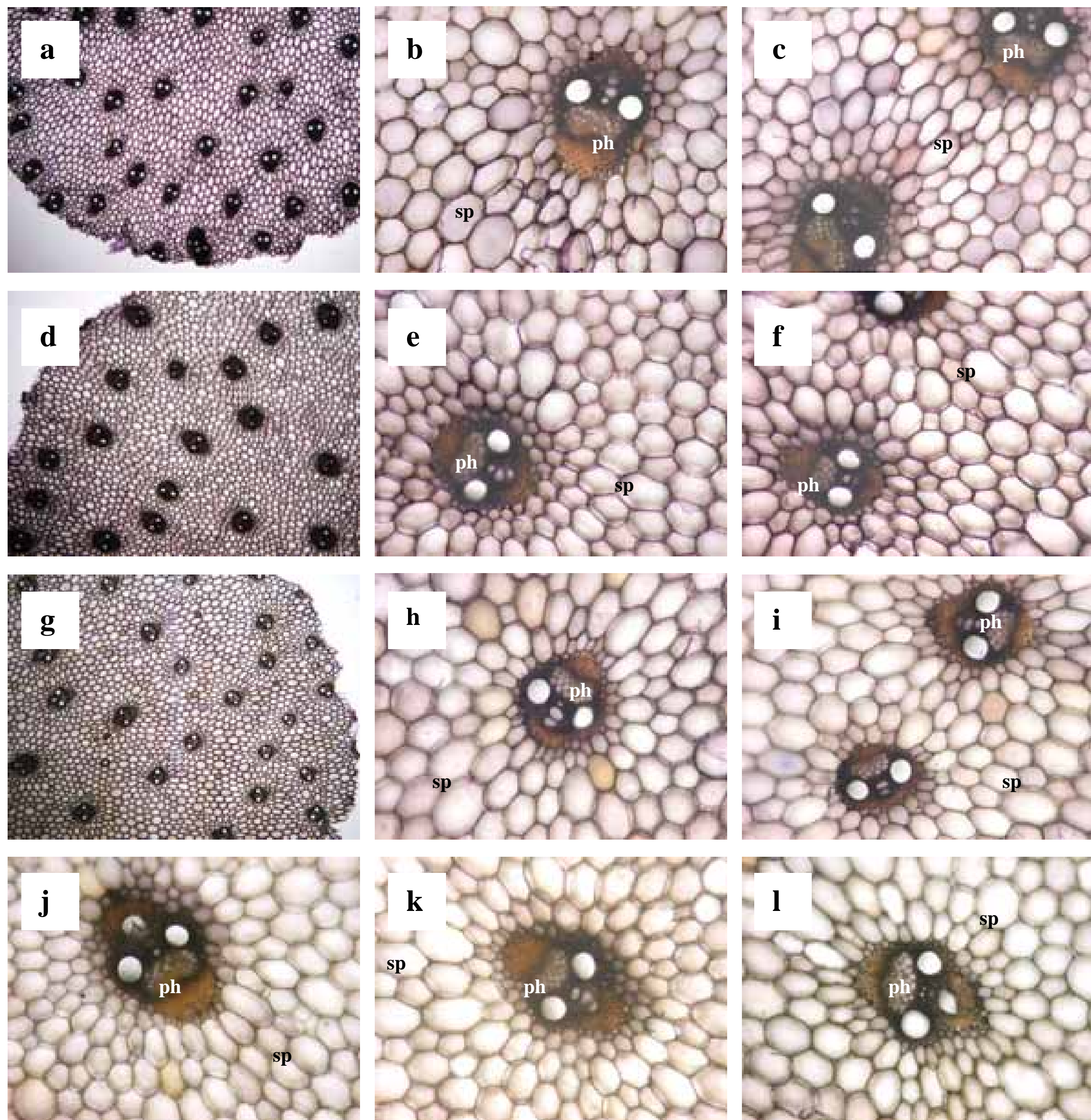

Figure 4.3 Distribution of neutral invertase mRNA in young (internode 3, a-c), maturing (internode 6, d-f) and mature (internode 13, g-i) sugarcane culm. Young, maturing and mature tissue hybridised with sense probe showed no blue-purple precipitate (j, k and l respectively); b, e and h represent core tissue; c, f and i represent peripheral tissue; ph, phloem; sp, storage parenchyma; a, d and g, 40x; b, c, e, f, h -l, 100x.

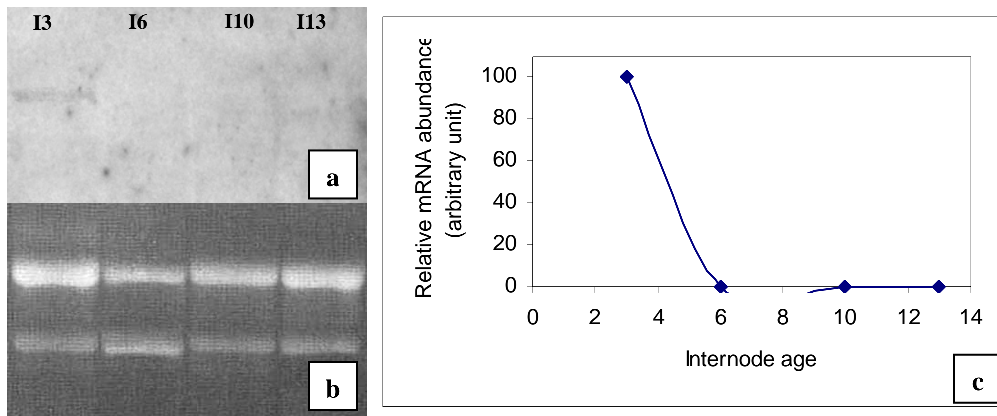

Figure 4.4 RNA blot analysis of acid invertase expression in internodes 3, 6, 10 and 13 (a), with corresponding gel of total RNA loaded (b). Signal intensity quantified and expressed as an arbitrary unit of relative RNA in the different internodes (c).

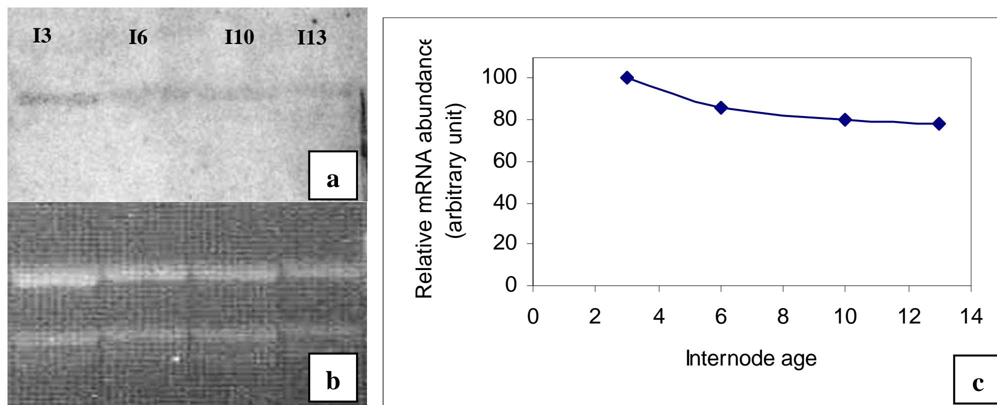

Figure 4.5 RNA blot analysis of cell wall invertase expression in internodes 3, 6, 10 and 13 (a), with corresponding gel of total RNA loaded (b). Signal intensity quantified and expressed as an arbitrary unit of relative RNA in the different internodes (c).

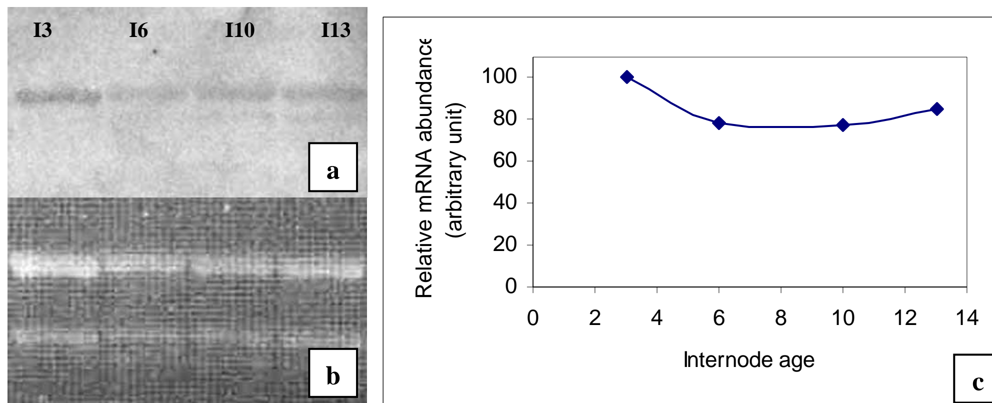

Figure 4.6 RNA blot analysis of neutral invertase expression in internodes 3, 6, 10 and 13 (a), with corresponding gel of total RNA loaded (b). Signal intensity quantified and expressed as an arbitrary unit of relative RNA in the different internodes (c).

4.4 Discussion

The results presented here show that the sugarcane invertase isoforms have very similar tissue specificity, and display only subtle differences in developmental expression patterns. Transcript for soluble acid invertase was present in immature tissue but dropped below detectable levels as the tissue matured, which was evident from Northern blot analysis. This trend is similar to previous work on soluble acid invertase transcript levels (Zhu *et al*., 2000) and activity levels (Vorster and Botha, 1999) in sugarcane, although certain varieties seem to maintain significant amounts of invertase even in mature tissue (Vorster and Botha, 1999). In such cases acid invertase could be involved in a cycle of synthesis and degradation (Sacher *et al*., 1963), although this raises the question of whether such cycling occurs in tissue with very low levels of acid invertase, such as that of the variety used in the current study. The observed decrease in acid invertase transcript could ultimately contribute to the increased sucrose accumulation rate typical of maturing tissue (Whittaker and Botha, 1997). Previous work has shown that in variety N19, increased accumulation seems to be achieved by increased synthesis of sucrose, since a highly significant correlation exists between SPS activity (specific and total) and sucrose accumulation rate (Botha and Black, 2000). The results of the current study in fact suggest that sucrose accumulation in this variety might be achieved by a combination of this and reduced sucrose breakdown, due to the decreased levels of acid invertase observed.

Acid invertase mRNA was present in the storage parenchyma and in the phloem tissue of immature tissue. This is in contrast to acid invertase in grape berries (Famiani *et al*., 2000), cucumber petioles (Kingston-Smith *et al*., 1999) and barley leaves (Kingston-Smith and Pollock, 1996), where acid invertase mRNA was localised to the vascular regions alone. However, differences in localisation between species are not surprising in light of the fact that even within a single species there are likely to be genetically different forms of source organ invertase and sink organ invertase, with distinct biochemical differences (Quick and Schaffer, 1996). Elongation of the sugarcane stem as a whole is mostly achieved by the immature tissue, where cells are actively dividing and expanding (Jacobsen *et al*., 1992). This metabolically intensive phase would require

cleavage of sucrose so that the products could be directed into respiration and other energy-requiring processes. The results of this study show that acid invertase is almost ubiquitous in the immature sugarcane stem tissue, and this coupled with the fact that it declines to undetectable levels in fully elongated internodes would suggest that acid invertase serves to provide energy for metabolism during elongation. This is in agreement with previous work, in which internode elongation rate was correlated with acid invertase activity (Lingle, 1999).

Cell wall invertase transcript, like that of soluble acid invertase, was found to be present in the storage parenchyma and the phloem tissue, and also decreased as tissue matured. This trend is similar to previous work which showed cell wall invertase activity to decrease with increasing tissue age (Vorster and Botha, 1999). However, the magnitude of the observed decrease appeared small, and appreciable levels of cell wall transcript were still detectable in fully mature tissue. This persistence throughout development, combined with the presence of transcript in the phloem tissue, would suggest a function in phloem unloading. Sucrose leaked from the sieve tubes would presumably be cleaved by cell wall invertase in the apoplast, and the resulting hexose sugars taken up by the vascular parenchyma surrounding the phloem tissue. Putative hexose transporters have been identified in sugarcane, and have been localised to the companion cells and vascular parenchyma (Casu *et al*., 2003). Cleavage of sucrose in the apoplast would also increase partitioning to storage by steepening the gradient between source and sink. This has been previously suggested, although increased sucrose unloading was proposed to occur by increasing the levels of sugar transport proteins in the plasma membrane (Walsh *et al*., 1996). Whether it is cell wall invertase or the putative transporters, or both, that control sucrose unloading requires further investigation.

Storage parenchyma of sugarcane accumulate molar concentrations of sugars during maturation, and the potentially high osmotic pressure of such a build-up would appear to be kept low by an accumulation of solutes in the apoplast (Moore and Cosgrove, 1991). The observed ubiquitous expression of cell wall invertase in the sugarcane parenchyma of the present study, added to the fact that it is present in maturing and fully mature tissues,

suggests it could play some role in regulating turgor. This would presumably be achieved by cleavage of sucrose unloaded into the apoplasm, which in turn would draw more sucrose into the extracellular space. Apart from reducing the potentially injurious osmotic pressure within storage parenchyma, unloading to the apoplast of the storage cells would also contribute to the overall pull of assimilate into the sink tissue.

Neutral invertase displayed a similar expression pattern to acid and cell wall invertase, in that it was present in the storage parenchyma and phloem tissue. Transcript levels were highest in young tissue and declined during tissue maturation, with the exception of fully mature tissue in which transcript levels appeared to increase again. This is in contrast to a previous study, in which neutral invertase activity in both leaves and stem of sugarcane showed relatively little variation between tissues of differing age (Albertson *et al*., 2001). However, it is similar to other work on the same sugarcane variety as used in the present study, in which neutral invertase activity was shown to decrease as tissue matured (Rose and Botha, 2000).

It is interesting to note that neutral invertase levels in this study appear to be lowest during the period of reportedly highest sucrose accumulation (Botha and Black, 2000). However, previous work has consistently failed to find a correlation between neutral invertase activity and sucrose accumulation (Zhu *et al*., 1997; Ebrahim *et al*., 1998; Rose and Botha, 2000). Invertase-mediated turnover of sucrose has been proposed to occur at all stages of maturation, and based on extractable activities and labelling studies it has been suggested that neutral invertase will significantly contribute to this turnover (Vorster and Botha, 1999). While the results of the current study do not confirm or refute this theory, the observed widespread expression of neutral invertase throughout the stem tissue suggests that it is not impossible. Alternatively, neutral invertase could function to channel sugars into respiration, since total cellular respiration increases with increasing sucrose accumulation (Botha *et al*., 1996). A third possibility is that neutral invertase might be involved in controlling cellular sugar levels, the results of which would affect the expression of sugar-responsive genes and lead to different physiological responses (Sturm and Tang, 1999). This might explain why past studies have failed to find any

definite correlations between neutral invertase and sucrose accumulation in sugarcane: perhaps neutral invertase does significantly affect sucrose accumulation, albeit indirectly through complex sugar signalling pathways.

The results of this study reveal a lack of variation in tissue specificity between the invertase isoforms, and this makes it difficult to define any precise functional relationships. In particular, the role of neutral invertase remains frustratingly unclear. Despite this, the results presented do add weight to some of the functions previously suggested for acid and cell wall invertase. Acid invertase would appear to provide energy for internode elongation in immature tissue, and may even be a minor determinant of sucrose accumulation in this specific variety. Cell wall invertase would seem to be responsible for turgor maintenance in the storage tissue and, more importantly, would appear to be involved in phloem unloading. This has significant implications for future work, as this enzyme could potentially represent a point of control for sucrose accumulation.

CHAPTER 5

General discussion and future directions

A thorough understanding of sucrose accumulation in sugarcane requires knowledge of all enzymes potentially involved, particularly the sucrolytic enzymes. This includes knowledge of their biochemistry, kinetics, regulation, and localisation within the plant. The latter of these has been neglected in the past, and in fact to date there is not a single report of the *in situ* localisation of any sucrolytic enzymes in sugarcane (see chapter 2). While extraction studies have helped elucidate the subcellular locations of enzymes based on pH optima, such methods have failed to indicate whether such enzymes are specifically expressed in the storage or vascular tissue, or indeed both. In an effort to resolve this, the current project had two objectives: firstly, to develop an *in situ* hybridisation technique that could be used to investigate gene expression in sugarcane culm tissue, and secondly, to use this technique to identify the tissue-specific expression patterns of three major invertase isoforms at a variety of culm developmental stages.

Although *in situ* hybridisation has been routinely used for gene expression studies in plants for a number of years now (Burgess, 1992; Busch *et al*., 1994; Engler *et al*., 1998), the technique has only recently been applied to sugarcane tissue (Casu *et al*., 2003). However, the method used by those authors only allowed visualisation of gene expression in younger tissue, and clearly a technique which can be applied to any developmental stage would be advantageous. The technique developed in this study (chapter 3) can be applied to culm tissue of any age, and in addition it is quick and non-hazardous. Yet as with all preparative techniques, this method is a compromise between a number of factors. The use of thick hand-cut sections, as opposed to thin sections cut from wax-embedded tissue, allows the method to be applied to young and old tissue alike. However, this does somewhat reduce the resolution of the final image. The technique described in this study is quick due to the preclusion of prolonged wax infiltration and sectioning, but the drawback is that fixed tissue must be used immediately and cannot be stored. Finally, the technique is not hazardous due to the non-radioactive

nature of the probe, but the disadvantage is that the hybridisation signal cannot be quantified. While it is tempting to conclude that the technique requires further development, it is perhaps wiser to conclude that the development of a particular technique depends on the desired outcomes. The method developed here was suited to the second objective of the project because it provided a qualitative view of invertase expression. If, however, it had been necessary to evaluate expression quantitatively in order to compare it with expression in another variety, then it might have been more appropriate to develop a method using radioactive probes.

While *in situ* hybridization techniques have obvious advantages, they do not give unequivocal evidence that a gene is transcribed into protein, since post-transcriptional regulation might occur (Sergeeva and Vreugdenhil, 2002). Localization of proteins using immunocytochemistry would seem the next logical step towards understanding invertase expression in sugarcane. However, once again the presence of a protein does not necessarily prove its activity *in situ*, since it might be activated or inactivated after translation. Sergeeva and Vreugdenhil (2002) describe a potent method for visualizing enzyme activity *in situ*. This method involves the coupling of NAD reduction to the reduction of NBT, which results in a blue precipitate forming at the site of enzyme activity. The authors used this method to localize activity of a number of enzymes in various tissues, among them being invertase activity in potato stems. The one major drawback to this method is that it fails to differentiate between the invertase isoforms, and so future studies should aim to find a way to couple the precipitate-forming reaction specifically to the different isoforms. This method would be able to show precisely where and when a particular invertase-mediated reaction occurs. Another future consideration would be the use of radioactive *in situ* hybridization methods. While these methods may be more hazardous and time-consuming than non-radioactive methods, they do possess the advantage of allowing signal quantification by means of densiometric analysis of the autoradiographs (Wilkinson, 1998).

Using the *in situ* hybridisation technique developed in chapter 3, expression patterns of acid, cell wall and neutral invertase were investigated in young, maturing and mature

sugarcane internodes (chapter 4). The results revealed that transcript of all three isoforms was present in the storage parenchyma, as well as in the phloem tissue. While cell wall and neutral invertase appeared to be constitutively expressed in all aged tissues, acid invertase transcript was only detectable in young, actively-growing tissue. This, together with previous work in which acid invertase activity was correlated with internode elongation rate (Lingle, 1999), suggests that acid invertase is a major factor in internode elongation. Whether it is specifically the down-regulation of acid invertase that signals the cessation of elongation, or vice versa, is not yet known. The results also suggest that once moved into the vacuole the sucrose would be stable. However, this means that remobilisation of sucrose from the vacuole would then be dependent on either induction of soluble acid invertase or the presence of a symporter (H^+ and sucrose) in the tonoplast.

Perhaps most interesting of all the results is the presence of cell wall invertase in the phloem tissue at all developmental stages, which would imply a crucial role in phloem unloading. However, there is evidence that phloem unloading follows a symplastic route in sugarcane stalk tissue, as a result of intense lignification of almost all of the stem tissue (Welbaum *et al*., 1992; Jacobsen *et al*., 1992; Walsh *et al*., 1996). So what is the purpose of cell wall invertase in the phloem apoplast? The localisation of putative sugar transporters in companion cells (Casu *et al*., 2003) suggests at least some sugar transfer occurs between the symplasm and apoplasm of the vascular tissue. Perhaps the action of cell wall invertase here represents an overflow mechanism: in the presence of a bottleneck at the plasmodesmata, excess sucrose could be unloaded via the sugar transporters to the apoplast, where cleavage by cell wall invertase would ensure maintenance of the sucrose gradient. Apart from preventing a back-up in the unloading process, such partitioning to and cleavage in the apoplast could be a way of avoiding large and potentially harmful fluctuations in osmotic pressure. So cell wall invertase does indeed appear to play an important role in phloem unloading, albeit an indirect one.

Neutral invertase has previously been suggested to significantly contribute to sucrose cycling (Rose and Botha, 2000), and its homogenous expression observed in the parenchyma in the present study, added to its presence throughout development, would

seem to support this. The results of this study, together with previous findings that sugars are present in appreciable amounts in both the cytoplasm and the apoplast (Moore and Cosgrove, 1991; Welbaum *et al*., 1992), would also suggest that sugarcane cell wall invertase is involved in turgor maintenance by exerting a continuous pull on sucrose into the apoplastic space. Since this would be an on-going process, with sugars entering and leaving the apoplast, it could theoretically be considered as another form of sucrose cycling. Originally thought of as wasteful of energy, it is now recognised that such cycling allows plants to respond in a highly sensitive manner to small changes in the supply-demand balance of carbohydrate metabolism (Moore and Maretzki, 1996). Hence it is possible that cell wall and neutral invertase represent crucial control points in sucrose metabolism. This is further supported by sugar-sensing research, which shows that both glucose and sucrose can modulate gene expression (Koch, 1996; Chiou and Bush, 1998; Roitsch, 1999). Since both the substrate and products of these invertases represent pivotal signalling molecules, it is evident how their activity can have far-reaching effects on metabolism. Dissecting the exact mechanisms involved, however, remains elusive.

The interpretation of gene expression patterns relies increasingly on localizing expression *in situ*. This is not to say that traditional methods involving extraction and assays *in vitro* will become obsolete; rather, these methods should be used in conjunction with *in situ* methods to obtain a holistic picture of gene expression. This study has shown the value of *in situ* hybridization, yet at the same time has demonstrated its limitations. While it does not completely clarify the functions of the invertase isoforms in sugarcane, it has paved the way for tissue-specific promoter design and future *in situ* studies of the sugarcane invertases.

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