SUCROSE TRANSPORTERS AND SUCROSE UPTAKE MECHANISMS IN SUGARCANE

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 part submitted at any university for a degree.

______________________ ________________

Charlene H A Titus Date

To my parents

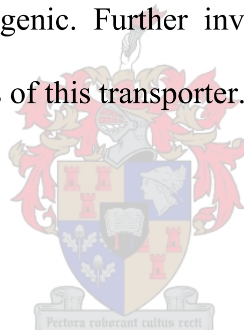
ABSTRACT

The process of sugar accumulation and transport in sugarcane is still poorly understood. Understanding the processes involved in sucrose transport are important, since membrane transport might be important control points in this pathway. The goals of this project were to unravel the mechanisms of sugar transport in sugarcane culm tissue by using ^{14}C-sugar analysis as well as molecular techniques to identify possible sucrose transporters.

Developing (internode 2 and 4) and maturing (internode 8 and 15) culm tissue of sugarcane (*Saccharum* hybrid) commercial variety N19 was used for all tissue disc experiments. Tissue discs from internodes of different developmental stages were cut from field grown sugarcane plants (cv. N19) and the uptake of ^{14}C-labelled glucose, fructose and sucrose measured. The uptake rates were measured at varying pH, temperature and concentrations of sugars. Hexoses were found to be the major sugar taken up and sucrose was only important when little hexose was available, as was found in the mature ripe internodes. Sucrose uptake differs between tissues and our study showed that sucrose was taken up rapidly at pH 5, similar to the pH optimum of most sucrose transporters Inhibition studies with TRIS (2-amino-2- (hydroxymethyl)-1,3-propanediol) and PCMBS (*p*-chloromercuribenzenesulphonic acid) indicated that more than one sucrose transporter activity may be present in the sugarcane system at different sucrose concentrations.

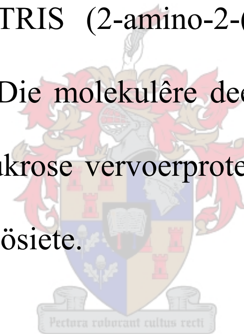
To date work on sugarcane sucrose transporter expression on DNA and RNA level has been limited. Only recently a sucrose transporter from *Saccharum* hybrid sugarcane stem cDNA libray, ShSUT1 (*S*accharum hybrid Sucrose Transporter) was isolated and functionally characterized in the yeast strain SEY 6210 (Rae *et al.,* 2004). In an effort to understand sucrose transport in sugarcane culm tissue, a partial sucrose transporter cDNA, ScSUT1(p) from *Saccharum* hybrid sugarcane a bud cDNA library was isolated, and cloned from a bud cDNA library. The clone was designated ScSUT(p) as a partial

Sugarcane Sucrose Transporter. The ScSUT1(p) sequence showed 94% identity to ShSUT1 on nucleotide level over 1258 nucleotides and had an estimated open reading frame of 419 amino acids. Southern blot analysis indicated that the transporter had a low copy number and the ScSUT1(p) transcript expression was constitutive in sucrose accumulating and sucrose storing stem tissue, but was less abundant in immature tissue such as internodes 2 and 3 and in lateral buds. It was concluded that the primary function of ScSUT1(p), was not phloem unloading but that the transporter may be involved in phloem loading, as it is abundant in mature source leaves. ShSUT1 cDNA was obtained from Dr C Grof and the functionality of ShSUT1 as a sucrose transporter in *Xenopus leavis* oocytes was confirmed. However, electrophysiological measurements on the oocytes demonstrated no measurable current associated with sucrose challenge to the oocytes indicating that the transporter activity was either very low or possibly non-electrogenic. Further investigation is required to characterise the specific mechanism and kinetic properties of this transporter.



OPSOMMING

Die proses van suikerakkumulering en -vervoer in suikerriet word steeds baie vaag verstaan. 'n Deeglike begrip van die prosessewat betrokke is in die vervoer van sukrose is baie belangrik omdat transmembraan vervoer moontlik een van die belangrike beheerpunte in metabolisme mag wees. Die doelwitte van die studie was om 'n beter begrip te bekom van die meganisme wat betrokke is by die vervoer en berging van sukrose in suikerriet. Die projek is in 'n fisiologiese en 'n molekulêre afdeling verdeel. In die fisiologiese afdeling is stingelweefsel van 'n *Saccharum* hybried (variëteit N19) van verskillende stadiums van ontwikkeling (internodes 2-4, internode 8 en internode 15) gebruik. Opname van radioaktiewe (^{14}C) sukrose, glukose en fruktose is as analise metode gebruik vir die suikeropname eksperimente. Die invloed van pH, suiker konsentrasie en inhibitore soos PCMBS (p-chloromercuriphenylsulfonic acid) en TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) op die tempo van suikeropname is ondersoek. Die molekulêre deel fokus hoofsaaklik op die identifisering, isolering en karakterisering van nuwe sukrose vervoerproteine in suikerriet, met behulp van PCR en heteroloë uitdrukking in *Xenopus laevis* oösiete.



Die ^{14}C - opname eksperimente het tot die volgende gevolgtrekkings gelei: Heksoses speel die belangrikste rol in die vervoer van suiker in die riet as daar min of geen sukrose teenwoordig is nie. Sodra daar sukrose in groot mate teenwoordig is soos in die geval van ontwikkelde, ryp internodes, is die rol van sukrose egter belangriker. Sukrose is die maklikste opgeneem by pH 5, wat naby die pH optimum van die meeste sukrose vervoerproteïene is. TRIS en PCMBS het beide 'n inhiberende effek op sukrose opname gehad, maar die invloed was groter by die laer sukrose konsentrasies.

Tot onlangs was daar baie min inligting oor sukrose vervoer in suikerriet op DNA en RNA vlak. Die eerste sukrose vervoerprotein uit suikerriet, ShSUT1 (*Saccharum* Hibried Sukrose Transporter) is eers onlangs uit 'n stingel - cDNA biblioteek geïsoleer (Rae *et al*., 2004) en die funksionering daarvan is in

‘n gisras (SEY6210) getoets. In my pogings om sukrose vervoer te verstaan is ‘n gedeeltelike cDNA, naamlik ScSUT(p) (partial Sugarcane Sucrose Transporter) van 1258 nukleotiede, uit cDNA afkomstig van suikerrietbotsel geïsoleer. Die nukleotiedvolgorde stem 94% ooreen met ShSUT1 en kodeer vir ‘n moontlike oopleesraam van 419 aminosure. Southern analises het aangedui dat ScSUT(p) ‘n lae kopie getal het, in ooreenstemming met wat vir ander sukrose vervoerproteïene gevind is. Northern analises het getoon dat die uitdrukking van ScSUT(p) konstitutatief is in sukrose akkumulerende sowel as sukrose bergingsweefsel. Jong weefsel (internode 2 en 3) het baie lae uitdrukking getoon, met die hoogste uitdrukking in blaarweefsel. Uit die resultate is afgelei dat ScSUT(p) ‘n rol in floeëmlading en -ontlading mag speel.

Xenopus laevis oösiete, is as ‘n heteroloë uitdrukking sisteem gebruik om te bevestig dat ShSUT1 as ‘n sukrose vervoerproteïen funksioneer. Elektrofisiologie het nie daarin geslaag om ShSUT1 se spesifieke werkingsmeganisme te identifiseer nie. Aanduidings is egter gevind dat ShSUT1 moontlik nie as ‘n H^+/sukrose simportsisteem werk nie, maar by gefasilliteerde vervoer van sukrose betrokke mag wees. Verdere navorsing is noodsaaklik om die meganisme van ShSUT1 se werking te verstaan.

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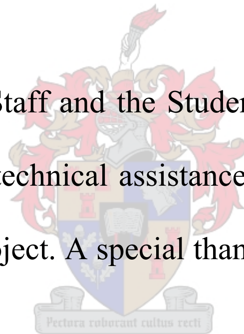

- To all my friends especially **Carlo, Bernadette** and **Charmell** for listening to all my “stories”, thank you for your support, understanding and encouragement while completing my studies.

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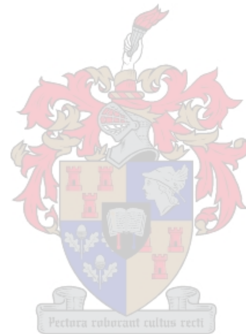

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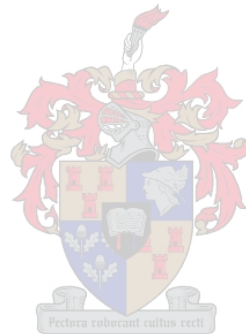

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
bp	nucleic acid base pair
cDNA	complementary deoxyribonucleic acid
^{14}C	radiolabelled carbon
CWI	Cell wall acid invertase
ddH_20	double distelled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribo nucleic acid
DTT	1,4-dithiothreitol
EDTA	ethylenediamminetetraacetic acid
Excl.	excluding
FW	fresh weight
x g	gravitational force
G6PDH	glucose-6-phosphate dehydrogenase (EC1.1.1.49)
gDNA	genomic DNA
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanasulphonic acid
HK	hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1)
K_m	substrate concentration producing half maximum velocity
MES	2-[N-morpholino] ethanesulfonic acid
NAD^+	oxidised nicotinamide adenine dinucleotide
NADP	reduced nicotinamide adenine phosphate dinucleotide
NI	neutral invertase (β-fructofuranosidase, EC 3.2.1.26)
PCMBS	*p*-chloromercuriphenylsulfonic acid

RNA	ribonucleic acid
RnaseA	ribonuclease A
rpm	revolutions per minute
SAI	soluble acid invertase (β-fructofuranosidae, EC3.2.1.26)
SDS	sodium dodecyl suphate
SE	standard error
SPS	sucrose phosphate synthase (UDP-glucose:D-fructose-6-P 2-α-D-glucotransferase, EC 2.4.1.14)
20 X SSC	3 M NaCl, 300 mM $Na_3C_6H_5O_7$ (pH 7.0)
Suc	sucrose
SUC	sucrose carrier
SuSy	sucrose synthase (UDP-glucose:D-fructose 2-α-D-glucosy;-transferase, EC 2.4.1.13)
SUT	sucrose transporter
TBE	tris borate/EDTA electrophoreis buffer
TE	tris/EDTA buffer
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol)
UDP	uridine 5'diphosphate
UV	ultra violet
v	volume
V	Volt
Vmax	Maximum velocity
w	weight

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CHAPTER 1

General Introduction

The South African Sugar Industry is one of the leading cost competitive producers of sugar. It is a diverse industry combining the agricultural activities of sugarcane cultivation with the industrial factory production of molasses, raw and refined sugar (www.sasa.org.za). The industry generates both direct income and employment in the regions within which it operates and indirect economic activity because of the many backward linkages that exist between the sugar sector and the core businesses that supply the sugar industry (www.sasa.org.za). Based on revenue generated through sugar sales on the local market, it is estimated that the industry contributed R2 billion to the country's foreign exchange earnings in 2001-2002. Employment within the sugar industry amounts to approximately 85 000 jobs. Direct and indirect employment is estimated at 350 000 people with approximately one million people dependant on the sugar industry. In addition there are more than 50 000 registered cane growers comprised of approximately 2 000 large-scale farmers, farming freehold property, and approximately 48 000 small-scale (South African Sugar Association Annual report 2001/2002). From the above analysis it is evident that the sugar industry is socio-economically important in South Africa.

Sucrose is the most abundant low-molecular weight carbohydrate in higher plants (Hart *et al*., 1963; Hawker, 1985; Komor, 2000a). Together with starch it is the dominant assimilation product in leaves. Sucrose has five fundamental and interrelated roles in plants: 1) It is a principal product of photosynthesis and can account for most of the CO_2 absorbed by the plant during photosynthesis (Singh and Malhotra, 2000; Kruger, 1997); 2) Sucrose is the major compound translocated in the phloem to the non-photosynthetic and storage tissue (Komor, 2000b); 3) Sucrose is a common storage compound in many plants although usually at lower storage density compared to starch

(Moore, 1995); 4) It is an important metabolic substrate (Kruger, 1997); 5) Is also important in metabolic signalling (Lalonde *et a*l., 1999).

Commercial varieties of sugarcane (interspecific hybrids derived from crosses between *Saccharum officinarum* L. and *S. spontaneum* L.) are unusual because, in comparison to other plants, they have high sucrose concentrations in the culm and a virtual absence of starch. This has been the reason for the economic value of sugarcane for more than two thousand years. Furthermore, sugarcane is of interest for agronomists and plant physiologists because it is suitable for the study of the mechanisms involved in the regulation of sucrose accumulation and storage.

Higher plants represent a functional network separating different tasks to different organs. Mature leaves provide photo-assimilates and act as source tissue. These photo-assimilates are exported in the form of sucrose to sink organs such as roots, fruits, flowers, stems and developing leaves (Hellman *et al*., 2000). Sucrose does not only function as a transport metabolite but also contributes to the osmotic driving force for phloem translocation and serves as a signal to activate or repress specific genes in different tissues (Koch *et al.,* 1992; Koch, 1996). The transporters involved in the allocation of assimilates include both sucrose and hexose transporter families (Hellman *et al*., 2000). The long distance transport of sucrose and hexose in the phloem is mediated by a family of proteins that function as transmembrane sucrose carriers (Büttner and Sauer, 2000). Research into sucrose transport in sugarcane has been of limited scope, and to date the progress has been minimal. In contrast, sucrose transporters have been identified in dicot species such as *Arabidopsis thaliana* (Sauer and Stoltz, 1994), *Solanum tuberosum* (Riesmeier *et al.,* 1993) and in monocots such as *Zea mays* (Aoki *et al*., 1999) and *Oryza sativa* (Hirose *et al*., 1997).

Various enzymes associated with sugar metabolism have been studied in sugarcane such as invertase, sucrose phosphate synthase (SPS) and PFP to investigate which of the enzymatic steps is

limiting to sucrose accumulation (Grof and Campbell, 2001; Rohwer and Botha, 2001). To date the limiting enzymes have not been identified. Models of enzyme kinetics and metabolite fluxes in the culm have predicted that transport of hexoses and sucrose to storage cells and sucrose into the vacuole, as well as the rate of sucrose hydrolysis by cytoplasmic invertase and subsequent metabolism of the hexose carbon skeletons are likely to be limiting steps (Rohwer and Botha, 2001). This is an indication that sugar transporters are a very important component in the accumulation of sucrose in plants.

In this study the mechanism of sucrose transport within/into sugarcane culm cells will be investigated by using ^{14}C labeled sucrose, glucose and fructose at different sugar concentrations and at different internodes of *S. officinarum*, identifying sucrose transporter(s) in sugarcane with PCR techniques and characterising the sucrose transporter(s) in *Xenopus laevis* oocytes.

CHAPTER 2

Sucrose transporters and sucrose

2.1 Introduction

Sugar transport proteins play a crucial role in the cell-to-cell and long-distance distribution of sugars throughout the plant. A proton-sucrose symporter is well established as the key transporter in apoplastic phloem loading (Ward *et al*., 1998), and hexose transporters have long been associated with sugar uptake in many sink tissues (Thorne, 1985 and Patrick, 1997). This review will focus on the role of transporters in influencing sucrose metabolism, sucrose storage and mechanisms of sucrose transport within plants, beginning with the different membrane transport mechanism plants use to transport solutes, the synthesis of sucrose in the source tissue, the path it follows through the phloem and moving on to the fate of sucrose in the sink tissue, as detailed in Figure 2.1. Sinks can be divided into utilisation and storage sinks. The utilising sinks include meristems, growing roots and developing leaves that import photo-assimilates mainly for catabolism to sustain growth and development of the respective organ. The storage sinks are organs such as growing tubers, tap roots, seeds and fruits whose primary function is to store imported carbohydrate such as sugars, starch or oil.

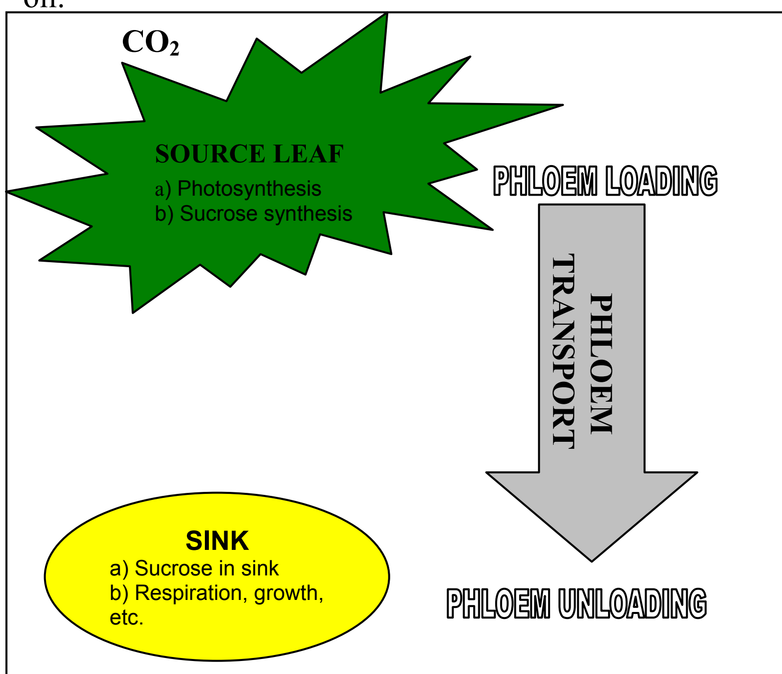


Figure 2.1 Diagram of path sucrose follows from being synthesised in the source leaves, phloem loading and then the metabolism and storage within the sinks.

2.2 Membrane transport

The difference in electrical potential between two aqueous media separated by a biological membrane is called the membrane potential. All living cells exhibit a membrane potential that is due to asymmetric ion distribution between the inside and outside of the cell. The typical membrane potential across plant cell membranes ranges from –60 to –240 mV, with the negative sign indicating that the inside of the cell is negative compared to the outside. Two forces drive the passive transport of ions across membranes: 1) the concentration gradient of the ion and, 2) the effect of the membrane potential on the ion. Higher observed than predicted internal ion concentrations calculated by the Nernst equation indicates active uptake. Lower than predicted internal concentration of ionic solutes indicates active extrusion. This can be easily explained when a molecule is charged, but the situation is less clear when the molecule has no charge, as in the case of sucrose. There are three possible transport mechanisms: 1) facilitated diffusion refers to the movement across membranes through a channel totally dependent on the concentration gradient; 2) using an antiport system; 3) using a symport system. The vacuole and cytosol are the most important intracellular compartments that determine ionic relations. In plants the vacuole occupies up to 90% of the cell volume and contains the bulk of cell solutes.

In most plants, sucrose is the main transport form of photo-assimilates, in contrast to hexoses that do not circulate over long distances as they do in the animal kingdom (Williams *et al*, 2000). Sucrose moving from source to sink organs has to pass through several membranes implicating specific sucrose carriers, unless the sucrose is hydrolysed to hexoses prior to transport of hexoses with subsequent re-assembly on the other side of the membrane. Sucrose transporters in plants are assumed to be of three types: 1) Plasma membrane influx carriers responsible for entry of sucrose into cells that are of the H^+/Suc symporter type (Logan *et al*., 1997); 2) Tonoplast carriers have been proposed to work as H^+/Suc antiporters (Briskin *et al.,* 1985, Getz and Klein, 1995) as the

vacuole is acidic compared to the cytoplasm and 3) Plasma membrane efflux carriers responsible for unloading of sucrose in sink organs or for sucrose exit from the mesophyll cells in close vicinity to the phloem (Bush, 1993). To date only plasma membrane influx sucrose transporters on the plasma membrane have been cloned and characterised (Lemoine, 2000).

2.3 Sucrose and source tissue

In higher plants, CO_2 fixation occurs in the Calvin cycle in chloroplasts of leaf mesophyll cells, mainly in the palisade parenchyma of mature leaves (Singh and Malhotra, 2000). These are net exporters of sugars and are known as “carbon sources”. Net products of this cycle are triose phosphates that can supply several biosynthetic pathways including synthesis of starch, sucrose, lipids and amino acid synthesis in the cytosol (Kühn *et al*, 1999). Various biosynthetic pathways in different compartments of the mesophyll cells compete for triose phosphates (Schultz *et al.,* 1993). Triose phosphates are converted to hexose phosphates in the pentose phoshate pathway, which is then used for sucrose synthesis in the cytosol of mesophyll cells.

Two enzymes, sucrose phosphate synthase (SPS) and sucrose phosphatase (SP), are associated with sucrose synthesis in higher plants. These enzymes occur in the cytoplasm and cell walls (Avigad, 1982; Hawker, 1985; Huber and Huber, 1992). Sucrose is derived from hexose phosphates through combined activities of UDP-glucose pyrophosphorolase, SPS and SP. After synthesis, sucrose can pass the entire route from the mesophyll cells to the sieve element - companion cell complex (SE-CC) either symplastically, moving from cell to cell via plasmodesmata (Ward *et al*., 1998) or apoplastically through the release from the mesophyll cells and then active loading from the apoplast into the SE-CC (apoplastic loading). Sucrose is predominantly exported from the cells, probably by facilitated diffusion, and subsequently taken up by the phloem complex via a specific H^+/Suc co-transport mechanism (Frommer and Sonnewaldt, 1995). Several sucrose transporters from plant species such as *Arabidopsis thaliana* (Sauer and Stoltz, 1994)*, Plantago major* (Gahrtz

et al., 1994), *Daucus carota* (Shakya and Sturm, 1998), *Oryza sativa* (Hirose *et al*., 1997) and *Saccharum officinarum* (Rae *et al*., 2004) has been identified. Most of these clones were predominantly expressed in the vascular system (Truernit and Sauer, 1995) and seem to be involved in loading sucrose into the phloem (Riesmeier *et al*., 1994; Stadler and Sauer, 1996)

Sucrose can also accumulate in the vacuole rather than being transported. The exact mechanism of sucrose uptake by vacuoles in source tissue is unclear. In leaves carbohydrates accumulate during the day, when loading capacity of phloem is limiting, and are exported during the night. Leaf vacuoles must be equipped with a transport system enabling rapid accumulation and export of sucrose as a function of the physiological status. Experiments using isolated barley leaf vacuoles showed that uptake occurs by facilitated diffusion (Kaiser and Heber, 1984), a transport mechanism that allows rapid equilibration between the cytosol and vacuole. The permease had a low affinity for sucrose (K_m 20-30 mM, a concentration which is easily attained in some plants during the daytime) and was found not to be inhibited by hexose. Facilitated diffusion of sucrose rather than active transport of sucrose has also been observed for vacuoles isolated from sugarcane cell cultures, which accumulated sucrose at concentrations comparable to those in stalk tissue (Preisser and Komor, 1991) and tomato fruit vacuoles (Milner *et al*., 1995). To date no active sugar transporter has been identified on the tonoplast of vacuoles. Chiou and Bush (1996) reported the cloning and the vacuolar localisation of a putative sugar transporter from *B. vulgaris*. However the authors did not demonstrate transport activity, and more detailed localisation studies are needed to demonstrate that the gene product codes for a vacuolar sugar transporter.

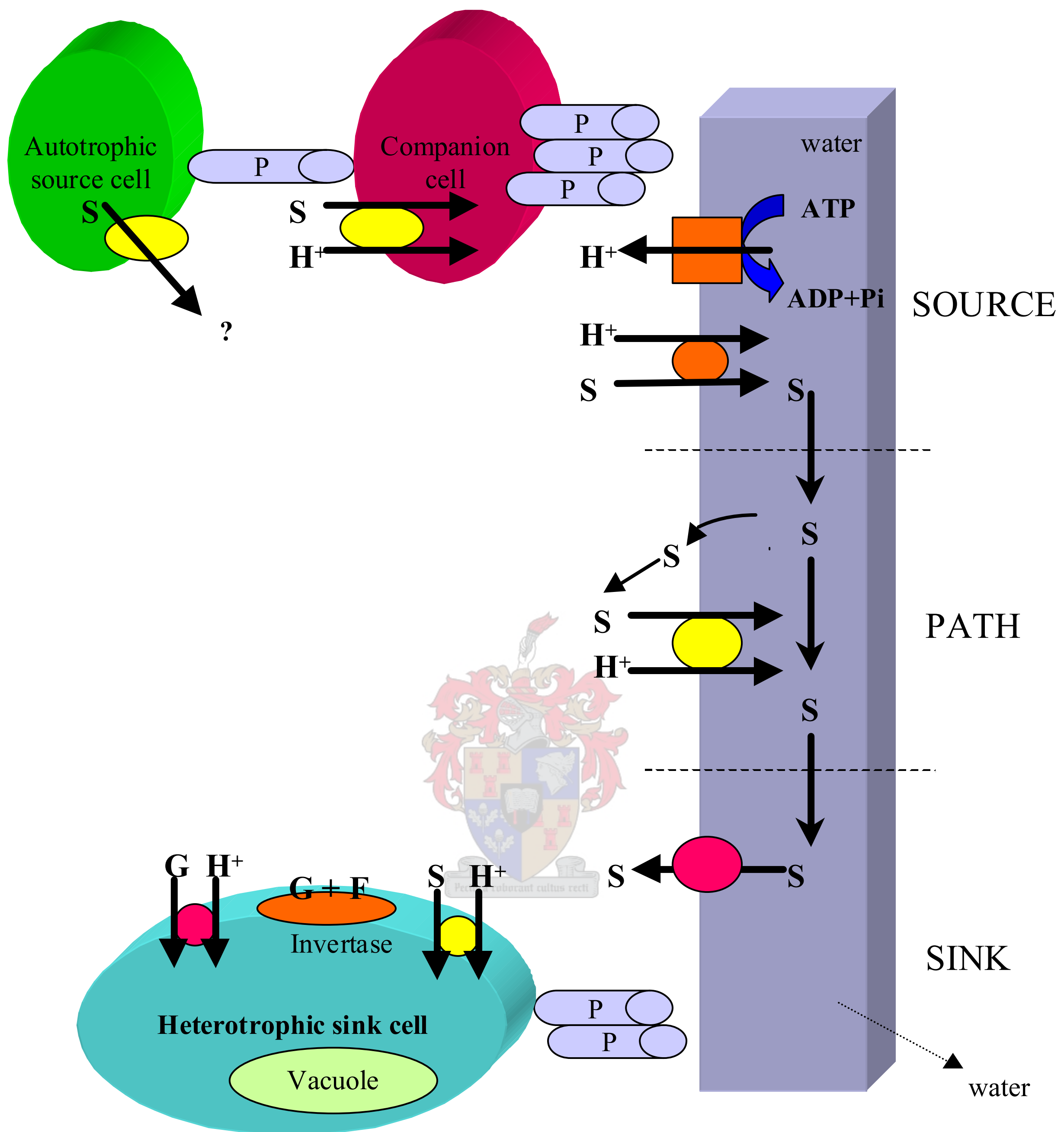


Figure 2.2 Possible locations of sucrose transporters in plant cells (modified from Williams *et al.*, 2000). Transport of sucrose (S) between sources and sinks occurs in sieve elements of the phloem by bulk flow. In the source leaf, sucrose moves entirely symplastically, (plasmodesmata, P). However, in many species, sucrose leaves the symplast, possibly via sucrose efflux carriers, and is actively accumulated from the apoplast into sieve elements and/or companion cells by plasma membrane H+-sucrose symporters. Energisation is via PM H1-ATPase. Passive influx of water into the sieve tubes presumably occurs via water channels. Sucrose carriers along the path are related retrieval of sucrose leaked from the phloem. Unloading of sucrose into sink cells might occur symplastically via plasmodesmata or sucrose might be delivered to the apoplast via a sucrose efflux carrier. Here, it is either taken up directly by plasma membrane sucrose transporters, or hydrolysed to glucose (G) and fructose (F) by the cell-wall invertase then taken up via plasma membrane hexose transporters.

2.4 Phloem transport

2.4.1 Phloem loading

In plants, sucrose is transported over long distances in the phloem sap. The flow of sap occurs in a specialised network of cells, called sieve elements. These sieve elements are connected to companion cells, which have high metabolic activity (Thorne, 1985). In most crop species the sieve element/companion cell complex (SE-CC) is isolated from surrounding cells but is closely linked with another by specific plasmodesmata (Patrick, 1997). The high solute content of the phloem sap (sucrose, amino acids, organic and inorganic ions and other compounds) and the high osmotic pressure (30 bar) of the SE-CC compared to the mesophyll cells (13 bar), has led to the concept of active phloem loading (Geiger *et al.,* 1973).

According to this concept, the high osmotic pressure in the SE-CC is due to active 'loading' of solutes, mainly sucrose in cells. However, this may not be universal as in some species such as willow, no solute concentration difference exists between the SE-CC and the surrounding cells (Turgeon and Medville, 1998) The plasmolysis of mesophyll cells was determined in comparison to SE-CC and found that both remained unplasmolysed in osmoticum > 1.2 M. Usually much lower concentrations of osmoticum severely shrink the protoplast of the surrounding mesophyll indicating that total solute levels in SE-CCs are much higher than in the mesophyll cells. This difference in plasmotic response between phloem and mesophyll was not found in willow (Turgeon and Medville, 1998).

The loading of sucrose into the phloem results in the movement of sap in the phloem through mass flow (Horwitz, 1958). The driving force for this movement is the entry of sucrose and H_2O in the sieve tubes of the source organ and the unloading of solutes and water at the sink organs. The accumulation of sucrose in sieve tubes requires the presence of sucrose transporters to drive this

active accumulation. The existence of a carrier system specific for sucrose and responsible for entry of sucrose into phloem was postulated in the late 70's (Willenbrink and Doll, 1979). Evidence was also found when transgenic *Lycopersicon esculentum* and *Nicotiana tabacum* plants that express yeast invertase in the apoplast had severely stunted growth. This was presumably as a result of sucrose hydrolysis and the ensuing disruption in phloem loading (Schaewen *et al*., 1990; Dickinson *et al*., 1991). Since then the existence of carriers have been demonstrated (Bush 1993; 1999). The energy for this transport being proton gradient is established by a H^+/ATPase located in the plasma membrane (Bush, 1989; 1992; Lemoine and Delrot, 1989). The first H^+/Suc symporter gene SoSUT1 from *Spinacia oleracea* was isolated using a yeast complementation system (Riesmeier *et al*., 1992). Several lines of evidence indicate the essential role of SUT1 in phloem loading and long distance transport (Kühn *et al*., 1997). Antisense repression of SUT1 in transgenic plants inhibits sucrose export from leaves adding to the evidence (Riesmeier *et al*., 1994).

2.4.2 Phloem unloading

The discussion above illustrates the events leading up to the export of sugars from sources. The transport events from the sieve elements to the sites of utilisation within the recipient cells contribute to phloem unloading. The phenomenon links sink metabolism and/or compartmentation with phloem transport to, and partitioning between sinks. Since phloem unloading occurs along the entire length of the axial phloem path different mechanisms function in different sink tissue (e.g. vegetative apices, shoot apices, stem elongation zones, mature axial pathway, terminal vegetative storage sinks, reproductive storage sinks) (Patrick, 1997). Unloading can be symplastic or apoplastic. Symplastic transport would involve direct transfer of sucrose from the phloem to the storage parenchyma cells via plasmodesmata. In contrast, apoplastic unloading would involve the transport of sucrose into the apoplast of sink tissue via sucrose transporters, followed by uptake of sucrose or its cell wall invertase generated hydrolysis products into storage cells. The common unloading of phloem borne carbohydrates is symplastic with an apoplastic step at or beyond the

sieve element boundary. Plasmodesmal conductivity exerts the primary control over symplasmic transport that occurs by diffusion with bulk flow anticipated to be of increasing significance as import rate rises (Patrick, 1997). When sucrose is unloaded into the apoplastic space, it can be taken up as sucrose into the sink cells or cleaved by an invertase to hexoses that are transported by specific carriers (Büttner and Sauer, 2000). Sucrose is then used in metabolism for sink growth and development or can be stored in vacuoles. Studies using asymmetrically labelled sucrose have demonstrated that sugar obtained through translocation moves primarily through the symplast and was not cleaved into glucose and fructose (Patrick, 1990).

2.4.2.1 Symplasmic unloading

A diffusive efflux of assimilates from sieve elements can be driven by the large concentration differences of assimilates between sieve elements and importing sink cells (Wang and Fisher, 1995). Consistent with unloading by diffusion, phloem import was slowed when root tips were exposed to dilute sucrose solution (Schulz, 1994). Metabolism and intercellular compartmentation determine cytoplasmic concentrations of sucrose in sink cells (Patrick *et al*., 2001). High concentrations of assimilates may be transported over considerable distances through non-vascular symplasmic routes, a process that depends on assimilate retention in the symplasm by retrieval from the sink apoplasm (Patrick, 1997).

2.4.2.2 Apoplastic unloading

Mechanisms of assimilate release to the apoplasm along post sieve element pathways range from passive leakage to energy coupled membrane transport. The apoplastic route was probably used by developing seeds in which there are no protoplasmic connections between the maternal and embryonic tissues (McDonald *et al*., 1996a). Apoplastic sucrose cleavage by invertases maintains large transmembrane differences that favour passive sucrose release in *Lycopersicon esculentum* fruit (Ruan and Patrick, 1995), seeds of cereal and during the pre–storage phase of seed

development in Fava bean (Weber *et al*., 1995). In developing seeds, sucrose release from maternal tissue occurs by facilitated diffusion through carriers in cereals (Wang and Fisher, 1995) and non-selective pores in pea (De Jong *et al*., 1996). An energy dependant H^+/Suc antiport system was described for *Phaseolus vulgaris* and *Vicia faba* (Walker *et al*., 1995, 2000). Sucrose influx into fillial seed tissues of grain legumes (McDonald *et al*., 1996b; Tegeder *et al*., 1999) and *Hordeum vulgaris* (Weschke *et al*., 2000) was mediated by H^+/Suc symport mechanism.

The sugarcane culm is composed of storage parenchyma tissue permeated by numerous vascular bundles. A sheath of two or more layers of thick-walled, lignified sclerenchyma cells surrounds the vascular bundles whereas storage parenchyma cells become lignified at a later stage of development (Clements, 1980). *W*elbaum *et al*., (1992) and Jacobsen *et al*., (1992) indicate that two well separated apoplastic spaces occur in *S. officinarum*, the one in the bundle sheath, the other in the stem parenchyma. The current anatomical data for sugarcane indicate that regardless of whether sucrose is initially unloaded from phloem sieve tubes symplastically or apoplastically, transport to the storage parenchyma must occur via plasmodesmata connecting the numerous pits in the sheath cell walls (Jacobsen *et al*., 1992). In mature storage parenchyma, sucrose could move efficiently from cell to cell only through the symplast because a considerable barrier exists in the form of lignified, suberised cell walls. Sucrose in the storage cells could apparently move freely between the symplast and apoplast via the non-lignified, non-submersed cells. The symplastic pathway plays an increasingly greater role as the tissue undergoes lignification and suberisation.

Although understanding of phloem loading and unloading is increasing, there are still many questions that have to be answered such as the role of plasmodesmata in phloem loading and unloading at the molecular level. The availability of cloned genes for members of the SUT family as well as phloem-sap specific proteins provide the tools to further explore and understand the mechanism and regulation of long distance transport in plants.

2.5 Sucrose in sink tissue

Sinks can be divided into utilisation and storage sinks. The utilising sinks include meristems, growing roots and developing leaves that import photo-assimilates mainly for catabolism to sustain growth and development of the respective organ. The storage sinks are organs such as growing tubers, tap roots, seeds and fruits whose primary function is to store imported carbohydrate such as sugars, starch or oil (Herbers and Sonnewald, 1998). In *S. officinarum*, the major sites requiring import of photo-assimilates to support respiration and growth are the roots, the shoot apical region, and the developing leaves and shoot internodes. The apical meristem of the shoots and roots remain growth sinks, whereas the leaves undergo a sink to source transition and the internodes change from growth sinks to storage sinks.

2.5.1 Sucrose metabolism

As in most plants, sucrose is the sugar that is translocated in the phloem (Hatch and Glasziou, 1964) to sinks where it is used for growth and metabolism or storage in sugarcane (Hawker, 1985). Young internodes use incoming sucrose for growth while the older internodes store sucrose (Glasziou and Gaylor, 1972). In the older internodal tissue, sucrose can account for up to 50% of the total dry weight reaching a concentration of 500 mM (Bull and Glasziou, 1963).

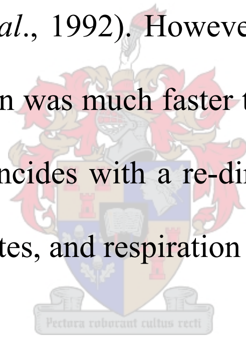
Once sucrose arrives in the stem it can be catabolised by Sucrose synthase (SuSy) or by one of the three invertases. Sucrose synthase (SuSy) which catalyses the readily reversible reaction of hydrolysing sucrose to UDP-glucose and fructose is important in sucrose degradation. Evidence from a range of tissues suggests that this enzyme is confined to the cytosol (Kruger, 1997). Invertase catalyses the irreversible hydrolysis of sucrose to glucose and fructose. Isoforms of invertase with different biochemical properties accumulate in the cytoplasm (neutral invertase), cell wall bound acid invertase and soluble acid invertase, which is found in the vacuole and apoplast (Sturm and Tang, 1999). Vacuolar and cell wall invertases have acidic pH optima of about 5 – 5.5

and are, therefore, referred to as acid invertases (soluble and insoluble acid invertase respectively). The pathway of sucrose may determine the contribution of these enzymes to the pathway of sucrose breakdown. After entry into the metabolic compartment of the parenchyma cells, the hexoses may be metabolised or resynthesised into sucrose by sucrose phosphate synthase (SPS) and sucrose phosphatase (SPase) (Hatch *et al*., 1963; Hatch and Glasiou, 1964). Botha *et al*. (2001) investigated the role of acid invertase in sucrose accumulation in *sugarcane* and found that acid invertase plays an insignificant role. This was concluded after the endogenous acid invertase activity of transgenics was reduced by up to 80%.

A model for sugar transport in parenchyma cells of sugarcane internodes has been described by Komor *et al*., 1996. According to this model young, growing internodes used a partially apoplastic mechanism of phloem unloading since the apoplastic barrier around the bundle sheath had not been fully developed. Also young growing tissue had a high apoplastic acid invertase activity, so that active transport systems were mainly hexose transporters. Here as much sugar as is available is used for growth and cell expansion. Sucrose transporters are unlikely to play a significant role in this stage of growth, since most sucrose is being metabolised and not stored. As the internode ripens phloem unloading through the bundle sheath becomes exclusively symplastic. Hexose transporters may still play an important role as a retrieval mechanism, but as the internodes mature the active transport activities decline and a linear phase of uptake becomes more prominent. If the linear phase represents a passive, equilibrating transport system, its major net transport direction will be from the symplast to the apoplast. As consequence an apoplastic concentration of sugars, especially of sucrose nearly as high as in the symplast will build up, with the result of low turgor in the storage cells and further promotion of symplastic bulk flow of solution into storage tissue.

Several lines of evidence demonstrate the presence of a cycle in which sucrose was synthesised and degraded simultaneously (Whittaker and Botha, 1997). Wendler *et al.,* 1990 found that in sugarcane

suspension cells the sucrose phosphate synthase (SPS) activity doubled during the phase when the cells were actively storing sucrose. Pulse experiments with [^{14}C] fructose also indicated that sucrose synthesis occurs not only during the storage phase, but also after storage had stopped and during rapid mobilisation of sucrose (Whittaker and Botha, 1997). The cells contained high activities of SuSy and alkaline invertase and these were both at a maximum when sucrose storage was occurring. The rapid cycling of sugars in non-photosynthetic cells has been referred to as "futile cycling" (Dancer *et al*., 1990) because the simultaneous synthesis and degradation of sucrose appears to involve energy being wasted. It is thought that these cycles allow cells to respond in a highly sensitive manner to small changes in the balance between the supply of sucrose and the demand for carbon respiration and biosynthesis (Moore, 1995). The rate of transport across the tonoplast may influence the duration of exposure of sucrose and hexose to the synthesis and degradation in the cytosol (Preisser *et al*., 1992). However, Wendler *et al.,* (1990) found that the rate of sucrose synthesis and degradation was much faster than the net uptake rate by vacuoles. The maturation of sugarcane internodes coincides with a re-direction of carbon from insoluble matter, amino acids, phosphorylated intermediates, and respiration to sucrose (Whittaker and Botha, 1997).

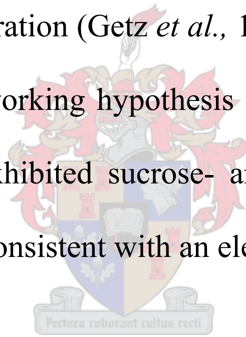


2.5.2 Sucrose transport across tonoplast

Sucrose concentration in the apoplast of Solanaceous species has been estimated at 2 to 5 mM, which was much lower than sucrose concentration in phloem (>100 mM) (Frommer and Sonnewald, 1995; Ward *et al.,* 1998), whereas in *S. officinarum* the sucrose concentration in the apoplast was almost as high as in the phloem (>200 mM) (Komor *et al*., 1996). Sucrose loading into the phloem and sink tissue therefore requires energy input and occurs by symport with H^+ (Bush 1993). Although *in vitro* studies using vesicles offer an insight into the capabilities of individual membranes to transport sugar, the *in vivo* environment experienced by the membranes is likely to be different from *in vitro* conditions (Getz, 1991). The concentration and ratio of sucrose and hexoses

were influenced by the presence of a number of enzymes such as acid invertase and sucrose synthase that were absent from vesicle systems (Salmon *et al*., 1995).

The role of the vacuole as storage compartment has been well documented (Wink, 1993), however, it is not known whether the concentration of compounds inside the vacuole is higher than in the cytosol in *S. officinarum*. Preisser *et al.* (1992) found evidence that sucrose is not concentrated in the vacuole of sugarcane suspension cells but that the concentration is the same as in cytosol, using NMR for compartmentation studies. Although sugarcane parenchyma cells store considerable amounts of sucrose, no active transport mechanism into isolated vacuoles has been described (Preisser and Komor, 1991; Maretzki and Thom, 1986; Williams *et al.,* 1990). Transport of uncharged molecules across the tonoplast of *B. vulgaris* has been described for intact vacuoles and mature plasma membrane vesicle preparation (Getz *et al.,* 1987). A H^+/Suc antiport mechanism was postulated by Doll *et al*. (1982) as a working hypothesis emerging from studies with isolated red beet root vacuoles. These vacuoles exhibited sucrose- and glucose-induced acidification of the vacuole suspension medium, which is consistent with an electrogenic mechanism.


Pectora roborant cultus recti

Essential criteria for an electrogenic carrier mediated sugar transporter coupled to a driver ion in plasma membrane vesicles are (a) voltage dependency, (b) similar saturation kinetics of sucrose and the co-transported ion, (c) substrate specificity, (d) similar sensitivity toward inhibitors of sucrose transport and sucrose induced ion movement, and (e) whole number stoichiometry between H^+ and sucrose (Slone and Buckhout, 1991). The existence of an electrogenic and substrate – specific H^+/Suc antiport was demonstrated by Briskin *et al*. (1985) with *B. vulgaris* light density membranes. Vacuoles from sugarcane stalk tissue and cell suspension cultures show different uptake mechanisms. Isolated vacuoles from sugarcane suspension cells take up sucrose at high rates without dependence on energisation of the tonoplast (Preisser and Komor, 1991). The uptake rate was pH dependent with an optimum at pH 7 and was inhibited by PCMBS. This supported the idea

of carrier mediated sucrose transport rather than diffusion or leakage. Passive carrier mediated sucrose transport with a high Km value had been reported for barley mesophyll vacuoles, but different kinetics were found for sucrose and glucose uptake. In vacuoles of sugarcane suspension cultures and tonoplast vesicles from sugarcane stalk tissue no evidence was found for an H^+/Suc antiport system (Williams *et al*., 1990; Preisser and Komor, 1991). The uptake kinetics in the stalk vesicles showed a saturable phase at lower sucrose concentration, which was not found in the suspension cells (Williams *et al*., 1990). Getz *et al*., (1991) found evidence that pointed to a carrier mediated sucrose uptake by an ATP dependent H^+/sucrose antiport system similar to that described for *B. vulgaris* taproot (Briskin *et al*., 1985) in tonoplast vesicles from sugarcane stalk tissue. However the low rate of proton transport found makes it difficult to demonstrate a sucrose concentration gradient with vesicle preparations, and without more significant sucrose specific transport it is not possible to say that an antiport mechanism is functioning in the sugarcane tonoplast. The possibility that a decreased pH on the inside of the membrane causes conformational changes in sucrose transport relevant proteins cannot be excluded as an explanation for these findings. The most common belief regarding sucrose accumulation in sugarcane vacuoles is that it occurs primarily via a system of facilitated transport (Preisser *et al*., 1992).

Functional sucrose transporter genes have been cloned from various plant species and plant organs, including sugarcane, *S. officinarum*. The functionality of ShSUT1 was determined in *Saccharomyces cerevisiae* and a *c.* K_m of 200 mM for sucrose was measured (Rae *et al*., 2004). Also a H^+/ glucose symporter has been cloned from sugarcane leaf tissue and is thought to be located on the plasma membrane (Bugos and Thom, 1993). The functionally identified hexose and sucrose carrier genes from plants all code for an active transport system, no equilibrating, passive sugar transporters has been cloned from plants yet.

In conclusion sucrose storage in sugarcane stem parenchyma is an example of a highly regulated process, where anatomical features, metabolic reactions and transport through membranes interact closely.

2.6. The sucrose transporter families

The existence of specific carriers responsible for the crossing of sucrose through membranes has been postulated for many years. The first successful identification of a sucrose carrier was based on a yeast complementation approach. Riesmeier *et al.,* (1992) developed a yeast strain that could only grow on sucrose when it was complemented with a sucrose carrier. The secreted invertase of the yeast strain was mutated so that sucrose could not be cleaved outside the yeast cell. Sucrose synthase was then expressed in the cell so that when a sucrose carrier was expressed in this strain, sucrose would be able to enter the cell and be metabolised. The yeast strain was named (SUSY7) and was complemented with a *Spinacia oleraceae* leaf cDNA library and plated on a medium containing sucrose as the sole carbon source allowing for the isolation of the first identified plant sucrose carrier (SoSUT1). The same method was then used to identify the sucrose carrier (StSUT1) from *Solanum tuberosum* leaves (Riesmeier *et al.,* 1993). All the other identified carriers have been obtained by hybridisation and screening or PCR amplification from these initial sequences.

More than 30 different cDNAs encoding sucrose carriers have now been identified in species such as *Solanum tuberosum* (StSUT1)*, Arabidopsis thaliana* (AtSUC1 & AtSUC2)*, Plantago major* (PmSUC1)*, Zea Mays* (ZmSUT1)*, Triticum aevistum* (TaSUT1)*, Oryza sativa* (OsSUT1) *and Hordeum vulgaris* (HvSUT1), *Saccharum officinarum* (ShSUT1), *Daucus carota* (DcSUT1 & DcSUT2); (Riesmeier *et al.,* 1992; 1993; Sauer and Stoltz, 1994; Gahrtz *et al.,* 1996; Shakya and Sturm, 1998; Aoki *et al.,* 1999; Aoki *et al*., 2002; Hirose *et al.,* 1997; Weschke *et al*., 2000). For many of these species two or more SUT genes have been reported (Lemoine, 2000, and Williams *et al*., 2000). From the results obtained from *A. thaliana* it is thought that in each species sucrose

transporters belong to a gene family, since five SUT genes have been functionally characterised by expression in yeast cells (Sauer and Stoltz, 1994; Meyer *et al*., 2000, Schulze *et al*., 2000, Weise *et al*., 2000; Ludwig *et al*., 2000). In addition four further putative SUT sequences from *A. thaliana* are present in public databases. A sucrose transporter gene family was also described in rice OsSUT1, 2, 3, 4 and 5 (Aoki *et al*., 2003) based on sequence similarities.

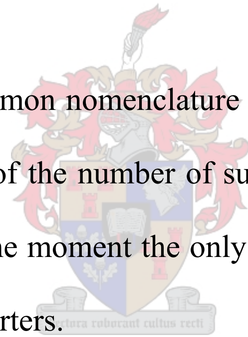
Based on phylogentic analysis of deduced peptide sequences from dicotyledon these SUTs have been classified into three groups, SUT1, SUT2 and SUT4 type (Barker *et al*., 2000; Weise *et al*., 2000). The nomenclature in the literature for plant sucrose transporters is confusing with SUT and SUC used for Sucrose Transporter and Sucrose Carrier, respectively. Also the sucrose transporters are classified into 3 classes Type I, Type II and Type III which will be elaborated on in following paragraphs.

Type I is called the dicotyledon SUT1 subfamily a large group of high affinity transporters, which is required for phloem transport (Riesmeier *et al*., 1993; Kühn *et al*., 1996; Bürkle *et al*., 1998; Gottwald *et al*., 2000). The classification is also confusing within this family since transporters like NtSUT3, AtSUT2, PmSUC2, AtSUC5 and AtSUC1 were included. The name of the transporter does not indicate in what subfamily the transporter is classified.

Type II is also referred to as the dicotyledon SUT2 or cereal SUT1 subfamily (Aoki *et al*., 2003), which were localised to sieve elements in *Lycopersicon esculentum.* SUT2 differs structurally from the other SUTs (Lalonde *et al*., 1999; Barker *et al*., 2000) by having extended domains at the N-terminus c. 30 amino acids longer than the sucrose transporters already identified and a central cytoplasmic loop that was c. 50 amino acids longer. Thus SUT2 shows structural analogies to metabolite sensors in yeast (Özcan *et al*., 1996) and this has led to the hypothesis that SUT2 family members may function in sucrose sensing (Barker *et al*., 2000). The AtSUT2 and LeSUT2 genes

were unable to complement a yeast mutant that was deficient in sucrose uptake, and its expression in yeast may cause toxicity in yeast (Barker *et al*., 2000). However later reports gave evidence that AtSUT2/AtSUC3 can mediate sucrose transport in yeast (Meyer *et al*., 2000; Schulze *et al*., 2000) and the sensor theory was modified. It was proposed that SUT2/SUC3-type transporters might represent flux sensors that measure the transport rates of sucrose across the membrane (Schulze et al., 2000)

Type III is also called dicotyledon SUT4 or cereal SUT2. This is a smaller subfamily of low affinity/high capacity sucrose transport system playing a role in phloem loading in minor veins, (Lalonde *et al*., 1999; Weise *et al*., 2000). Examples are OsSUT2, HvSUT2, DcSUT1A, VvSUC11 and StSUT4.



It is evident from the above that a common nomenclature needs to be found since the name of the transporter may only be an indication of the number of sucrose transporter found in a species and not indicate what the function is. For the moment the only way to determine the role is to compare the sequences of already known transporters.

In solanaceae SUT2 co-localises with high- and low-affinity sucrose transporters SUT1 and SUT4, respectively (Reinders *et al*., 2002b). A model for the function of the three SUT proteins in plasma membrane sieve elements was proposed in which SUT2 functions as a receptor for extra cellular sucrose and regulates the relative activities of the high affinity SUT1 transporter and the low affinity SUT4 transporter. This was suggested to occur either by controlling protein turnover or through signal transduction, resulting in transcriptional activation/repression in the companion cell (Weise *et al*., 2000). In contrast to the situation in solanaceous plants, PmSUC3 which is also a SUT2/SUC3-type sucrose transporter does not co-localise with PmSUC2, the source-specific phloem-loading sucrose transporter in Plantago (Barth *et al*., 2003).

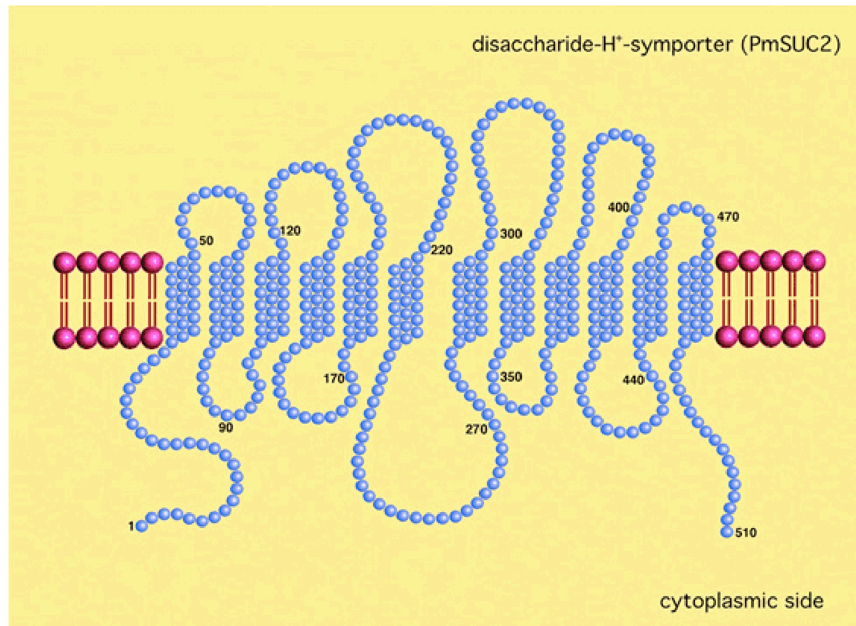


Figure 2.3. Topological model of a disaccharide H^+- symporter, PmSUC2 showing the 12 transmembrane spanning loops (from Williams *et al.*, 2000). Disaccharide transporters have predicted molecular masses of 55 kDa and hydrophobicity analysis indicates that they are highly hydrophobic integral membrane proteins.

The sequences of the sucrose transporters were highly conserved and carriers display the typical 12 trans-membrane helices (Figure 2.3) and a cytosolic orientation of N and C termini as has been described for several cation/substrate co-transporters (Henderson, 1990, Stoltz *et al.*, 1999). This is the characteristic feature of the family of the major facilitator superfamily (MFS) described by (Marger and Saier, 1993). The second loop of the transporters characteristically contains a highly conserved motif (RXGRR), which is found at the equivalent position in the *Escerichia coli* lactose permeases (Henderson, 1990). This indicates that this amino acid sequence could be important for transport. A chimeric protein was constructed between AtSUT2 and the high affinity StSUT1 in which the N-terminus and the central loop of AtSUT2 were exchanged with those domains of StSUT1 and *vice versa* (Schulze *et al.*, 2000). AtSUT2 showed significantly lower affinity for sucrose compared to chimeras containing the N-terminus of StSUT1. The results indicate a significant function of the N-terminus but not of the central cytoplasmic loop in determining

substrate affinity. Even though the amino acid sequences were relatively similar, the properties of sucrose transporters can be very different and have different functions and locations within plants.

2.6.1 Diverse locations for sucrose transporters

Disaccharide transporters are thought to be specific to plants whereas monosaccharide transporters are found in bacteria, fungi and mammals (Williams *et al*., 2000; Bush, 1999). Only a few sucrose transporters have been precisely localised at the membrane level (Kühn *et al*., 1997; Bick *et al*., 1998). It is often assumed that these proteins are localised at the plasma membrane since they are targeted to the plasma membrane in transgenic yeast. Sucrose transporters have been localised in the phloem, roots and floral organs. For the purpose of this study the focus will be on phloem associated and sink tissue (e.g. root) transporters.

2.6.1.1 Phloem associated sucrose transporters

In apoplastic phloem loading, a sucrose transporter at the plasma membrane of phloem cells accumulates sucrose in the sieve element-companion cell complex (SE-CCC) to drive long distance transport. In some plants such as *N. tabacum, L. esculentum* and *S. tuberosum,* SUT1 sucrose transporters have been detected in sieve elements (Kühn *et al*., 1997). Analysis by *in situ* hybridisation showed that StSUT1 mRNA was localized mainly in the SE plasma membrane and was preferentially associated with sink tissue (Kühn *et al*., 1999). StSUT1 expression was inhibited and it was found that the reduced expression did not have an effect on the above ground organs but led to a reduced fresh weight accumulation during early stages of tuber development, indicating that SUT1 plays an important role in sugar transport. Other transporters such as PmSUC2 and AtSUC2 were specifically localised in the companion cells (Stadler *et al*., 1995; Stadler *et al*., 1996). Analysis by electron microscopy indicated that SUT1 transcripts were present in the orifices of the plasmodesmata between companion cells (CC) and sieve elements (SE) (Kühn *et al*., 1997). Thus, the simplest explanation is that *SUT1* mRNA is synthesized in the CC, the mRNA is translocated

through plasmodesmata by specific targeting mechanisms to the SE, and subsequent translation occurs in the SE. Alternatively, SUT1 mRNA or protein (or both) were already present in SE-CC mother cells before division, and both RNA and protein are stable for several months (that is, the life span of SEs). Young SEs still containing a nucleus already expressed SUT1 protein. However, *SUT1* mRNA levels were highest in mature leaves where SE development is complete (Riesmeier *et al.,* 1993). According to above it is seems that sucrose transporters, at least SUT1 from *Solanaceae* species, result from gene transcription/translation in the companion cell and must be targeted to their final destination.

Anti-sense repression demonstrated that, the high affinity transporter, SUT1 was essential for long distance transport in *S. tuberosum* and *N. tabacum* (Riesmeier *et al*., 1994; Kühn *et al*., 1996; Bürkle *et al*., 1998). The anti-sense plants had retarded growth phenotype, and their source leaves were found to export less carbohydrate. As a result of this, carbohydrates accumulated in the leaves and the sink organs were malnourished, resulting in a dramatically reduced tuber yield in the transgenic *S. tuberosum* plants. This was also confirmed in *N. tabacum*, where strong antisense plants had a dwarf phenotype and sugar export from source leaves was drastically impaired. However, this was not the case with the rice sucrose transporter, OsSUT1 where there were no differences between wild-type and anti-sense plants in there carbohydrate content and photosynthetic ability of the flag leaves in the vegetative growth stage, suggesting that OsSUT1 may not play an important role in carbon metabolism in flag leaves (Ishmaru *et al*., 2001). These observations clearly show that sucrose transporters in the phloem were essential for carbohydrate partitioning, at least in *N. tabacum* and *S. tuberosum*, both members of the *Solanaceae* family.

2.6.1.2 Root associated sucrose transporters

Roots represent heterotrophic carbon sinks and can be divided into zones of cell division, elongation and maturation. Sucrose transporters (DcSUT2) have been identified in developing

taproots from *Daucus carota*. DcSUT2 was expressed in storage parenchyma tissue of *D. carota* taproots where it seems to import sucrose for storage (Shakya and Sturm, 1998). The mRNA of DcSUT2 was mainly expressed in sink organs and was not restricted to the phloem but expressed in xylem. The amino acid sequence of DcSUT2 is closely related to an H^+/Suc symporter from Fava bean which facilitates sucrose uptake in cotyledons of developing seeds indicating a possible common function (Weber *et al*., 1997).

2.6.2 Kinetic properties of sucrose transporters

Heterologous expression of sucrose transporters in yeast and in *X. laevis* oocytes have indicated both high and low affinity kinetic characteristics. Known sucrose transporters were also found to be all influx carriers that co-transport sucrose and protons with a Km for sucrose uptake that ranges from 0.2 to 11 mM (Lemoine, 2000; Weise *et al*., 2000; Schulze *et al*., 2000). The best characterised sucrose transporter subfamily is the SUT1 subfamily, which is required for phloem transport. Studies done on AtSUC1 and StSUT1 sucrose transporters expressed in *X. laevis* oocytes demonstrated H^+-sucrose symport activity (Boorer *et al*., 1996; Zhou *et al*., 1997). PmSUC1 and PmSUC2 were also functionally charaterised using *X. laevis* oocytes (Zhou and Miller, 2000) and the sucrose affinities of AtSUC1, PmSUC1 and PmSUC2 at pH 7.0 were higher than that of the *S. tuberosum* sucrose transporter, StSUT1 (Boorer *et al*., 1996).

The kinetics described for plant sucrose transporters (PmSUC2, AtSUC1, SoSUT1, StSUT1) in yeast, oocytes and plants are similar. The Km values at pH 5.5 for PmSUC2 and AtSUC1 expressed in oocytes were similar to those obtained for sucrose carriers expressed in yeast cells under similar conditions. For example in yeast at pH 4 to 5.5 the reported values for the Km's for sucrose were 1.5, 1.0 and 0.7 mM for SoSUT1, StSUT1 and AtSUC1, respectively (Riesmeier *et al* 1992; 1993; Sauer and Stoltz, 1994). When measured in plant membrane preparations (Lemoine and Delrot, 1989; Bush, 1990; Buckhout, 1994) or estimated *in planta* (Hitz *et al*., 1986), the values of Km

were found to be *ca* 1 mM. There is good evidence that all of these transporters are localised in the phloem and that there main function is the loading of sucrose from the apoplast into the specific cells of the phloem (Gahrtz *et al*., 1996). Most of the sucrose transport carriers characterised to date including StSUT1, show a pH and voltage sensitive Km for sucrose, with the Km value decreasing at more negative voltage and more acidic pH's.

Heterologous expression of PmSUC1 and AtSUC1 sucrose transporters in yeast indicated that they were different from other transporters as they were found to be relatively insensitive to changes in the extra-cellular pH and transport with almost constant uptake rates over a pH range 4.5-6.5 (Sauer and Stoltz, 1994; Gahrtz *et al*., 1995). The similarity of the properties and the pattern of tissue distribution of PmSUC1 and AtSUC1 suggest that they perform similar roles in both species (Zhou and Miller, 2000). AtSUC1 may have a role in anther development and pollen tube growth and PmSUC1 was expressed during seed development (Ghartz *et al*., 1996; Stadler *et al*., 1999)

A subfamily SUT4 was isolated from *A. thaliana* (AtSUT4), *L. esculentum* (LeSUT4) and *S. tuberosum* (StSUT4). These transporters show only 47% similarity on amino acid level to the SUT1 subfamily. AtSUT4 did not saturate at low sucrose concentrations in contrast to the rates for the high affinity sucrose transporter, StSUT1 (Weise *et al*., 2000). Expression of AtSUT4 and StSUT4 in yeast conferred low affinity saturable sucrose uptake activity, SUT4 appears to represent a Low affinity high capacity (LAHC) sucrose transporter system which is localised in SE of sink tissue. The Km for sucrose uptake of AtSUT4 was 11.6 ± 0.6 mM, 10 fold greater than for SUT1 transporters. The StSUT4 had similar properties to the AtSUT4 (Weise *et al*., 2000).

The SUT2 subfamily also shows different kinetic properties from SUT4 and SUT1 transporters. LeSUT2 and AtSUT2 were unable to complement the yeast mutant that was deficient in sucrose uptake (Barker *et al*., 2000). Schulze *et al*., (2000) characterised the transport activity of AtSUT2

directly using ^{14}C-labeled sucrose uptake using yeast cells expressing sucrose transporters and chimeras. This approach was more sensitive, since the Km of *S. tuberosum* sucrose synthase, which cleaves sucrose internally in the strain SUSY7/ura3, was rather high (65 mM) (Salanoubat and Belliard, 1987). Therefore high sucrose uptake rates were necessary to enable the SUSY7/ura3 strain to grow on sucrose as the sole carbon source. The sucrose transporter, AtSUT2 was found to be a low affinity sucrose transporter with a Km of 11.7 mM at pH 4 (Schulze *et al*., 2000). Sucrose uptake decreased rapidly at less acidic pH with no uptake at pH 6.

In Solanaceae, the three types of transporters SUT1, SUT4 and SUT2 differing with respect to kinetic properties co-localise in mature sieve elements (Barker *et al*., 2000, Reinders *et al*., 2002b and Weise *et al*., 2000). The split-ubiquitin system (SUS) was developed in which an interaction of two membrane proteins forces reconstitution of two halves of ubiquitin, leading to cleavage and release of a coupled transcription factor that activates a reporter gene expression. Using this system Reinders *et al*. (2002a) showed that the sucrose transporters SUT1 and SUT2 have the potential to form homo-oligomers and that SUT1, SUT4 and SUT2 were able to interact with each other. The N-terminal half of the low affinity SUT2 interacts functionally with the C-terminal half of SUT1. Since the N-terminus of SUT2 determines affinity for sucrose, the reconstituted chimera has lower affinity than SUT1. The *in vivo* interaction between functionally different Suc transporters indicates that the membrane proteins were capable of forming oligomeric structures, similar to mammalian glucose transporter complexes, that might be of functional significance for regulation of transport.

2.6.3 Sucrose transporter regulation.

Membrane transport activities are qualitatively important for eukaryotic cells, which invest about 12% of their genomic information in transport proteins (Tanner and Caspari, 1996). One of the questions in assimilate partitioning was how plants regulate the allocation of photosynthate between competing sink organs. Sugar transporters might be important control points for the allocation of

carbohydrates and it is therefore to be expected that the activities of these transporters are controlled in a tight and complex way. Transporters can be regulated developmentally and environmentally through transcriptional and post-translation regulation.

2.6.3.1 Developmental regulation

The young leaves import their assimilates symplastically and, during the sink/source transition, a number of events take place that allow apoplastic transport and phloem export. In dicotyledonous plants the transition from photo-assimilate sink to source status begins shortly after the leaf begins to unfold (Turgeon, 1998). There is a decrease of plasmodesmatal density between cells which resulted in the progressive symplastic isolation of the sieve tube/companion transfer cell complex (Borquin *et al*., 1990).

Changes in the expression levels of a *S. tuberosum*, StSUT1, sucrose transporter occured when leaves underwent a sink-to–source transition (Riesmeier *et al*., 1993, Lemoine *et al.,* 1997). A sucrose transporter, VfSUT1 was regulated during seed development in *Vicia faba* (Weber *et al*., 1997). The application of exogenous sugars to developing cotyledons of *V. faba* suppressed both transfer cell differentiation and symporter gene expression, thereby coupling symporter gene expression to the differentiation of a highly specialised cell.

2.6.3.2 Enviromental factors (Biotic and abiotic)

The expression of sucrose transporters is regulated by diurnal factors, salt stress, wounding, ageing and pathogen attack. Light may control the expression and activity of transporters either directly as a physical signal involving specific receptors and/or because it affects the nutrient status of the cells, in particular the sugar content, through photosynthesis (Delrot *et al*., 2000). Sucrose transporters were diurnally regulated in *S. tuberosum*, *L. esculentum*, *N. tabacum and D. carota*. The SUT1 transcript levels and the SUT1 protein in *S. tuberosum* decreased during the dark phase

and increased in the light phase (Kühn *et al*., 1997). In carrot, DcSUT1, which was found only in the green parts of the plant, was diurnally regulated while DcSUT2 which was found in the storage tissue (sink organs) was not regulated diurnally (Shakya and Sturm, 1998).In contrast to the above results, OsSUT1 transcripts were higher in etiolated rice seedlings than in the light grown seedlings (Hirose *et al*., 1997). However, when the expression of OsSUT1 was tested in the rice embryo it was found that light in combination with exogenous sugar induced the expression of OsSUT1 after 36 hours light (Matsukura *et al*., 2003). In comparison to SUT1 transcripts, the diurnal cycle did not cause a significant variation in PmSUC1 and PmSUC2 transcript levels, indicating that all transporters do not behave in the same manner (Stadler *et al*., 1999).

Plants are subjected to a variety of mechanical stress due to wind, herbivory, agricultural practices (pruning, grafting, mowing, etc.) and attacks by pathogens. Cutting (or wounding) involves breakage of some cells and modifications of the apoplectic compartment. Cutting and ageing of leaves from *Beta vulgaris* (Sakr, *et al*., 1997) increased the symporter transcript level of BvSUT1. Cutting stimulated protonmotive force driven sucrose transporter activity in isolated plasma-membrane vesicles, whereas ageing had little effect. In contrast protein ELISA assays indicated that ageing increased the amount of symporter protein present in the plasma membrane. Thus both transcriptional and post-translation regulation of the sucrose transporters occurs (Sakr, *et al*., 1997).

In a follow up experiment by Roblin *et al.* (1998) the effects of okadaic acid which is an inhibitor of protein phosphotase on sugar uptake in *Beta vulgaris* leaf discs and plasma membrane vesicles was investigated. In both systems sucrose uptake was inhibited by 44% in comparison with the controls. An ELISA assay indicated that there was no significant difference in the okadaic acid treated and the control tissue, suggesting that okadaic acid does not affect the synthesis and targeting of the sucrose transporter in the plasma membrane. This indicated that posttranslational control of a *Beta*

vulgaris sucrose transporter involves its phosphorylation status and that sucrose uptake could be inhibited by maintaining the sucrose transporters in the phosphorylated form.

Even though knowledge of transporters is increasing the main focus so far was description and isolation of new transporter clones. There is still a lot to be investigated regarding biochemical and physiological regulation of these transporters

2.7 Sugar sensing

Sugars can also act as regulatory signals that influence gene expression and hence plant development (Koch, 1996; Williams *et al.,* 2000). In relation to resource allocation, the ability to sense altered sugar concentrations could have important advantage by allowing the plant to tailor its metabolism in source tissues to meet demands in sinks. There is great interest in dissecting the signalling processes that are involved in sugar sensing systems in plants.

Evidence for the existence of three different sugar-sensing systems in plants has been obtained in recent years. These are a) a hexose-kinase (HXK) sensing system, b) a hexose transport-associated sensor and c) a sucrose-specific pathway, which may involve a signalling sucrose transporter. The exact mechanism by which plants sense sugar and initiate signal transduction is still largely unknown. Sugars might regulate plant metabolism via sucrose and glucose specific sensor molecules, the nature of which remain unclear. Because plant cells appear to measure the influx of sucrose and glucose into the cytosol, rather than their actual concentrations, it has been postulated that plasma membrane-located sugar transport proteins in conjunction with hexokinase are involved (Smeekens and Rook, 1997). Plant cells were also able to sense rapid changes in sucrose-to-hexose levels either in the apoplast or in the endomembrane system. A specific threshold level of hexose is required before gene expression is induced (Herbers, 1996). The sensor appears to be membrane located, feeding signals toward expression of genes for defense. Long-term shifts in the sucrose-to-

hexose ratio have marked effects on cell differentiation and plant development. Cell wall and vacuolar invertase appear to be involved, but whether the sugar ratio is measured at a membrane or in the cytoplasm is not clear (Sturm and Tang, 1999).

A sucrose transporter like gene, SUT2, has been identified in solanaceous species, which has extended cytoplasmic domains and structurally resembles the yeast sensors RGT2 and SNF3 (Barker *et al*., 2000). This putative sugar sensor, co-localises with SUT1 and SUT4 in the enucleate sieve elements, differs from their kinetic properties and potentially regulates the relative activity of the low and high affinity sucrose transporters in the sieve elements.

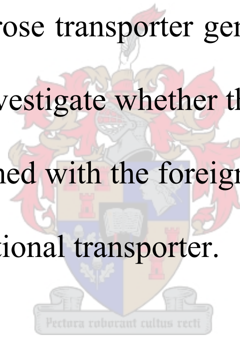
However there may be differences between solanaceous (tomato and potato) and non-solanaceous plants (Plantago and Arabidopsis). PmSUC3 which also has an extended cytoplasmic domain does not co-localise with high and low affinity transporters it is thus unlikely that it will regulate the activity of these transporters directly (Barth *et al*., 2003). The recent discovery of a family of SUT2/SUC3 like transporters in rice, at least one of which includes cytoplasmic extensions similar to this in PmSUC3 and AtSUT2/SUC3 (Aoki *et al*., 2003), broadens the scope for further investigation of the family of sucrose transporters.

2.8 Techniques for characterisation of transporters

Analysis of gene function is of central importance for the understanding of physiological processes. Expression of genes in heterologous organisms has allowed the isolation of many important genes (e.g. for nutrient uptake and transporters) and has contributed a lot to the functional analysis of the gene products. The most common heterologous expression systems used for the expression of transport proteins in plants include yeast complementation studies (Frommer and Ninneman, 1997) and *Xenopus* oocytes (Miller and Zhou, 2000).

2.8.1 Yeast as expression system

In 1978, the development of yeast transformation provided a new way to isolate eukaryotic genes (Hinnen A *et al*., 1978). Shortly thereafter, the development of shuttle vectors allowed complementation of the yeast leu2 mutation with E.coli DNA (Beggs, 1978). The first gene of a higher eukaryote isolated from a cDNA library complementation was the human homologue of a yeast gene controlling the cell cycle (Lee and Nurse, 1987). Since then functional expression has been used frequently to prove the function of genes or to isolate new genes. Complementation of yeast mutants deficient in defined solute transport activities and metabolic functions by plant cDNAs ('suppression cloning') has been a extremely efficient technology to identify and characterize transport protein genes from various plants. This strategy is employed to isolate novel transporters in plants. The principle of this strategy is that yeast is grown on media containing a carbon source (e.g. sucrose). If the sucrose transporter gene of yeast is mutated, the yeast cell will not be able to grow on the media. To investigate whether the isolated gene is functional as a sucrose transporter the mutant yeast is transformed with the foreign gene. The yeast cell will now be able to grow if the foreign gene encodes a functional transporter.


Pectora roborant cultus recti

Advantages of yeasts are that stable expression can be induced by a particular substrate if an appropriate promoter is used. Yeast cells can also be subjected to radiolabeled tracer uptake experiments and large amount of yeast cultures can be grown over a short period of time. The one disadvantage is the size of yeasts cells but patch clamp analysis of heterologous membrane proteins have been done on *Saccharomyces cerevisiae* cells (Bertl *et al*., 1995)

2.8.2 *Xenopus laevis* oocyte as expression system

The basic principle of heterologous expression in oocytes is as follows (Fig. 2.4): Foreign genetic material is introduced into a denuded oocyte by microinjection. The injected material may be message RNA (mRNA) (from tissue extracts), synthetic mRNA (from in vitro transcription of

cDNA), or cDNA. DNA is microinjected directly into the nucleus (located under the animal pole of the oocyte) where it is transcribed and follows the normal pathway to produce a functional protein. The mRNA is microinjected directly into the cytoplasm of the vegetal pole, where it is translated by the endogenous cellular machinery. Oocytes have been shown not only to successfully express injected DNA and RNA, but also to carry out post-translational modifications and target protein products to the plasma membrane as appropriate (Fraser and Djamgoz, 1992).

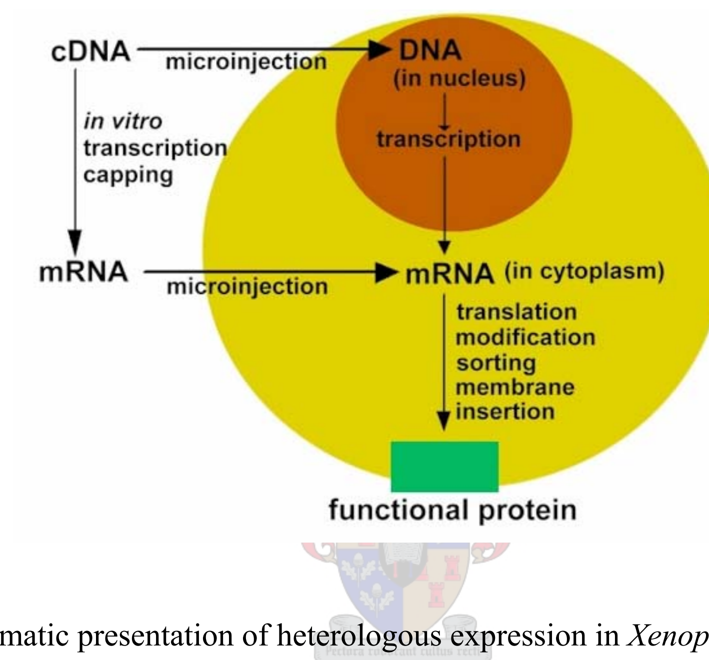


Figure 2.4 Diagramatic presentation of heterologous expression in *Xenopus laevis* oocytes.

The *Xenopus laevis* oocyte has become a major heterologous expression system because of its large size, which makes it easy to handle and to transfer genetic information (Frech *et al.,* 1989; Miller and Zhou, 2000; Zhou *et al*., 1997). Both soluble enzymes and integral proteins have been expressed or cloned into oocytes. A high proportion (>98%) of cells express the genetic information after injection. Post-translational modification and targeting of expressed proteins is possible within this system and little endogenous activity transport activity occurs.

Oocytes are ideal for studying transport processes because they are amenable to tracer uptake studies and electrophysiological analysis. The oocyte can also be used to identify unknown genes if screening systems, such as uptake of radioactive tracers, are available. Alternatively changes in ion

flow can be determined by two electrode voltage clamp analysis, provided the substrate is charged, co-transporters a charged ion, or induces endogenous currents upon uptake. This technique is efficient, because transport can be measured with mixtures of substrates or substrates that are difficult to label (Frommer and Ninneman, 1995).

However the oocytes system also has disadvantages including: a) oocytes show seasonal variation in quality and alteration in the ability of the individual cells to express a foreign message. For this reason, it is useful to test the protein synthetic ability of each batch of oocytes by injecting a number of the oocytes with a test message, which is known to be translated in oocytes, b) Further problem are the short transient expression period which does not last longer than about 2 weeks, c) relatively small number of cells that can be handled in a single experiment, d) heterologously expressed proteins may require co expression of additional subunits that are not available in the oocyte, e) although there is little endogenous transporter activity in oocytes, foreign transporters may interact with or be obscured by endogenous systems

The first successful cloning of a plant sucrose transporter (Riesmeier *et al.,* 1992) was achieved by functional complementation of an engineered *S. cerevisiae* strain unable to efficiently transport and hydrolyse external sucrose, but also unable to metabolise it once it has been taken up. In this way, a cDNA, SoSUT1 (for *Spinacia oleracea* sucrose transporter, Riesmeier *et al.,* 1992) and potato cDNA StSUT1 *Solanum tuberosum*, (Riesmeier *et al.,* 1993) was cloned. The predicted sequences of SoSUT1 and StSUT1 encoded polypeptides (ca 55kDa) were 68% identical. Riesmeier *et al.,* (1992, 1993) found that the predicted amino acid sequences revealed 12 putative transmembrane segments separated by a central hydrophobic loop of 40 amino acids. Analysis of sucrose uptake in the engineered yeast strains expressing SoSuT1 or StSUT1 revealed that these systems do mediate sucrose transport (Km1-1.5 mM). Competition analysis suggested that both systems were specific for sucrose. Sucrose transport activity of both systems is inhibited by protonophores, and stimulated

by decreasing external pH, supporting the hypothesis of proton symport mechanism. Northern blot analysis indicated that the StSUT1 is highly expressed in source tissue (mature leaves) whereas sink tissue (young developing leaves) shows low mRNA. The oocyte system was successfully used to characterise the PmSUC1, PmSUC2 and AtSUC1 transporters (Zhou and Miller, 2000).

Classical molecular genetics has been developed for bacteria, especially for *E.coli*, and suitable expression vectors and hosts are available. The advantages of heterologous expression in *E.coli* include the availability of well-established molecular tools and defined mutants, as well as high growth rates and high yield of overproduced protein. As early as 1976 (Struhl *et al*., 1976), an *E.coli* histidine auxotrophy was complemented by yeast DNA. Disadvantages of the bacteria are the lack of organelles and cellular modification mechanisms responsible for the types of RNA and protein processing found in eukaryotes. Furthermore, eukaryotic polypeptides expressed in *E.coli* often denature and aggregate (Marston, 1986), and many membrane proteins, even from *E.coli* itself, were toxic when over-expressed in bacteria (Schertler, 1992).

Heterologous expression systems are powerful tools for isolating new genes and for characterising proteins from all organisms. The major expression system for plant genes are yeasts and *X. laevis* oocytes. The genes identified so far represent only a small fraction of transporters present in plants. Combinations of these heterologous expression systems are very important to allow better understanding of how plants work.

CHAPTER 3

cDNA Cloning and Tissue-specific Expression of a Putative Sucrose Transporter Gene from Sugarcane

3.1 Abstract

The cloning of a partial sucrose transporter gene (ScSUT1(p)) from sugarcane (*Saccharum hybrid)*, and the expression of a full length putative sucrose transporter gene from sugarcane, ShSUT1 in *Xenopus laevis* oocytes is reported. ScSUT1(p) was isolated from a sugarcane bud cDNA library in this project and ShSUT1 clone was isolated from a stem cDNA library and obtained from Dr C Grof (Plant Industry, CSIRO, Australia). Genomic Southern hybridisation, revealed that the ScSUT1(p) gene is present at a low copy number. The highest level of ScSUT1(p) expression was obtained in the leaves, while within the culm ScSUT1(p) expression was constitutively expressed except for immature internodes 2 and 3. Expression in the lateral buds was also very low. This suggested that ScSUT1(p) expression was higher in source than sink tissues and could be an indication that ScSUT1(p) has a role in both phloem loading and unloading, since it was expressed in leaves as well as sucrose-accumulating and sucrose-storing tissue. When ShSUT1 was expressed into *X. laevis* oocytes, the oocytes accumulated ^{14}C-sucrose, which indicated that it was functional as a sucrose transporter. However, electrophysiological measurements on the oocytes demonstrated no measurable current associated with sucrose challenge to the oocytes indicating that the transporter activity was either very low or possibly non-electrogenic.

3.2 Introduction

Sucrose is a major end product of photosynthesis and is the main transport carbohydrate in higher plants. In the source leaves sucrose is loaded into the phloem cells apoplastically and/or symplastically and translocated to sink tissues via the sieve tubes of the phloem (van Bel, 1993). Sucrose can then either be stored or metabolised. In the process of apoplastic phloem loading, a

H^+/sucrose symporter protein plays an important role in the active uptake of sucrose from the companion cell-sieve tube complex (Ward *et al.,* 1998). Carrier-mediated and proton-coupled sucrose transport activities have been identified in a number of plant species (Lemoine, 2000). The first plant sucrose transporter gene cloned, SUT1, was obtained using a yeast complementation system (Riesmeier *et al.,* 1992).

A proton sucrose co-transport system has been well characterised in dicotyledonous plants (dicots) such as *Arabidopsis thaliana* (Sauer and Stoltz, 1994), *Solanum tuberosum* (Riesmeier *et al.,* 1993), *Daucus carota* (Shakya and Sturm, 1998), *Fava* bean (Weber *et al.,* 1997) and in the monocotyledonous plants (monocots) *Hordeum vulgaris* (Weschke *et al.,* 2000), *Zea mays* (Aoki *et al.,* 1999) and *Oryza sativa* (Hirose *et al.,* 1997). Using *in situ* hybridisation, immunolocalisation and promoter-Gus fusion, the majority of these sucrose transporters have been shown to be expressed in the phloem and particularly in the companion cells (Riesmeier *et al.,*1993; Stadler *et al.,* 1995; Stadler and Sauer, 1996). *Xenopus laevis* oocytes have also been used extensively for the heterologous expression and characterisation of plant sucrose transporters from plants such as *Arabidopsis thaliana* and *Plantago major* (Zhou and Milller, 1997). The oocytes are useful for determining whether transport is electrogenic or non-electrogenic, since the oocytes are large enough to impale and perform electrophysiological measurements with (Miller and Zhou, 2000).

Results from antisense suppression of sucrose transporter levels in transgenic potato and tobacco indicated that sucrose transporters are important for photoassimilate export from source leaves in *Solanaceae* family (Kühn *et al.,* 1996; Riesmeier *et al.,* 1994; Lemoine *et al.,* 1996; Bürkle *et al.,* 1998). Although the antisense repression experiments gave clear evidence for the importance of sucrose transporters in phloem loading of sucrose in source leaves, gene expression analyses revealed sucrose transporters are also expressed in sink tissue such as roots, flowers, pollen, anthers as well as developing seeds (Truernit and Sauer, 1995; Gahrtz *et al.,* 1996; Skakya and Sturm,

1998; Stadler *et al.,* 1999; Lemoine *et al.,* 1999). These results imply that sucrose transporters play a role in sucrose loading in source tissue and in the unloading and/or post phloem transport in sink tissue.

Sugarcane accumulates high concentrations of sucrose in the culm. Young culm internodes use incoming sucrose for growth while the older internodes store sucrose (Glasziou and Gaylor, 1972). In older internodal tissue, sucrose can account for up to 50% of the total dry weight. The first sucrose transporter from sugarcane, ShSUT1, has recently been cloned and functionally characterised in *Saccharomyces cerevisiae (*Rae *et al*., *2004)*. The estimated K_m for sucrose of ShSUT1 was 2 mM and it was expressed predominantly in mature leaves of sugarcane that are exporting sucrose and in the culm tissue that are actively accumulating sucrose. A proposed role for ShSUT1 is for sucrose partitioning between the vascular tissue and sites of storage in the parenchyma cells of sugarcane stem internodes.

In the present study the cDNA cloning of a partial sucrose transporter sequence ScSUT1(p) from a sugarcane bud cDNA library, the RNA expression of this fragment in source and sink tissue as well as the functional activity of a full length sucrose transporter, ShSUT1 in *X. laevis* oocytes is reported.

3.3 Materials and methods

3.3.1 cDNA cloning of a sugarcane sucrose transporter

Field grown mature sugarcane stalks of the variety N19 was used in all experiments. All restriction enzymes used were from Promega, Madison, USA unless otherwise stated. A cDNA library from N19 bud tissue was used (gift from S. Rutherford South African Sugarcane Experiment Station, Durban, South Africa). To isolate the sugarcane sucrose transporter cDNA, PCR primers were designed according to the conservative domains of sucrose transporters from *H. vulgaris* (Weschke

et al., 2000, AJ272309*)*, *O. sativa* (Hirose *et al.,* 1997, (D87819)) *and Z. mays (*Aoki *et al.,* 1999, (AB008464). PCR was performed in a Perkin Elmer cycler on a bud cDNA library using the forward primer: **STF2**, 5'- CTCACTTCATTCATGTGGCTATGCGGTC - 3'; and the reverse primer **STR3,** 5' GG/ideoxyl/ACG ATG/ideoxyl/GATGTT/ideoxyl/AG -3'. The template (1 ng) was combined with 1 X Taq polymerase Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 1U Taq DNA polymerase (Promega, Madison, USA). The first step was one min 94°C, followed by 30 cycles of three steps: 45 s at 94°C, 45 s at 55°C, 2 min at 72°C; followed by 7 min at 72°C and 5 min at 15°C. A 1258 bp fragment designated ScSUT1(p), was amplified and cloned into pGEM-T Easy vector (Promega) and sequenced using the ABI Prism ™ Big Dye Terminator Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The T7 and T3 primers were used to generate sequences for isolated cDNA. Cycle sequencing was performed in GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) and sequence analysis was done using an ABI Prism 373 Genetic analyser (Applied Biosystems, Foster City, CA). The partial sequence was designated ScSUT1(p) for partial sugarcane sucrose transporter. This was a partial cDNA since no ATG and STOP codon was amplified with 3'5' RACE. ScSUT1(p) could not be used for characterisation in *Xenopus laevis* oocytes.

3.3.2 DNA extraction and Southern blot analysis

For Southern blot analysis genomic DNA was extracted from young green leaves of sugarcane N19 variety according to the method of Sambrook *et al.* (1989). Genomic DNA (10 μg per lane), of sugarcane leaf tissue was digested with *Eco*RI, *Bam*HI, *Hind*III and *Pst*I and separated on a 0.8% (w/v) agarose gel. The DNA was denatured by incubating the gel in denaturation buffer containing 1.5 M NaCl and 0.5M NaOH for 15 min, followed by a 15 min neutralisation step in a buffer containing 1 M Tris-HCl (pH7.4) and 1.5 M NaCl. The gel was equilibrated in 10 X SSC (equivalent to 1.5 M NaCl and 0.3 M sodium citrate) and transferred to a nylon membrane (Hybond

N^+ membrane, Amersham Life Science) by upward capillary transfer overnight in 10 X SSC. The DNA was crosslinked to the membrane by UV radiation (1200 mJ m^{-2}) for 2.5 min.

Probe preparation and hybridisation

Probes were prepared by random primer incorporation of α^{32}P-dCTP (Prime it II Random Labelling kit, Stratagene, CA, USA). After preparation, unincorporated label was removed using Qiagen quick spin columns (QIAGEN, CA, USA). The membrane was hybridised with the ^{32}P radiolabelled ScSUT1(p) fragment using the Ultrahyb kit (Ambion inc., Austin, USA) according to manufacturer's instructions at 65°C for 12 h. The hybridised membrane was washed twice with 2 X SSC (150 mM NaCl, 15 mM sodium citrate), 0.1% (m/v) SDS at 42°C for 5 min and twice with 0.1 X SSC, 0.1% (m/v) SDS at 42°C for 15 min. The blot was exposed to a Supersensitive (ST) Cyclone Storage Phosphor Screen (Packard Bioscience, Meriden, US) for 1 to 16 h. The screen was then analysed using a Cyclone Phosphor imager (Packard Bioscience, Meriden, US).

3.3.3 RNA extraction and Northern blot analysis

For Northern blot analysis total RNA was isolated by the guanidine thiocyanate/CsCl method of Chirgwin *et al.* (1979). For analysis of internode specific expression, a combination of different internodal tissue (internode 2 and 3; 4 and 5; 6 to 8; 9 to 11; 12 to 15 and 16), buds and leaves were used in the experiment. Total RNA (20 µg per lane) was separated by electrophoresis in a 1.0 % (w/v) agarose gel at 120 V until the dye font had run 7 cm. The gel was trimmed and stained in ethidium bromide solution (approximately 0.4 µg ml^{-1}) for 15 min and the RNA viewed under a UV light. The gel was then equilibrated in 10 X SSC for 10 min and the RNA was transferred onto the membrane by upward capillary blotting overnight at room temperature using 10 X SSC. After transfer, the membrane was removed, rinsed in 5 X SSC and RNA cross-linked to the membrane by exposure to UV on both sides for 2.5 min at 1200 mJ cm^{-2}. Probe preparation, prehybidisation of

membranes, washing steps, exposure and developing of films was carried out as described for Southern blot analysis (section 3.3.2).

In order to quantify the levels of mRNA expression during sugarcane internode development, the relative pixel unit of the highest units determined for each of the transcripts used as the reference point and given a percentage value of 100. By comparing the highest value with the respective experimental samples, a series of percentages were obtained. These percentages were then used to normalise the experimental data (in relative pixel units).

3.3.4 Expression of ShSUT1 in *Xenopus laevis* oocytes

3.3.4.1 Sub-Cloning of ShSUT1 into Xenopus Expression vector pT_7T_S

Expression levels of heterologous protein in oocytes can be increased by poly-adenylation of the injected RNA to add a poly-(A) "tail" of up to 75 residues and by using an expression vector containing untranslated regions (UTRs) of the *X. laevis* β-globin gene (Miller and Zhou, 2000). The *X. laevis* expression vector pT_7T_s (Krieg and Melton, 1984) was provided by Dr A.J. Miller (Rothamsted Research, UK). This vector is based on the plasmid pGEM4Z (Promega), and has been widely used in heterologous expression studies. The vector contains 5' and 3' β-globin UTRs and a poly- (A) coding region.

Dr C. Grof (Plant Industry, CSIRO, Australia) provided ShSUT1, the full-length cDNA of a putative sucrose transporter from sugarcane, in pGEM-T Easy (Promega, Madison, USA). The clone was designated as ShSUT1 as it was isolated from sugarcane (Saccharum hybrid) stem cDNA library.ShSUT1 fragment consists of 1600nt (Rae *et a*l., *2004*). The pGEMShSUT1 construct, was digested with *Eco*RI to release the ShSUT1 insert. The pT_7T_S vector was dephosphorylated using Shrimp alkaline phosphatase (SAP) (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. The fragment was then blunt-cloned into *Eco*RV site of pT7Ts. To do

this, the *Eco*RI-digested insert was first treated with Klenow DNA polymerase according to manufacturer's instructions. After inactivation of the enzyme at 75°C for 10 min, the blunt-ended insert was ligated with the *Eco*RV digested vector. The ligation products were used to transform DH10Bα competent cells and correctly orientated inserts were verified by restriction digest of isolated plasmid DNA from bacteria. This construct (designated pT_7T_SShSUT1 was used to produce cRNA for oocyte injection.

***3.3.4.2 In vitro* cRNA production**

The construct pT_7T_SShSUT1 was linearised by digestion with *NotI* (Promega, Madison, USA), as positive control AtSUC1 an *Arabidopsis thaliana* sucrose transporter gene in pBluescript (obtained from A.J. Miller, Rothamsted Research, UK) was linearised with *Eco*RI and the cRNA transcribed and capped using an Ambion T7 mRNA mMachine (AMS Biotechnology, Oxford, UK) *in vitro* transcription kit according to the manufacturer's instructions.

Large, sexually mature female *Xenopus laevis* frogs were supplied by Blades Biological (Edenbridge, Kent, UK). Frogs were maintained and oocytes harvested and microinjected with prepared RNA according to Theodoulou and Miller (1995). Single-barrelled micropipettes were produced using unfilamented borosilicate glass capillaries (OD/ID 1.0/0.58 mm, "Kwik-Fil", WPI). Batches of micropipettes were baked at 200°C for 6 h to remove RNAse contamination. The micropipette tip was broken back slightly against a sterile plastic pipette tip before being connected to a microinjector (PLI-100 Pico-Injector, Harvard Apparatus Inc., Holliston, USA) adjusted to deliver approximately 50 nl of liquid per injection. The micropipette was mounted in a micromanipulator and loaded with 1 µl of 1 µg μl^{-1} cRNA or DEPC-water. The vegetal pole of oocytes was injected with 50 nl of either cRNA or DEPC-water (controls) and the injected oocytes were returned to MBS and incubated at 18°C for 5 days before assaying for foreign gene expression.

3.3.4.3 Assaying heterologous expression

Two techniques were used to investigate RNA and water injected oocytes: these were the measurement of sucrose dependent membrane potential depolarisations and uptake of ^{14}C radiolabelled sucrose.

Membrane Potential Measurements

Sucrose elicited membrane potential depolarisations were measured with single barrelled microelectrodes attached to WPI model FD223 high impedance electrometer amplifier (World Precision Instruments, Inc, Florida, USA). Oocytes were clamped in a perspex chamber and perfused with MBS. DEPC-water, AtSUC1 and ShSUT1-injected oocytes were impaled and the membrane potential allowed to stabilise before changing the perfusion solution to MBS containing 5 mM sucrose and monitoring the effects.

Uptake of radiolabelled ^{14}C sucrose.

Oocytes were injected with AtSUC1, ShSUT1 cRNA and DEPC water as negative control. AtSUC1 has been shown to be highly expressed in *X. laevis* oocytes and was therefore used as positive control. Injected oocytes were incubated for 18 to 20 h at 20°C in MBS (pH 7.4) supplemented with 5 mM sucrose to which had been added ^{14}C-labelled sucrose (Amersham Life Science, UK) to give a final concentration of 0.1 µCi ml^{-1}. At the end of the incubation, individual oocytes were washed 5 times in ice cold MBS supplemented with 5 mM sucrose to remove ^{14}C-sucrose from the surface. Washed oocytes were then transferred to scintillation vials containing 250 µl 5% SDS and vortexed to disrupt the cells. The ^{14}C was counted in 4 ml scintillation cocktail (Ultima Gold, Packard Bioscience Ltd, Pangbourne, UK) using a Packard Tricarb 2100 TR liquid scintillation analyser (Packard Bioscience, Meriden, US). To investigate the effect of pH on sucrose uptake the oocytes were incubated in MBS at pH 6, 6.5, and 7.5 and treated the same way for analysis.

3.4 Results

3.4.1 ScSUT1(p) sequencing and verification

A partial cDNA fragment ScSUT1(p) of 1258 bp and *c.* 419 amino acids (Fig 3. 1) was isolated and the sequence was found to show high identity with other sucrose transporters sequences such as *Zea mays* (ZmSUT1), *Hordeum vulgaris* (HvSUT1), *Triticum aevistum* (TaSUT1A) *Oryza sativa* (OsSUT1) and *Arabidopsis thaliana* (AtSUC1) reported in the NCBI database (Table 3.1). ScSUT1(p) nucleotide sequence showed 94% identity to the *ShSUT1* sequence obtained from Dr C Grof (CSIRO, Australia).

Table 3.1 Summary of results obtained from sequence analysis from of ScSUT1(p) followed by alignment with other known sucrose transporters using ClustalW. The identity of ScSUT1(p), 1258 bp with other sucrose transporters. Sequences were obtained from Genebank (NCBI).

Species	**Sucrose transporter**	**Identity (%)**	**Genebank accession numbers; reference**
Zea mays	*ZmSUT1*	88	AB008464; Aoki et al. (1999)
Hordeum vulgaris	*HvSUT1*	78	AJ272309; Weschke *et al*. (2000)
Triticum aestivum	*TaSUT1A*	77	AF408842; Aoki *et al*. (2002)
Oryza sativa	*OsSUT1*	78	D87819; Hirose *et al.* (1997)
Arabidopsis thaliana	*AtSUC1*	56	NM105846; Sauer and Stoltz (1994)
Saccharum hybrid	*ShSUT1*	94	AY780256; Rae *et al*. (2004)

```
ShSUT1   1    MARGDGELELSVGVRGAGAAAADHVAPISLGRLILAGMVAGGVQYGWALQLSLLTPYVQT
                                                                ...:||.::
ScSUT(p) 1    ................................................CGYAVLTSFMWL

ShSUT1   61   LGLSHA.....LTSFMWLCGPIAGLVVQPLVGLYSDRCTARWGRRRPFILTGCILISLAV
               ||.       |||||||||||||||||||||||||||||||||||||||||||||||||
ScSUT(p) 13   CGLTSFMWLCGLTSFMWLCGPIAGLVVQPLVGLYSDRCTARWGRRRPFILTGCILISLAV

ShSUT1   116  IVVGFSSDIGAALGDTKEHCSLYHGPRWHAAIVYVLGFWLLDFSNNTVQGPARAMMADLC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ScSUT(p) 73   IVVGFSSDIGAALGDTKEHCSLYHGPRWHAAIVYVLGFWLLDFSNNTVQGPARAMMADLC

ShSUT1   176  GHHGPSAANSIFCSWMALGNILGYSSGSTNNWHKWFPFLKTNACCEACANLKGAFLVAVV
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ScSUT(p) 133  GHHGPSAANSIFCSWMALGNILGYSSGSTNNWHKWFPFLKTNACCEACANLKGAFLVAVV

ShSUT1   236  FLVICLAVTLIFAKEVPYRGNENLPTKANGEVEAEPTGPLAVLKGFKNL.PPGMPSVLLV
              |||||||||||||||||||||||||||||||||||||||||||||:..  ||| ..||||
ScSUT(p) 193  FLVICLAVTLIFAKEVPYRGNENLPTKANGEVEAEPTGPLAVLKGLQRTCPPGCRPVLLV

ShSUT1   295  TGLTWLSWFPFILYDTDWMGREIYHGDPKGSNAQISAFNEGVRVGAFGLLLNSIILGFSS
              ||||||||||||||||||||||||||||||.|||||||||||||||||||||||||||||
ScSUT(p) 253  TGLTWLSWFPFILYDTDWMGREIYHGDPKGTNAQISAFNEGVRVGAFGLLLNSIILGFSS

ShSUT1   355  FLIEPMCRKLGPRVVWVTSNFMVCVAMAATALISYWSLKDYHGYVQDAITASTNIKAVCL
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ScSUT(p) 313  FLIEPMCRKLGPRVVWVTSNFMVCVAMAATALISYWSLKDYHGYVQDAITASTNIKAVCL

ShSUT1   415  VLFAFLGVPLAILYSVPFAVTAQLAATKGGGQGLCTGVLNISIVIPQVIIALGAGPWDAL
              |||||||||||||||| ||||||||||||||||||||||||.|  |.
ScSUT(p) 373  VLFAFLGVPLAILYSVLFAVTAQLAATKGGGQGLCTGVLNIPIRHPN.............

ShSUT1   475  FGKGNIPAFGVASGFALIGGVVGVFLLPKISKRQFRAVSAGGH

ScSUT(p) 420  ...........................................
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Figure 3.1 Comparison of the amino acids sequence deduced from the cDNa clones ShSUT1 and ScSUT1(p). The amino acid sequences are in the one letter code. Identical amino acid residues are indicated by (|) and the highlighted regions represent putative membrane spanning domains.

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3.4.2 Southern blot analysis

A genomic Southern blot analysis was carried out to estimate the number of genes coding for sucrose transporters in the sugarcane genome related to ScSUT1(p). The restriction sites used to digest the genome DNA were not present in the 1258 bp ScSUT1(p) fragment used as a DNA probe. The probe hybridised to a single band in all cases (Fig. 3. 2).

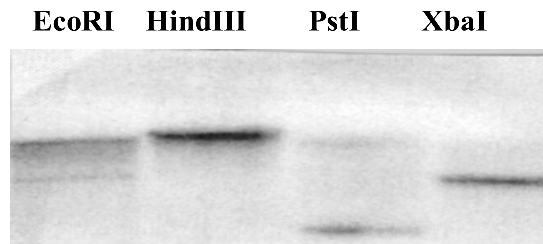

Figure 3. 2 Southern blot analysis of ScSUT1(p) gene in sugarcane genomic DNA. 10 µg of DNA per lane from the sugarcane leaves was digested with the restriction enzymes indicated and separated on a 0.8% (w/v) agarose gel. The partial sequence, ScSUT1(p) cDNA fragment 1258 bp fragment was used as a probe.

3.4.3 Northern blot analysis

Northern blot analyses were performed to determine the tissue specific expression of ScSUT1(p) (Fig. 3.3a, b, c). Very low expression of ScSUT1(p) was found in internodes 2 to 3 and in lateral buds. ScSUT1(p) expression was constitutively expressed in sucrose accumulating and sucrose storing internodes. Of the tissues analysed, leaf tissue exhibited by far the highest expression levels.

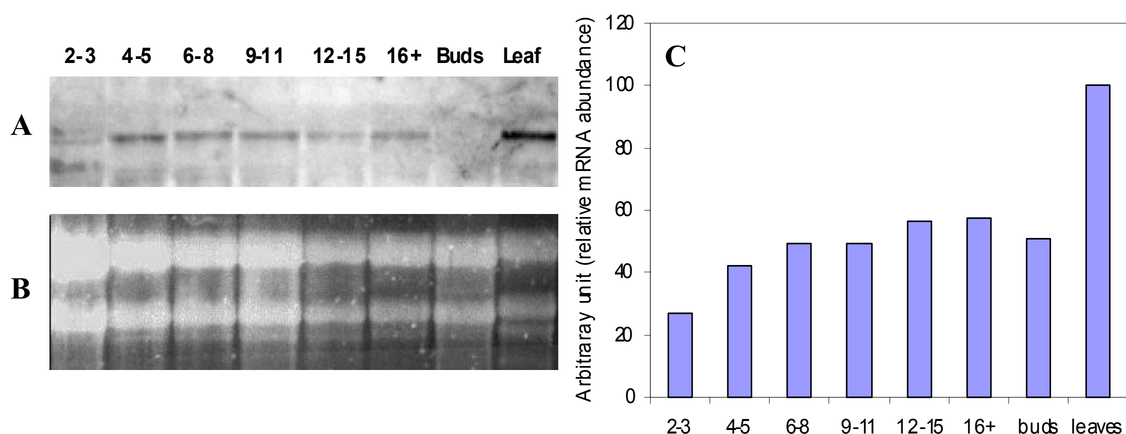

Figure 3.3 (A) Tissue specific expression of a ScSUT1(p), in internodes 2-3, 4-5, 6-8, 9-11, 12-15, 16-18, buds and leaves (B) with a corresponding gel of total sugarcane RNA loaded (20 µg).
(C) Signal intensity was quantified with a phospho-imager and expressed as an arbitrary unit on the basis of total RNA.

3.4.4 Electrophysiological measurements

As a control to measure oocyte health and responsivenes, uninjected oocytes were incubated in 10 mM NH_4Cl (Fig. 3.4A). Oocytes have an inherent non selective cation channel through which NH_4^+ passes. A clear response was visible with addition of 10 mM NH_4Cl. No significant effect on the membrane potential of the DEPC-water-injected oocytes (controls) was observed upon exposure to MBS supplemented with 5 mM sucrose. A depolarisation of 40 mV was observed within a minimum of 10 min after exposure to MBS supplemented with 10 mM NH_4Cl (Fig. 3. 4A). Thereafter the membrane potential recovered slowly, returning to the resting membrane potential level after an additional 15 to 20 min. This indicates that the oocytes were physiologically responsive. AtSUC1 cRNA was used as a positive control since it has been shown to have high expression levels in *X. laevis* oocytes (Fig. 3.4B) (Zhou *et al.,* 1997). An almost immediate depolarisation was observed when MBS supplemented with 5 mM sucrose was added to the bathing solution. The membrane potential depolarised by 60 mV and recovered 10 to 15 min after the sucrose was removed and replaced with MBS. A very slight (5 mV) depolarisation was observed when ShSUT1 injected oocytes were subjected to MBS supplemented with 5 mM sucrose (Fig. 3.4C). After the sucrose was replaced with MBS solution the membrane potential returned to the resting membrane potential indicating that this was a physiological response and that the sucrose transporter was functioning.

Pectora roborant cultus recti

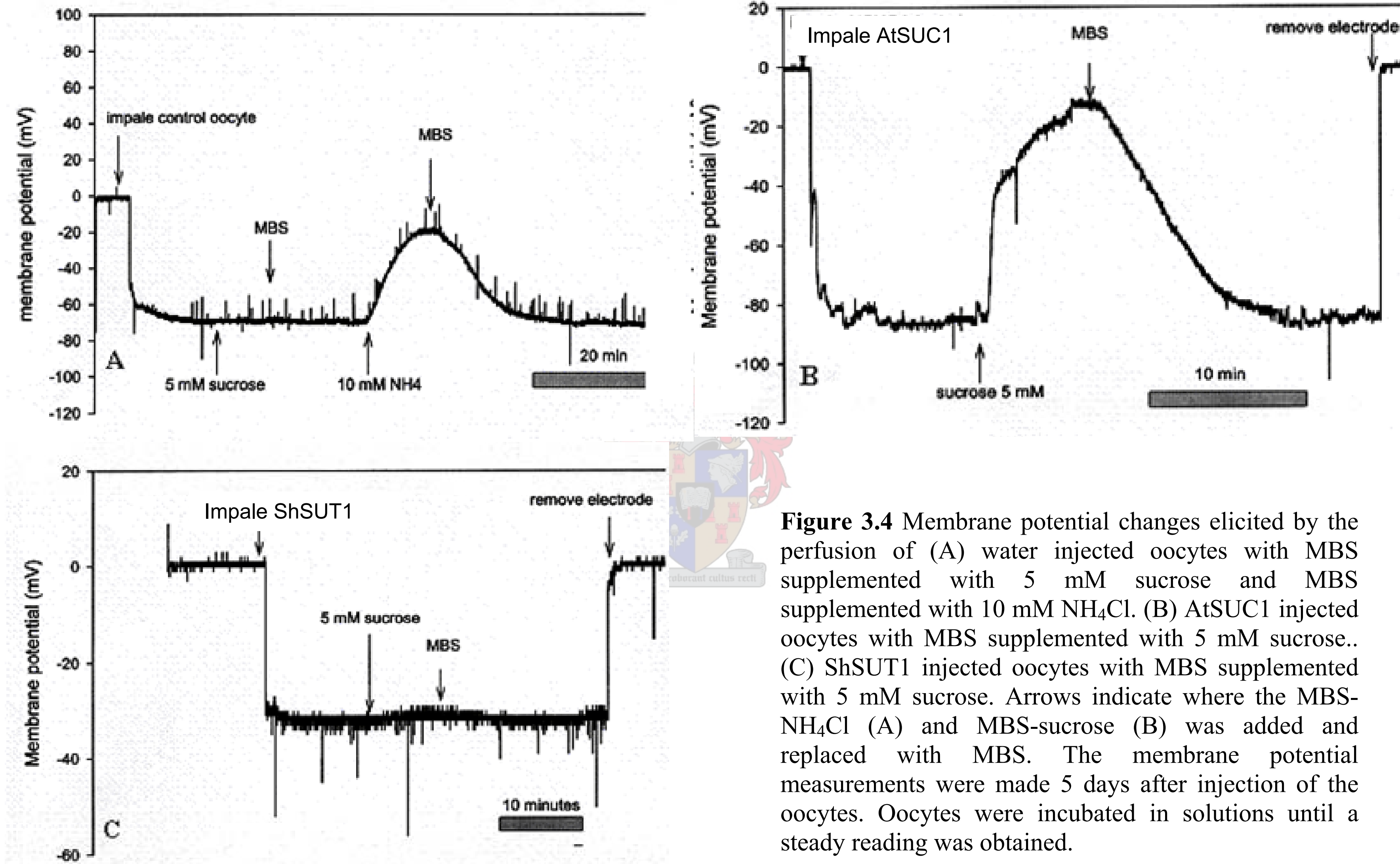

Figure 3.4 Membrane potential changes elicited by the perfusion of (A) water injected oocytes with MBS supplemented with 5 mM sucrose and MBS supplemented with 10 mM NH_4Cl. (B) AtSUC1 injected oocytes with MBS supplemented with 5 mM sucrose.. (C) ShSUT1 injected oocytes with MBS supplemented with 5 mM sucrose. Arrows indicate where the MBS-NH_4Cl (A) and MBS-sucrose (B) was added and replaced with MBS. The membrane potential measurements were made 5 days after injection of the oocytes. Oocytes were incubated in solutions until a steady reading was obtained.

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3.4.5 Uptake of radiolabelled [^{14}C] – sucrose

The efficacy of 10 mg ml^{-1} streptomycin sulphate and sodium penicillin sulphate was tested and it was found that the oocytes that were incubated in solution containing antibiotics yielded results that were less variable than those incubated without antibiotics, especially when incubation was over relatively long periods of time (10 to 18 h) (results not shown). For all work done thereafter, antibiotics were added to the incubation medium. Both cRNA's AtSUC1 and ShSUT1 were expressed in injected oocytes (Fig. 3.5). AtSUC1 was used as a positive control since it has been known to be highly expressed in *X. laevis* oocytes. The rate of sucrose uptake in the AtSUC1 injected oocytes was 3-fold higher than the uptake rate of water injected oocytes. The ShSUT1 injected oocytes also showed 2-fold higher uptake rates than that of the control oocytes. This experiment was repeated three times.

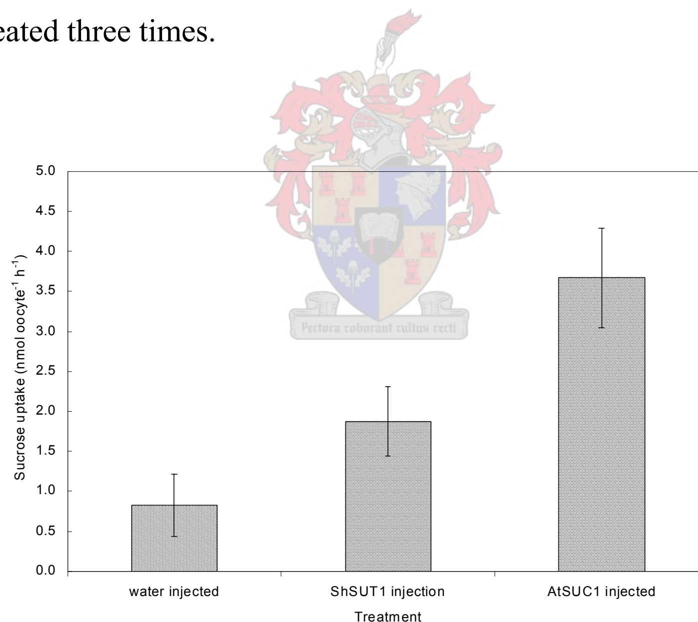
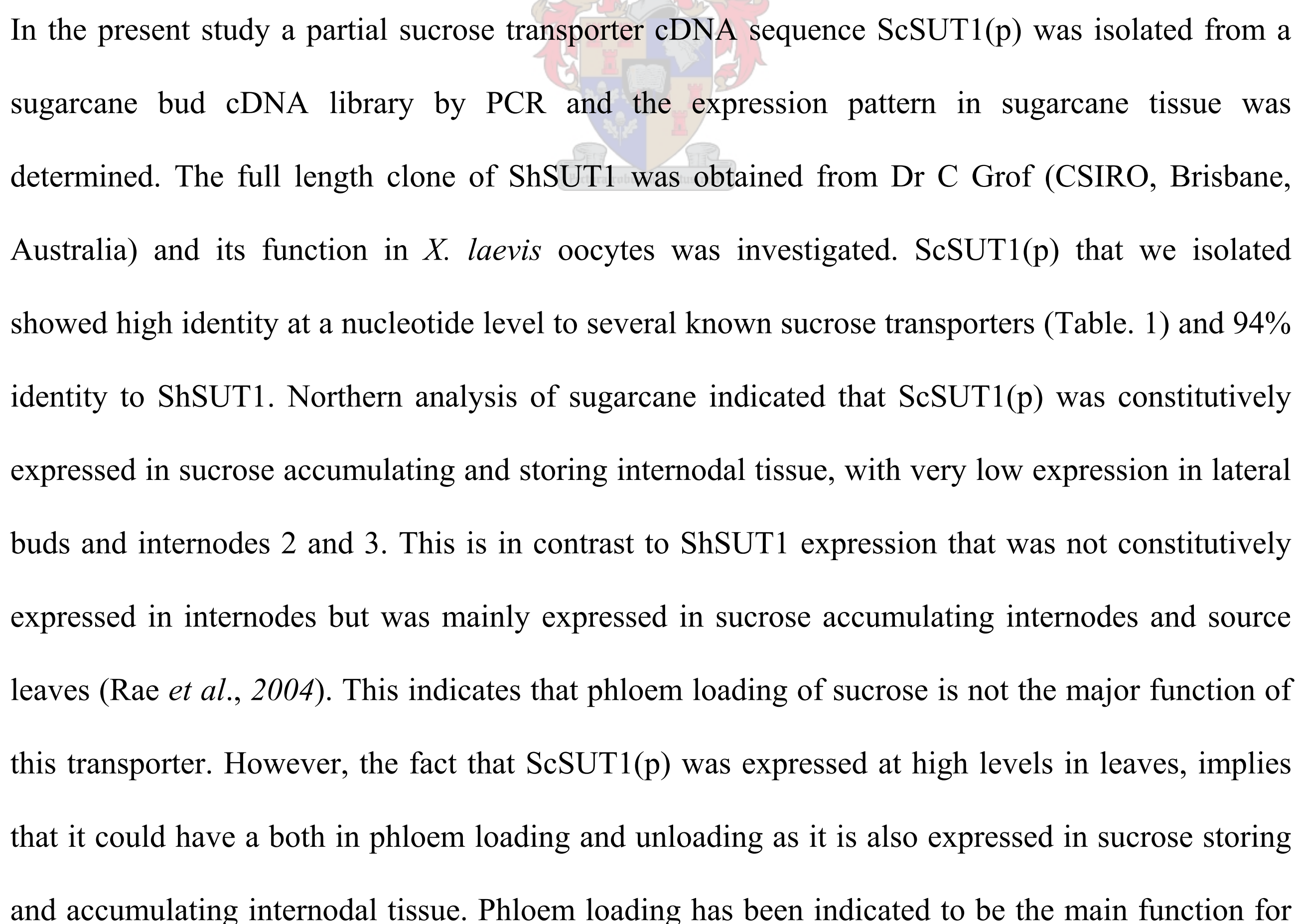


Figure 3.5 Uptake rate of 5 mM ^{14}C labelled sucrose in water injected (control), ShSUT1 cRNA injected and AtSUC1 cRNA injected oocytes. Oocytes were incubated in MBS containing 5 mM sucrose at pH 7.5 for 18 h at 18 °C. Bars indicate ± SE (n=10).

3.5. Discussion

Sugar transporters are key players in many fundamental processes in plant growth and are likely to be control points in the transport of sugars across pathways. Casu *et al.* (2003) identified several putative sugar transporters in an expressed sequence tag (EST) collection of sugarcane and analysed microarrays to correlate their expression with sucrose accumulation. A sequence survey of 7242 ESTs derived from the sucrose-accumulating, maturing stem revealed that transcripts for carbohydrate metabolism gene sequences (CMGs) are relatively rare in this tissue. However, within the CMG group, putative sugar transporter ESTs form one of the most abundant classes observed. Rae *et al*. (2004) isolated and characterised a sucrose transport gene, ShSUT1, from sugarcane, which corresponded to the EST designated PST6 (putative sugar transporter). ShSUT1 was functionally characterised in *S. cerevisiea* and had an apparent K_m for sucrose of 2mM that was higher than the K_m for high affinity sucrose transporters.



In the present study a partial sucrose transporter cDNA sequence ScSUT1(p) was isolated from a sugarcane bud cDNA library by PCR and the expression pattern in sugarcane tissue was determined. The full length clone of ShSUT1 was obtained from Dr C Grof (CSIRO, Brisbane, Australia) and its function in *X. laevis* oocytes was investigated. ScSUT1(p) that we isolated showed high identity at a nucleotide level to several known sucrose transporters (Table. 1) and 94% identity to ShSUT1. Northern analysis of sugarcane indicated that ScSUT1(p) was constitutively expressed in sucrose accumulating and storing internodal tissue, with very low expression in lateral buds and internodes 2 and 3. This is in contrast to ShSUT1 expression that was not constitutively expressed in internodes but was mainly expressed in sucrose accumulating internodes and source leaves (Rae *et al*., *2004*). This indicates that phloem loading of sucrose is not the major function of this transporter. However, the fact that ScSUT1(p) was expressed at high levels in leaves, implies that it could have a both in phloem loading and unloading as it is also expressed in sucrose storing and accumulating internodal tissue. Phloem loading has been indicated to be the main function for

sucrose transporters described in plants such as *N. tabacum* (Bürkle *et al*., 1998), *S. tuberosum* (Riesmeier *et al*., 1994, Kühn *et al*., 1997) and *P. major* (Gahrtz *et al*., 1994) but sucrose transporters have been localised in sink tissue tissue of *D. carota* (Shakya and Sturm,1998) as well as floral tissues (Gahrtz et al.,1996; Stadler et al.,1999). Further studies are required to confirm this result for sugarcane.

In dicotyledonous as well as monocotyledonous species, sucrose transporter gene copy number is low (Riesmeier *et al.,* 1993; Sauer and Stoltz, 1994; Gahrtz *et al.,* 1994; 1996; Hirose *et al*, 1997; Aoki *et al.,* 1999). For many of these species two or more SUT genes have been reported (Lemoine, 2000). Genomic Southern analysis, under low stringency conditions with a variety of enzymes and ScSUT1(p) as a probe gave a strong single band, indicating a low copy number of ScSUT1(p). All sucrose transporters identified to date in rice are members of a single gene family (Aoki *et al*., 2003) and similar characteristics would be expected for sugarcane sucrose transporters.

Both the membrane potential data and the labelled sucrose uptake experiment indicated that ShSUT1 was functional in *X. laevis* oocytes. However the expression of ShSUT1 was much lower than that of the positive control AtSUC1, but higher than the control oocytes. It has been found in Solanaceous species that three members of the sucrose transporter family with different properties co-localise in the same enucleate sieve element and can interact with each other to form hetero- and homo-oligomers (Reinders *et al.,* 2002b). The ShSUT1 protein showed very low electrogenic activity in *X. laevis* oocytes and this could be an indication that ShSUT1 might require more than one protein or RNA species to be active, or that the transporter is not electrogenic. This may be because the functional complex contains more than one protein subunit (Kroll *et al*., 1991), or because a second protein mediates correct assembly as in the case of the Na^+/K^+ ATPase (Noguchi *et al*. 1990). The phosphorylation status of sucrose transporter could also be a reason why the activity of ShSUT1 was so low. Roblin *et al*. (1998) presented evidence that sucrose uptake was

inhibited by maintaining the sucrose transporters in the phosphorylated form in sugarbeet. Stimulation of endogenous protein kinases in *X. laevis* oocytes changed the kinetic properties of plant carriers, indicating a role for phosphorylation in modifying the activity of carriers (Miller and Zhou, 2000).

There are several examples of plant genes that do not produce functional proteins when expressed in oocytes. For example, the Arabidopsis K^+ channel, AKT1, although successfully expressed in both yeast and insect heterologous expression system, does not produce functional protein in oocytes. In contrast, a related protein, KT3, has been successfully expressed in oocytes (Schachtman *et al*., 1992). There are several reasons that expression fails in oocytes: 1) oocyte quality can be variable between batches and developmental stages and shows seasonal variation; 2) heterologously expressed proteins may require co-expression of additional subunits that are not available in the oocyte; 3) although there is little endogenous transporter activity in oocytes, foreign transporters may interact with or be obscured by endogenous systems (Miller and Zhou, 2000); 4) standard oocyte saline contains higher NaCl (100 mM) than is usually encountered by plant cells, which may affect transporter expression and function.

Conclusion

^{14}C-sucrose uptake demonstrated that ShSUT1 is a functionally active gene for sucrose uptake, although expression levels of this gene in *X. laevis* were too low to characterise its activity. The results gives evidence against electrogenic transport by ShSUT1 or at least that AtSUC1 has a different H^+/sucrose stoichiometry to ShSUT1. Sucrose transporters isolated to date are all located on the plasmamembrane and are influx carriers. ShSUT1 might be localised on the tonoplast and could possible be a carrier involved in facilitated diffusion or a channel. A strong case for facilitated transport of sucrose across vacuoles in barley mesophyll was made by Kaiser and Heber (1984).

Further investigation is required to characterise the specific mechanism and kinetic properties of ShSUT1 as well as the membrane location *in planta*.

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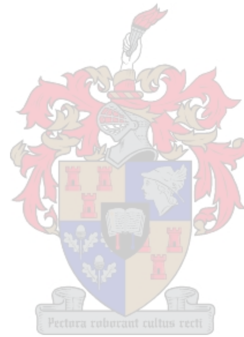


CHAPTER 4

Uptake of glucose, fructose and sucrose by sugarcane culm tissue.

4.1 Abstract

Cell wall invertase plays an important role in sucrose metabolism and the hydrolysis of sucrose into hexoses could significantly affect the sucrose uptake mechanism. Tissue discs from internodes of different developmental stages were cut from field grown sugarcane plants (cv. N19) and the uptake of ^{14}C-labelled glucose, fructose and sucrose measured. The uptake rates were measured at varying, pH, temperature and concentrations of sugars. Evidence for a diffusion and transporter component was described. PCMBS (*p*-chloromercuribenzenesulphonic acid) inhibited sucrose uptake at concentrations below 10 mM and this effect was more marked than TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) inhibition. Sugarcane cell wall invertase extracted from internodal and leaf extracts was not significantly inhibited by TRIS even at concentrations of 100 mM. The Q_{10} value, calculated between 25°C and 15°C, possibly indicated reduced dependence on respiratory metabolism for uptake at sucrose concentrations above 0.4 mM. Hexoses were found to be the major sugar taken up and sucrose was only important when little hexose was available, as was found in the mature ripe internodes.

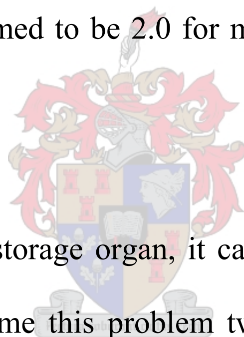
4.2 Introduction

Sucrose is the most important transport carbohydrate in many plants. In sugarcane, unlike in other plants, sucrose is also a major storage product in the culm rather than in the fruit or in the roots (Moore, 1995). The movement of photosynthate into and within sink regions of economically important plants such as sugarcane is one of several parameters that influence crop yield and productivity. Phloem unloading in storage tissue has been suggested to occur through both a symplastic and an apoplastic pathway (Patrick, 1997; Patrick, 2001). Symplastic transport would involve direct transfer of sucrose from the phloem to the storage parenchyma cells via

plasmodesmata. In contrast, apoplastic unloading would involve the transport of sucrose into the apoplast of sink tissue via sucrose transporters, followed by uptake of sucrose or its cell wall invertase generated hydrolysis products into storage cells. Sucrose transporters identified to date have K_m values for sucrose uptake ranging between 0.2 and 11 mM (Lemoine, 2000; Weise *et al*., 2000; Schulze *et al*., 2000). A sucrose transporter cloned from sugarcane (ShSUT1) had an apparent K_m for sucrose of 2 mM (Rae *et al*., *2004*). Several inhibitors of H^+/sucrose symport such as *p*-chloromercuribenzenesulphonic acid (PCMBS) and N-ethylmaleimide (NEM) have been used to determine whether sucrose uptake is linked to proton flux through a H^+/sucrose co-transport system (Thom and Maretzki, 1992; Bush, 1993) and H^+/ sucrose symporters have been identified in many plants (Lemoine, 2000). NEM and PCMBS bind to sulphydral groups (-SH) in the proteins and therefore may indicate a critical –SH group for function in the protein.

The uptake and release of sugars, mainly in the form of hexose, occurs through the plasmalemma of sugarcane culm cells. Furthermore, there is a cycle of synthesis and degradation that occur in these cells (Glasziou and Gayler, 1972). This has been confirmed and quantified in sugarcane suspension cells (Wendler *et al*., 1990). Both Lingle (1989) and Thom and Maretzki (1992) presented evidence that sucrose can be taken up by sugarcane cells prior to being cleaved to hexoses. Hexose levels have been shown to be positively correlated with neutral invertase levels but not with soluble acid invertase levels in mature sugarcane stem tissue (Glasziou and Gayler, 1972; Vorster and Botha, 1999; Botha *et al.,* 2001). Thus, sucrose synthesis from hexoses in the storage cells might be an essential component for control of the levels of sucrose storage. These findings suggest that the cleavage of sucrose by cell wall invertase, the uptake of hexoses by active transport systems, and the synthesis of sucrose in the cells from hexoses are important in determining sucrose storage. Komor *et al*. (1996) re-evaluated these findings and found that sucrose uptake in sugarcane is very low in comparison to that of hexose uptake, except in old ripe internodes, where all fluxes were small.

In previous studies, it was found that the growth of sugarcane plants at varying temperatures had a profound effect on several parameters of growth and sugar storage. The most prominent effect on growth and sugar storage was the inhibition of sugar translocation out of the leaves at low temperatures (15°C) and acceleration of sugar consumption at high temperature (45°C) (Ebrahim *et al*., 1998). An optimum glucose uptake rate was found at 45°C in sugarcane cell suspension cultures (Ebrahim *et al.,* 1999), but no temperature dependence was found for sucrose uptake. Sucrose accumulation in suspension cells was very low relative to the sucrose concentration in the medium. It was speculated that the low sucrose level in suspension cells was due to the low degree of vacuolisation and the substantially faster sucrose transport at the tonoplast of suspension cells compared to internodal tissue. The proportional change in respiration per 10°C in temperature is known as the Q_{10} and can be used to determine the metabolic component of respiration (Atkin and Tjoelker, 2003). The Q_{10} value is assumed to be 2.0 for metabolic processes and less than 2.0 for chemical processes such as diffusion.



Since the sugarcane culm serves as a storage organ, it cannot be sampled without sacrificing the entire plant (Moore, 1995). To overcome this problem two approaches have been used to study metabolism, namely a) cell suspension cultures derived from callus material (Komor *et al*., 1981; Wendler, 1990) and b) samples of internodal tissue (Bieleski, 1962; Glasziou and Gayler, 1972, Komor *et al*., 1996). Cell suspension cultures show atypical sucrose metabolism in that only 1 to 6% of total carbon is allocated to sucrose (Veith and Komor, 1993), compared to 66% in tissue discs of mature sugarcane tissue (Whittaker and Botha, 1997). Furthermore, vacuolisation is 40% lower in cell suspension cultures than in the culm tissue (Komor, 1994). The tissue disc system has its own limitations such as wound response caused by tissue slicing, long diffusion pathways of labelled sugars in tissue discs, and the inability to repeatedly and precisely associate morphological stage of internode development with sucrose levels (Moore and Maretzki, 1997; Lingle, 1989) Despite some of the limitations associated with the tissue disc system, it would appear to be the best

way to approximate the intact sugarcane culm. Careful selection of osmotic conditions for incubation medium and washing time of tissue after wounding (Whittaker and Botha, 1997; Lingle, 1989) significantly contributes to eliminating some of the potential problems with tissue discs. Short term labelling experiments on tissue discs (Vorster and Botha, 1999) can also help to reduce possible disruption of metabolism caused by depletion of substrate after long incubation periods.

In this study, *in vivo* ^{14}C uptake experiments were undertaken using tissue slices to determine the influence of temperature, pH, sugar concentrations and inhibitors on sucrose, glucose and fructose uptake in sugarcane (cv. N19). PCMBS and TRIS were used as inhibitors for sucrose uptake inhibition studies.

4.3 Materials and Methods

4.3.1 Biochemicals

All coupling enzymes, co-factors and substrate for sugar determinations were purchased from Sigma-Aldrich (USA), or Roche Biochemicals (Roche, Germany). The [U-^{14}C]-sucrose, glucose and fructose were from Amersham international (Amersham, UK).

4.3.2 Plant Material

Mature, non-flowering stalks from field grown sugarcane plants (cv. N19) were sampled between 9h00 and 10h00. The natural break point of the sugarcane stalk was defined as internode 3, and internodes below this were numbered sequentially. Internodes 2, 3 and 4 represent immature tissue, internodes 5 to 8 intermediate tissue, and internodes 9 to 15 represent mature tissues, which have accumulated high levels of sucrose. Leaf tissue of the same plants was used for the determination of cell wall invertase activity.

4.3.3 Sugar uptake [^{14}C] radiolabelling measurements

Internodes were excised and longitudinal cores were taken from internodes at different stages of maturity from mid-way between the core and periphery of the internode using a 5 mm diameter cork borer. One mm slices were sectioned from these cores using a hand microtome. The slices were washed for 15 min in ice-cold incubation buffer containing 250 mM mannitol, 25 mM K-MES (pH 5.7) and 1 mM $CaCl_2$ (Lingle, 1998; Whittaker and Botha, 1997) to remove solutes exuded from cut cells. Discs were then incubated at 25°C in different concentrations of radioactively labelled sugars in incubation buffer, keeping the osmotic strength constant at 250 mM by balancing sugar concentrations with mannitol. Tissue slices were removed from the incubation buffer at 10 min intervals and rinsed twice for 10 min in ice cold 250 mM mannitol, 25 mM K-MES (pH 5.7) and 1 mM $CaCl_2$. In the experiments in which pH was varied, the discs were incubated in the same buffer but at pH's 4, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5. Linearity of sucrose uptake after 10 min was tested (data not shown). After incubation, the tissue slices were removed and placed into scintillation vials containing 200 µl Soluene-350 (Packard Bioscience, Il, USA) for 4 h to disintegrate the tissue. After the tissue was solubilised, 4 ml of scintillation cocktail (Ultima Gold, Packard) were added and the samples were analysed in a Packard Tricarb 2100 TR liquid scintillation analyser (Packard Bioscience, Meriden, USA).

In order to inhibit sucrose uptake, 1 mM PCMBS was added to the discs 5 min prior to sucrose and incubated as described above. Substrate inhibition studies were done by adding 1, 10, 50 and 100 mM cold glucose to incubation buffer containing ^{14}C sucrose at 0.5 and 10 mM sucrose. For temperature dependent sucrose uptake experiments, the tissue was incubated at 15°, 25° and 45°C. The contribution of cell wall invertase to sucrose uptake was determined by incubating tissue slices in incubation buffer (pH 5.7) with unlabelled sucrose. Glucose concentration was determined spectrophotometrically according to Bergmeyer and Bernt (1974).

4.3.4 Cell wall invertase assay

Cell wall invertase was extracted according to the method of Albertson *et al*. (2001) with modifications. Leaf tissue and culm tissue from internode 5 were homogenised in liquid nitrogen. Tissue (1 g) was resuspended in 5 ml of extraction buffer containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) Triton X-100, 5 mM DTT, 0.2% (w/v) insoluble PVPP and 1 tablet of COMPLETE proteinase inhibitor (Roche). The extraction was mixed every 2 min for 10 min at 25°C and then centrifuged at 8000 g for 10 min at 4°C and the pellet was washed in 10 ml, extraction buffer and incubated for 10 min at 25°C with mixing every 2 min. After centrifugation at 8000 g for 20 min the pellet was resuspended in 900 µl 50 mM citrate/phosphate buffer (50 mM citrate, pH with 1 M Na_2HPO_4) (pH 3.5) and a 10 µl aliquot taken for later analysis. The reaction was initiated by the addition of 100 µl sucrose (100 mM) to the resuspended pellet and an 80 µl aliquot removed immediately. The aliquot was added to 160 µl of a 4 M imidazole solution and boiled for 3 min to terminate enzyme activity. The resuspended pellet was incubated on a shaker for 60 min at 37°C with three acid washed 5 mm glass beads. To account for acid hydrolysis of sucrose, a control of 100 mM sucrose in 50 mM citrate/phosphate buffer (pH 3.5) was analysed for glucose produced. Glucose formed by acid hydrolysis was subtracted from the glucose measured for invertase activity. Glucose concentration was determined as described above.

4.4 Results

4.4.1 Concentration dependence of sucrose uptake

Uptake of sucrose at sucrose concentrations between 0.2 and 250 mM exhibited linear kinetics (Fig. 4.1). The rate of sucrose uptake increased as the sucrose concentration increased. At sucrose concentrations from 0 to 20 mM sucrose uptake might saturate (Fig. 4.1 insert) and then continues to follow diffusion like kinetics above 20 mM sucrose. It is not possible to differentiate between the two components of sucrose uptake.

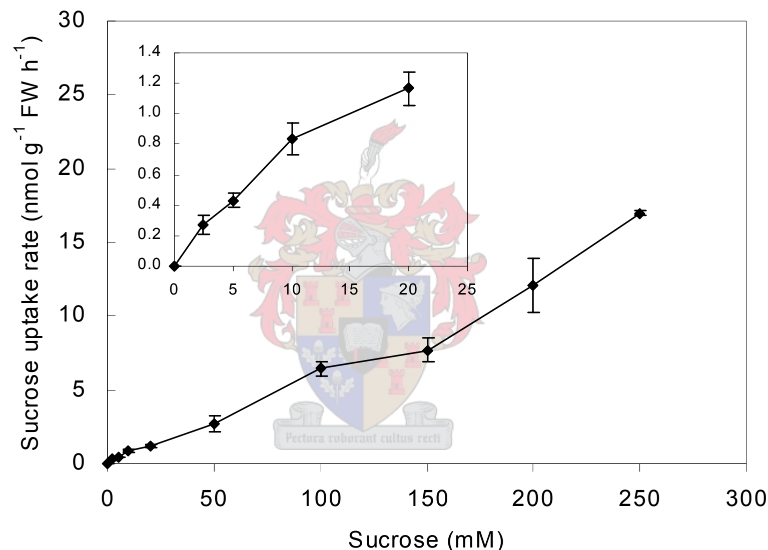


Figure 4.1. The rate of sucrose uptake by tissue discs cut from internodal parenchyma (internode number 5) and incubated in 0.2 to 250 mM ^{14}C-sucrose. Tissue discs were incubated for 10 min at 25°C in incubation buffer containing 250 mM mannitol, 25 mM K-MES (pH 5.7) and 1 mM $CaCl_2$ with the different sucrose concentrations. Bars indicate mean ± SE (n = 5). The inserted figure represents the sucrose uptake rate at concentrations between 0 and 20 mM sucrose

The young internodes took up the sugars at a much higher uptake rate at sugar concentrations ranging from 50 to 250 mM (Fig. 4.2A-C). The sugar uptake rate by the internodes was strongly dependent on the type of sugar. Glucose and fructose were taken up rapidly in comparison to sucrose. There was a decline in uptake rate of sugars as the internodal tissue matured. Hexoses were taken up rapidly at high external concentrations (50 to 250 mM) by young internodes, while sucrose

uptake was very slow (Fig. 4.2A-C). In the older internodes (internodes 4 to 15) the rate of sucrose uptake was similar to glucose and fructose at all sugar concentrations (Fig. 4.2A-C). All internodes studied appear to have linear uptake kinetics for sucrose as well as hexoses in sugarcane culm tissue up to 100 mM, whereafter a possible saturation occurs (Fig. 4.3A-D). However, the data could not be used to plot Michealis Menten kinetics since a clear saturation was not observed. A linear regression curve between 15 and 100 mM was plotted to simulate diffusion. The intercept was then used to approximate the V_{max} of active uptake. Thereafter the uptake was corrected by assuming that uptake is saturated at the calculated V_{max} . Using these calculation a hyperbolic curve was obtained and a K_m value of 2 mM and 3 mM was estimated for sucrose and glucose respectively (Fig. 4.4A-B).

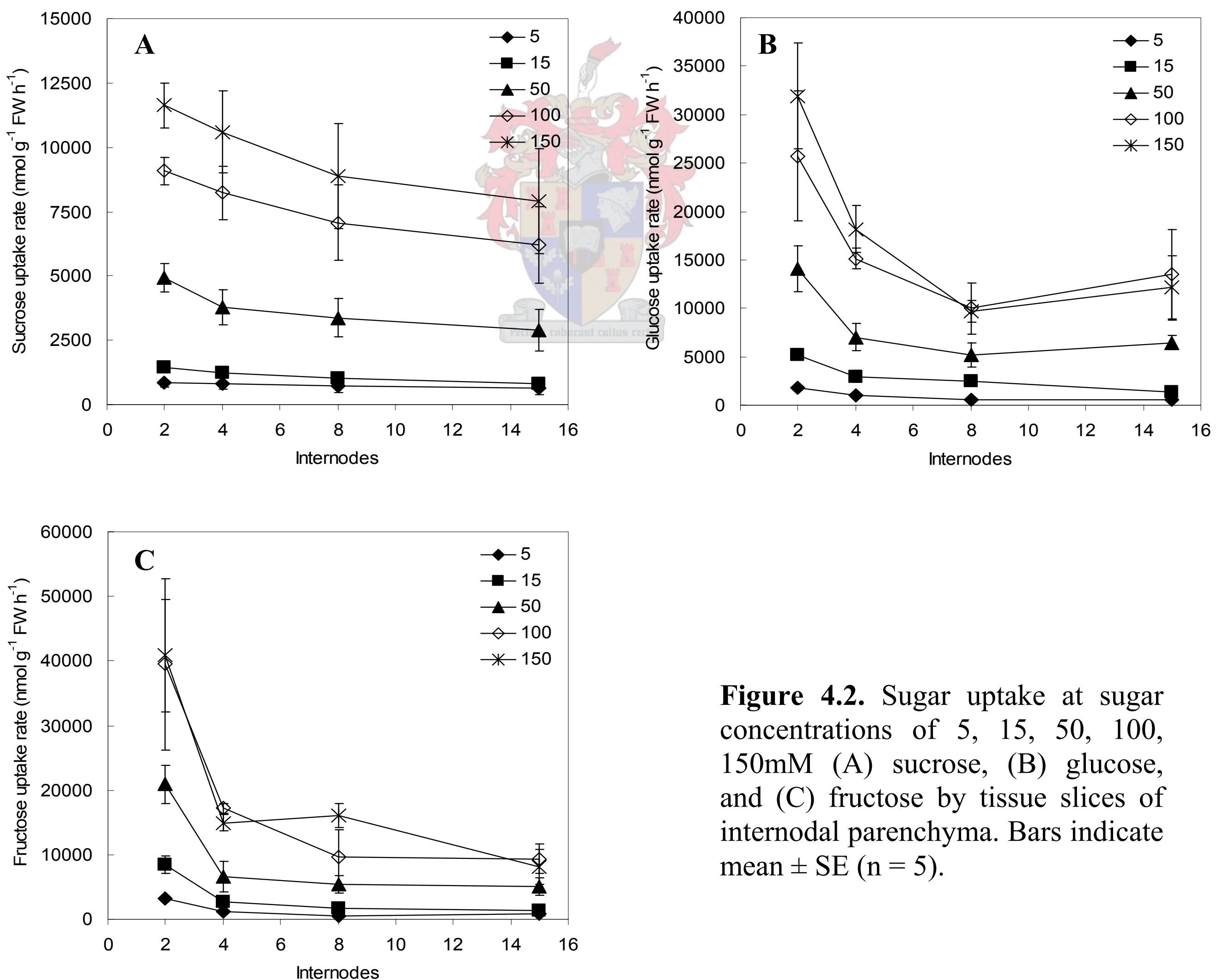


Figure 4.2. Sugar uptake at sugar concentrations of 5, 15, 50, 100, 150mM (A) sucrose, (B) glucose, and (C) fructose by tissue slices of internodal parenchyma. Bars indicate mean ± SE (n = 5).

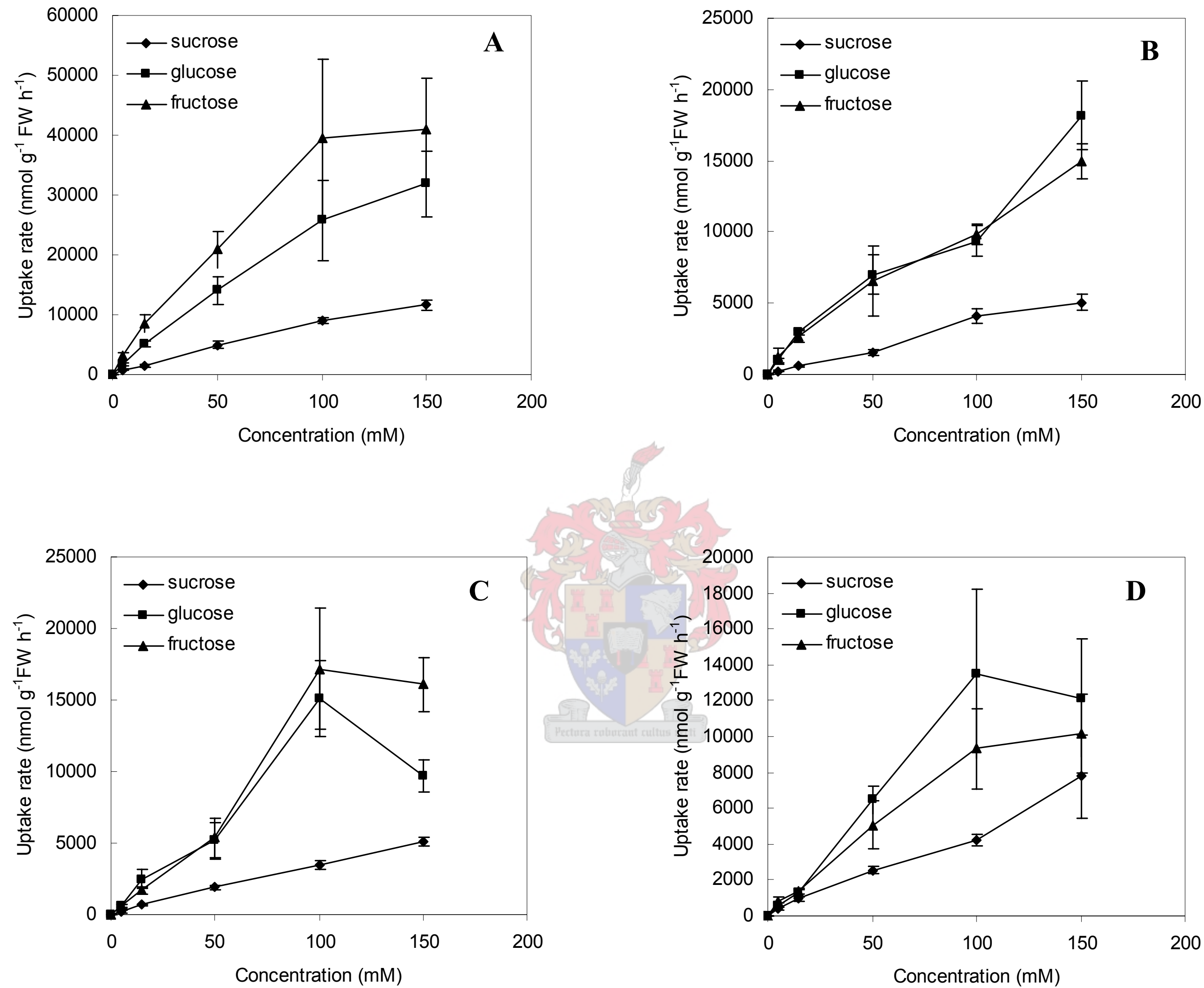


Figure 4.3. Sugar uptake at sugar concentrations of 5, 15, 50, 100, 150mM (A) internode 2 (B) internode 4 (C) internode 8 and (D) internode 15 by tissue slices of internodal parenchyma. Bars indicate mean ± SE (n=5)

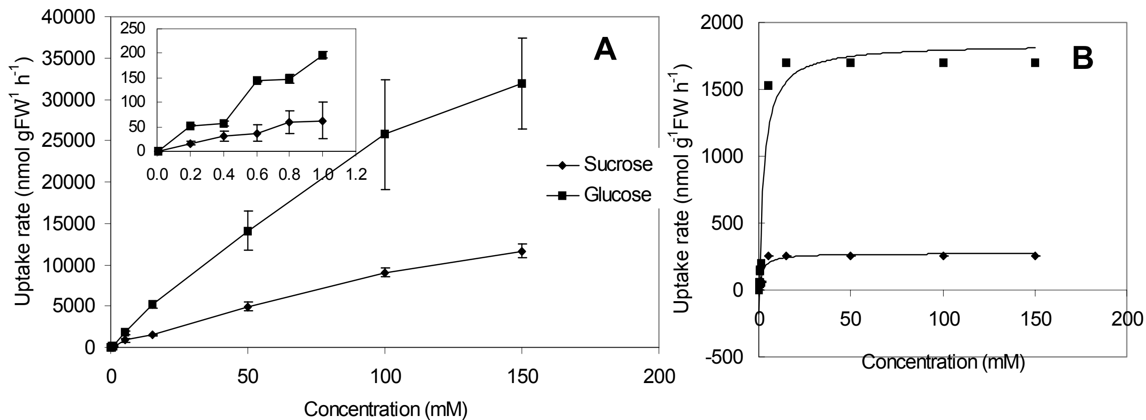


Figure 4.4 A) Sucrose and glucose uptake at internode 2 at 0, 0.2, 0.4, 0.6, 0.8, 1, 5, 15, 50, 100, 150 mM. The inserted figure represents the sucrose uptake rate at concentrations between 0 and 1 mM sucrose and glucose. B) Saturation kinetics of the sugar uptake, calculated by subtracting the diffusion component of uptake at internode 2. Bars indicate mean ± SE (n = 5)

4.4.2 pH and temperature dependence of sugar uptake

At pH 4.0 acid hydrolysis of sucrose was at a maximum, and decreased more than 2-fold as the pH was increased to 7.5 (Fig. 4.5A). The combined contributions of acid hydrolysis and enzymatic hydrolysis were determined for tissue discs from internodes 3 and 5 but no significant differences were found between the different pH's or ages of internodes (Fig. 4.5B). When the rate of acid hydrolysis was subtracted from the total enzymatic hydrolysis the data indicated that the major portion of glucose was produced by enzymatic hydrolysis (Fig 4.5C). The Pearson coefficient for the correlation between pH and glucose was not significant for internode 5. However, the coefficient for the correlation between pH and glucose for internode 3 was - 0.47 (P = 0.002). Indicating that more enzymatic activity occurred at a lower pH for internode 3.

Pectora roborant cultus recti

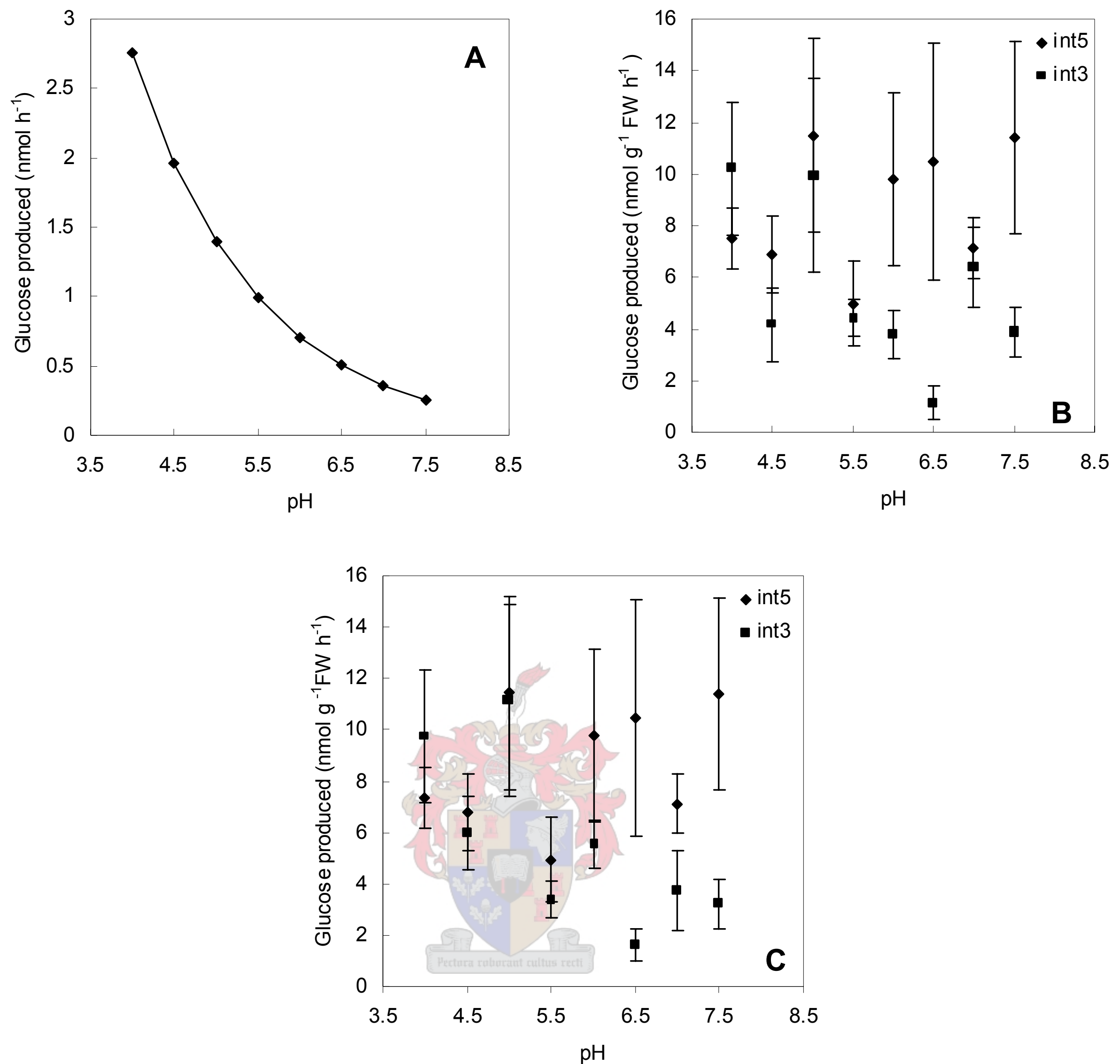


Figure 4.5 Glucose produced (A) as a result of non enzymatic sucrose acid hydrolysis. Sucrose (100 mM) was incubated in 50 mM citrate/phosphate buffer at pH's 4.0, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 (B) by tissue discs through a combination of enzymatic and acid hydrolysis. (C) Glucose produced by enzymatic hydrolysis. Tissue discs from internode 5 and 3 were incubated for 10 min in incubation solution (250 mM mannitol, 25 mM K-MES, 1 mM $CaCl_2$, 5 mM sucrose) of pH's 4.0, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5, and the amount of glucose produced measured spectrophotometrically with a NADPH linked assay (Bergmeyer and Bernt, 1974). Bars indicate ± SE. (P = 0.002 with Bivariate Pearson correlation coefficient, n=40).

There was no significant difference in the uptake rates of sucrose at the different pH's in internode 5. However, in internode 3 the uptake rate of sucrose reached a peak at pH 5. The rate of sucrose uptake decreased rapidly by two fold as the pH increased to 7.5. (Fig.4.6). The sucrose uptake rate was lower in internode 5 than in internode 3. Significance and detail is not noticeable in Fig. 4.2 because of difference in scale used.

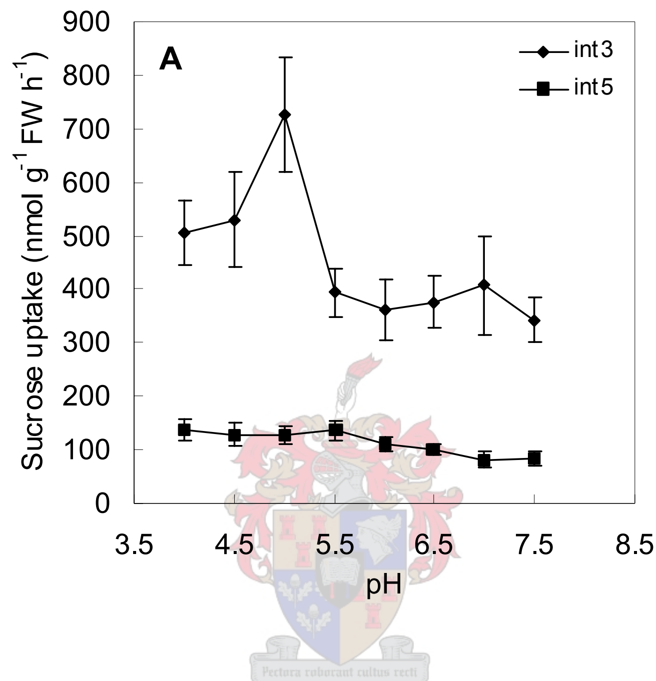


Figure 4.6. (A) The rate of sucrose uptake by immature (internode 3; int3) and maturing (internode 5; int5) internodes. Tissue discs were incubated in incubation buffer containing 250 mM mannitol, 25 mM K-MES, 1 mM $CaCl_2$, 5 mM ^{14}C sucrose at different pHs at 25°C. Bars indicate mean ± SE (n = 5).

Pectora roborant cultus recti

Sucrose uptake was temperature dependent and showed a decrease in uptake at 15°C, but no significant difference was observed between sucrose uptake of the tissue discs incubated at 25°C and 45°C (Fig. 4.7A). The Q_{10} value, calculated between 15° and 25°C, decreased rapidly form *ca.* 2 to 1.2 as the sucrose concentration increased (Fig. 4.7B).

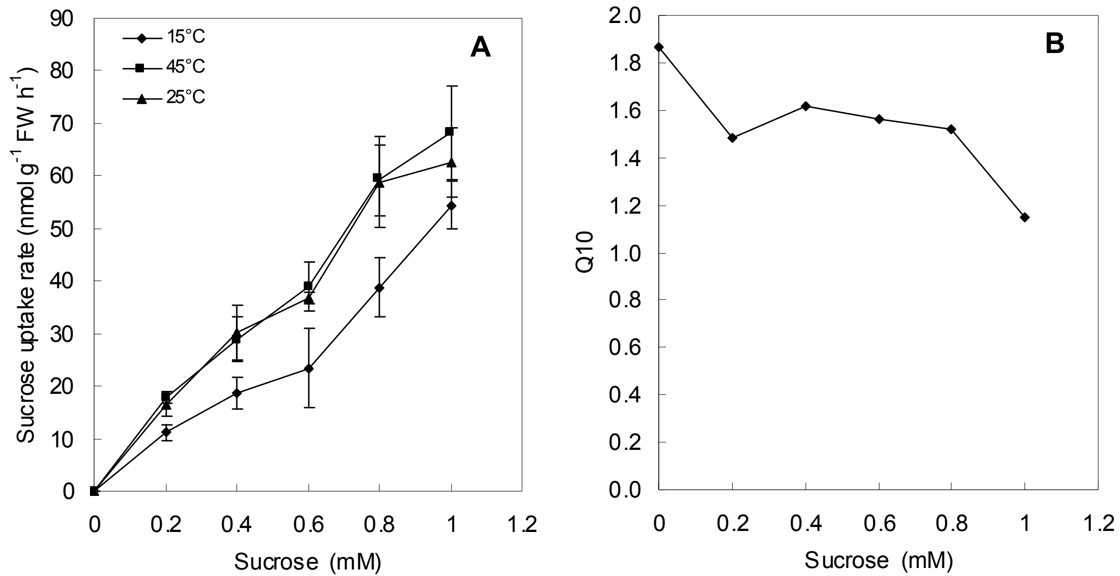
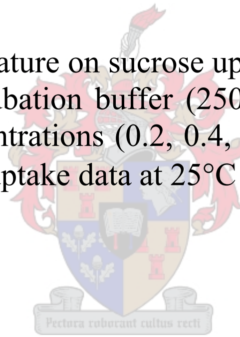


Figure 4.7 (A) The influence of temperature on sucrose uptake by tissue discs cut from internode 5. The tissue disc were incubated in incubation buffer (250 mM mannitol, 25 mM K-MES, 1 mM $CaCl_2$) with varying ^{14}C sucrose concentrations (0.2, 0.4, 0.6, 0.8, 1 mM) for 10 min at 25°C. (B) The Q_{10} value calculated from sucrose uptake data at 25°C and 15°C. Bars indicate mean ± SE (n = 5).



4.4.3 The Effect of PCMBS, TRIS and glucose concentration on sucrose uptake

Cell wall invertase (CWI) activity of internodal extracts from internode 5 was very low at pH 3.5, which is the optimum pH of CWI (Albertson *et al.*, 2001). Cell wall invertase activity in control leaves was 9-fold greater than that of the internodal invertase extracts (Fig. 4.8). TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) concentrations of 100 mM and 200 mM had no inhibitory effect on CWI extracted from leaves or from internodal tissue.

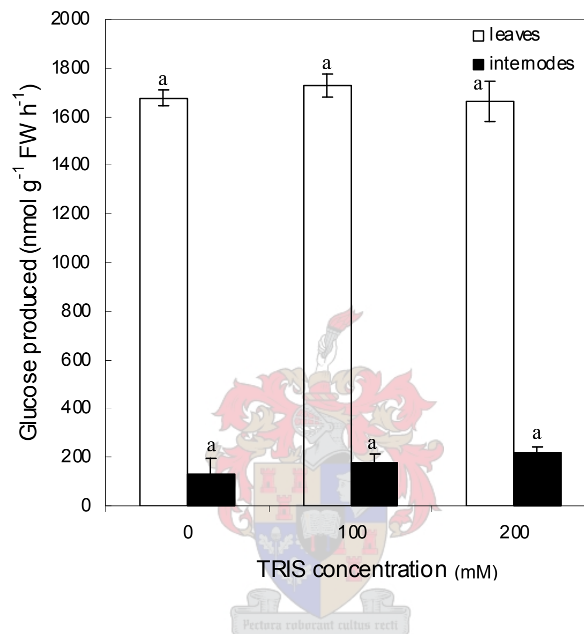


Figure 4.8. The influence of TRIS on cell wall invertase (CWI) activity in extracts from leaves and internode 5. Extracts were incubated in (50 mM citrate/phosphate buffer (pH 3.5), 100 mM sucrose at 37°C for 1 h with 0, 100 and 200 mM TRIS. The letters indicate whether the treatments had a significant effect (P < 005, ANOVA with post hoc LSD, n = 5).

Sucrose uptake was measured in the absence and in the presence of 100 mM TRIS (pH 5.7) and 1 mM PCMBS at different sucrose concentrations. Sucrose uptake increased significantly as the sucrose concentration increased and was inhibited by 100 mM TRIS as well as 1 mM PCMBS at all sucrose concentrations (Fig.4.9). At sucrose concentrations between 2.5 to 10 mM the inhibitory effect of TRIS and PCMBS was more apparent (Fig. 4.9 insert). A 50 % and 30% inhibition of sucrose uptake was observed in the presence of PCMBS at 10 mM and 250 mM sucrose respectively. Two way ANOVA indicated a significant interaction (P < 0.05) between sucrose concentration and the different treatments (control, TRIS, PCMBS). However PCMBS had a larger

inhibitory effect on sucrose uptake than did TRIS. Glucose concentration had no significant effect on sucrose uptake for either 0.5 or 10 mM sucrose (Fig. 4.9B). Two way ANOVA indicated that there was no interaction between glucose concentration and sucrose uptake.

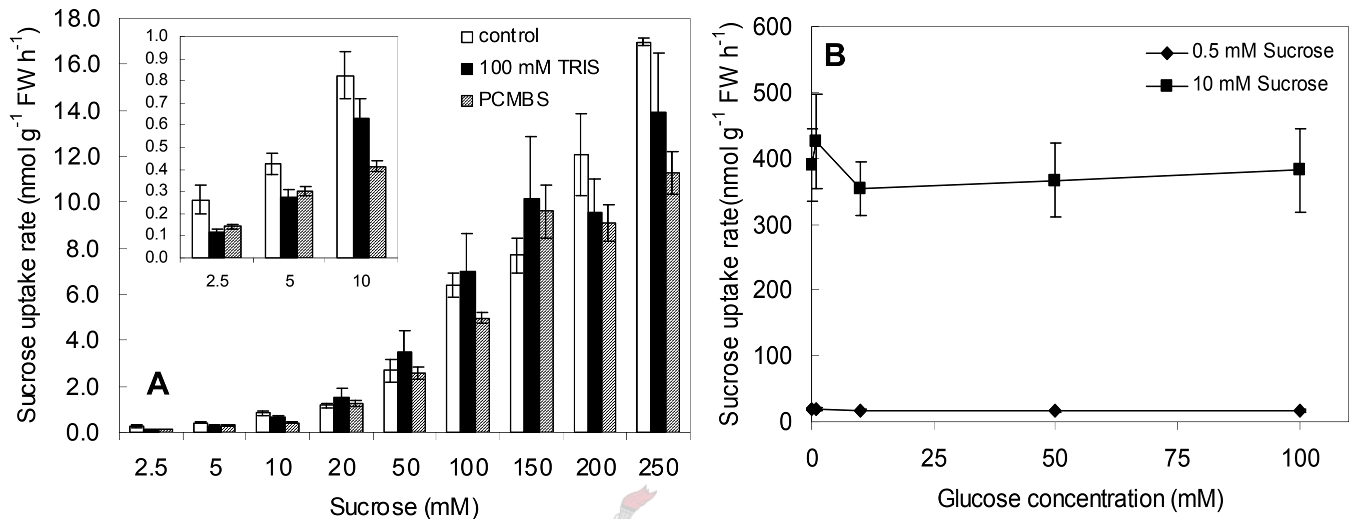


Figure 4.9 (A) The influence of 100 mM TRIS and 1 mM PCMBS on sucrose uptake in a range of sucrose concentrations in culm tissue disc of internode 5. Tissue discs were incubated in incubation buffer (250 mM mannitol, 25 mM K-MES, 1 mM $CaCl_2$) with varying ^{14}C sucrose concentrations (2.5, 5, 10, 20, 50, 100, 150, 200, 250 mM) for 10 min at 25°C. Two way ANOVA with post hoc LSD, n=5). The inserted figure represents the sucrose uptake rate at concentrations between 2.5 and 10 mM sucrose**.** (B) Sucrose uptake in the presence of increasing glucose concentrations, 0, 1, 10, 50 and 100 mM glucose at 0.5 and 10 mM sucrose. Tissue discs were incubated in uptake solution containing ^{14}C labelled sucrose in 250 mM mannitol, 25 mM K-MES and 1 mM. Bars indicate ± SE (n=5)

4.5 Discussion

Komor *et al.* (1996) investigated the rate of hexose and sucrose uptake in sugarcane (*S. officinarum*) cv L62-96 which is known to be a variety that accumulates little sucrose. In the present study, a high sucrose storing variety cv N19 was examined. Invertase catalyses the irreversible hydrolysis of sucrose to glucose and fructose. Isoforms of invertase with different biochemical properties accumulate in the cytoplasm (neutral invertase), cell wall bound acid invertase and soluble acid invertase, which is found in the vacuole and apoplast (Sturm and Tang, 1999). Vacuolar and cell wall invertases have acidic pH optima of about 5 – 5.5 and are, therefore, referred to as acid invertases (soluble and insoluble acid invertase respectively). Komor *et al.* (1996) did not take into account the activity of cell wall acid invertase (CWI) and the possible contribution of sucrose-derived hexose uptake, to the overall ^{14}C uptake rates. For this reason the activity of CWI was differentiated from leaf and internodal tissue in the present investigation. Using CWI extracts from leaf tissue and internodes to determine the maximum CWI activity, it was concluded that the contribution of CWI was negligible in internodal tissue in comparison to leaf activity. Vorster and Botha (1998) found that 10 mM TRIS inhibited neutral invertase (NI) activity in sugarcane by 85% while the present study showed that 100 to 200 mM TRIS had no significant inhibitory effect on CWI activity. Thus TRIS could not be used to effectively inhibit CWI. The sucrose uptake inhibition observed (Fig. 4.9) could be due to the inhibition of neutral invertase activity even though several washes were done to remove the soluble invertases.

The contributions of acid hydrolysis and enzymatic hydrolysis were determined in tissue slices from internode 3 and 5. Acid hydrolysis of 5 mM sucrose produced *ca.* 0.5 nmol h^{-1} glucose at pH 5.7, which indicated that the amount of glucose produced by acid hydrolysis was insufficient to explain the uptake of sucrose by the tissue discs supplied with 5 mM sucrose. Thus, although acid hydrolysis could have contributed to uptake by producing hexoses that could be taken up independently of sucrose, sucrose uptake must also have occurred. The pH of the uptake solution

played a significant role in determining sucrose uptake in internode 3, but had no significant effect on transport in internode 5 (Fig. 4.4A). Since it seems that sucrose hydrolysis is not important quantitatively in determining sucrose uptake, the influence of pH could be an indication that sucrose uptake occurred through the agency of a pH-sensitive transporter in young internodes. The pH optimum (4.5 to 5.5) for sucrose uptake was similar to that of sucrose transporters in other species (Lemoine, 2000). Further evidence that a H^+/sucrose symporter system was active in sugarcane was revealed since PCMBS, which is a potent inhibitor of H^+/sucrose symport (Bush, 1989; Slone and Buckhout, 1991), had a significant inhibitory effect on the sucrose uptake rate. The fact the PCMBS had a bigger effect on sucrose uptake at low sucrose concentrations does not exclude the possibility of additional sucrose permeation through channels at sucrose concentrations above 10 mM.

The major uptake system in suspension cells and in young growing internodal tissue from sugarcane is the high affinity hexose uptake system for glucose and fructose (Thom and Maretzki, 1992, Komor *et al*., 1996). Ebrahim *et al.* (1999) investigated these claims using sugarcane suspension cells and found that the glucose uptake rate increased continuously by a factor of 2.3 to 2.5-fold per 10°C increase in temperature from 15 to 40°C with an optimum at 45°C. These authors reported, however, that there was no temperature dependence for sucrose uptake. In contrast, in this study sucrose uptake increased significantly with an increase in temperature between 15 and 25°C. No significant difference was found between the sucrose uptake rate at 25°C and 45°C indicating that the optimum temperature for sucrose uptake probably lies between 25°C and 45°C. Sucrose is important for plant metabolism and may be transported by H^+/sucrose symporters or by facilitated diffusion, the latter of which are not dependent on respiratory metabolism. The H^+/symporters are dependent on a proton gradient provided by ATPase, which in turn is dependent on respiration for ATP provision. The decrease in Q_{10} value measured for sucrose uptake with increased sucrose concentration possibly indicates reduced dependence on respiratory metabolism for sucrose uptake

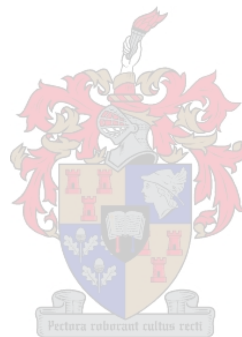
at sucrose concentrations above 0.4 mM. This could indicate that sucrose was being transported by passive influx or facilitated diffusion at the higher sucrose concentrations.

The results obtained when comparing glucose, fructose and sucrose uptake at different concentrations and in different internodes in this study are in agreement with the working model proposed by Komor *et al.* 1996. In the young internodes sugar uptake was very dependent on the sugar species. At low extracellular concentrations, hexoses were taken up rapidly, whereas sucrose was taken up at a lower rate. At high sugar concentrations, a diffusion like linear uptake was observed for all sugars, but sucrose uptake was still lower than hexose uptake. In the older internodes the uptake of glucose and fructose was strongly reduced compared to that in younger internodes for all sugar concentrations and sugar forms. The uptake rate for both hexoses and sucrose were similar in the older internodes. Thus, although sucrose uptake is likely to play a minor role in the younger internodes, in the older internodes it may be relatively more important.

At sugar concentrations above 10 mM it appears that the diffusion like component is more important. Sucrose uptake in the presence of different glucose concentrations provided evidence that sucrose uptake was not inhibited by the presence of glucose at both 0.5 and 10 mM sucrose. These results are in contrast to previous findings by Bieleski (1962) who found that glucose was a competitive inhibitor. The different finding could be as a result of different methodology since Bieleski (1962) used longer incubation times and different ^{14}C extraction methods from tissue, which could interfere with the sucrose metabolism and the reliability of results. The short assay times used in this investigation were selected to attempt to discriminate between ^{14}C sucrose uptake and respiration to some extent so that it would be able to quantify sucrose uptake. The use of tissue slices does not permit distinction between the intra- and inter-cellular uptake sites. It is possible that a sucrose saturable component of uptake exists in some tissue in the tissue slice (e.g. the phloem parenchyma) and a linear component in other compartments (e.g. the symplastic pathway). PCMBS

and TRIS inhibited sucrose uptake significantly across a range of different sucrose concentrations, indicating that a proton mediated system may be important. The effect of the inhibitor was more apparent at sucrose concentrations below 10 mM. This evidence is in agreement with above mentioned results (Fig.4.1) where possible sucrose saturation occurs at low sucrose concentration less than 20 mM and linear diffusion like kinetics follows.

In conclusion, the results indicate that sucrose uptake *in situ* plays an important role only when very little hexose is available and the sucrose concentration in the apoplast is high, as in the case of mature, ripe internodes. Evidence was also found for possible H^+/ sucrose symporter activity in sugarcane, as indicated by PCMBS inhibition studies.



CHAPTER 5

General Conclusion and Discussion

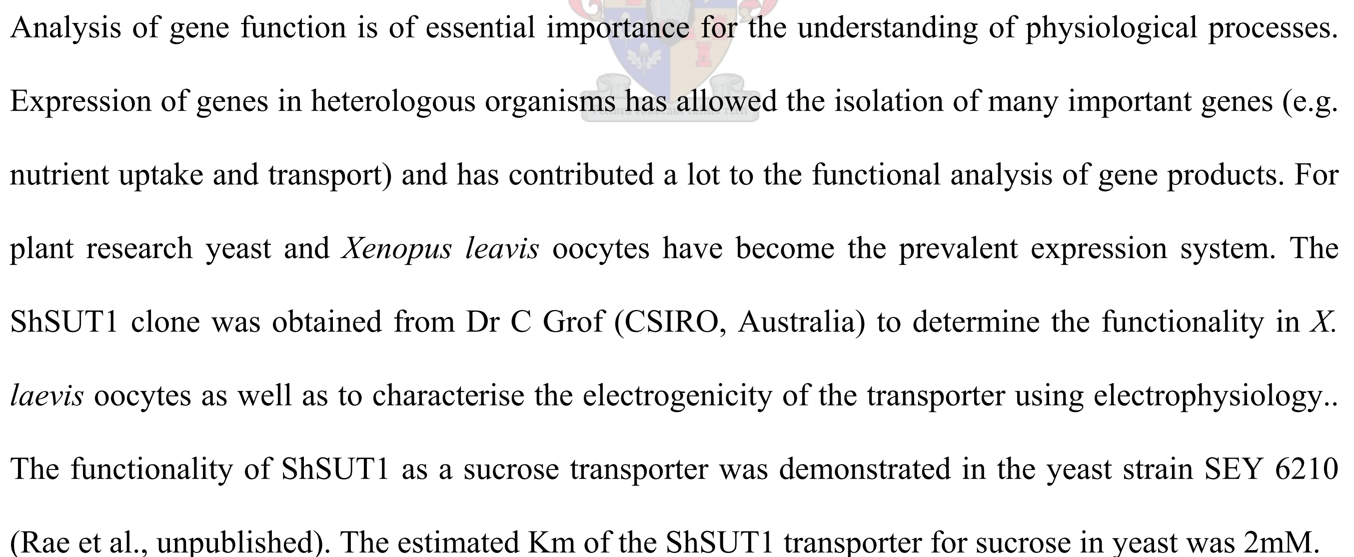
Sugarcane (*Saccharum* hybrid) can accumulate sucrose in the stem to levels exceeding 50% of the dry weight (Bull and Glasziou, 1963). Understanding the processes involved in sucrose transport and metabolism is necessary, since membrane transport events might be important control points in these pathways. The uptake and release of sugars, mainly in the form of hexose, occurs through the plasmalemma of sugarcane culm cells. Furthermore, there is a cycle of synthesis and degradation that occurs in these cells (Glasziou and Gayler, 1972). This has been confirmed and quantified in sugarcane suspension cells (Wendler *et al*., 1991). Both Lingle (1989) and Thom and Maretzki (1992) presented evidence that sucrose can be taken up by sugarcane cells prior to being cleaved to hexoses. Hexose levels have been shown to be positively correlated with neutral invertase levels but not with soluble acid invertase levels in mature sugarcane stem tissue (Glasziou and Gayler, 1972; Vorster and Botha, 1999; Botha *et al.,* 2001). Thus, sucrose synthesis from hexoses in the storage cells might be an essential component for control of the levels of sucrose storage. These findings suggest that the cleavage of sucrose by cell wall invertase, the uptake of hexoses by active transport systems, and the synthesis of sucrose in the cells from hexoses are important in determining sucrose storage. Komor *et al*. (1996) re-evaluated these findings and found that sucrose uptake in sugarcane is very low in comparison to that of hexose uptake, except in old ripe internodes, where all fluxes were small. To unravel the sucrose transport pathway at a physiological level in sugarcane, ^{14}C-labelled sugar uptake studies were done using sections of internodal tissue disc. The uptake of sucrose, glucose and fructose was investigated at different sugar concentrations and in different internodes of sugarcane (Chapter 4, section 4.4.1). There is some indication that sucrose uptake could saturate at *ca.*10 mM sucrose and thereafter follow linear uptake kinetics with increasing sucrose concentration indicating a diffusion based transport event. Hexoses were found to be the major sugar being taken up by sugarcane, while

sucrose was only important when very low amounts of hexoses were present as in the case of old, ripe internodes. These results are in agreement with the model presented by Komor *et al*. (1996).

Sucrose uptake differs between tissues and our study showed that sucrose uptake was taken up rapidly at pH 5, similar to the pH optimum of most sucrose transporters (Lemoine, 2000) and that sucrose uptake was inhibited at low temperature (15°C). Sucrose can be transported by H^+/sucrose symporters or by facilitated diffusion that are less dependent on energy. H^+/symporters are dependent on a proton gradient provided by the ATPase pump, which in turn is dependent on respiration for ATP. The Q10 value measured for sucrose uptake between 25°C and 15°C decreased as the sucrose concentration increased, possibly indicating reduced dependence on respiratory metabolism for sucrose uptake at sucrose concentrations above 0.4 mM (Chapter 4, section 4.4.2). This could indicate that sucrose was being transported by passive influx or facilitated diffusion at the higher sucrose concentrations. Inhibition studies with TRIS and PCMBS indicated that a H^+/symport mechanism is being used in sugarcane at sucrose concentration lower than 10 mM but that at sucrose concentration above 10 mM, sucrose is transported by means of diffusion or channels proteins (Chapter 4, section 4.4.3).

The first cloning of a sucrose transporter gene from spinach (Riesmeier *et al*., 1992) increased our understanding of the molecular mechanism of sucrose transporters. Since sugarcane is known to have a high ploidy level, the potential for transporters to belong to a multigene family is high. To date work on sugarcane sucrose transporter expression on DNA and RNA level has been limited. Only recently a sucrose transporter from *Saccharum* hybrid sugarcane, ShSUT1 was isolated and functionally characterized in the yeast strain SEY 6210 (Rae *et al., in press*). In an effort to understand sucrose transport in sugarcane culm tissue, a partial sucrose transporter cDNA, ScSUT1(p) was isolated, and cloned from a bud cDNA library. The ScSUT1(p) sequence showed 94% identity to ShSUT1 on nucleotide level over 1258 nucleotides and had an estimated open reading frame of 419 amino acids.

Within the sucrose transporter family, functional subgroups can be identified on the basis of kinetic properties. Recently an unknown maltose transporter, which is unrelated to known sugar transporter proteins, was discovered (Niityla *et al*., 2004). This maltose transporter encoded by the maltose excess1 (MEX1) gene is essential for the conversion of starch into sucrose in *Arabidopsis* leaves at night. It is possible that many genes involved in sucrose transport remain to be discovered. A sequence survey of 7242 ESTs derived from the sucrose-accumulating, maturing stem of sugarcane revealed that transcripts for carbohydrate metabolism gene sequence (CMGs) are relatively rare in this tissue (Casu *et al*., 2003). Within the CMG group putative sugar transporter ESTs form one of the most abundant classes observed. In sugarbeet, sucrose transport into the vacuole is associated with proton efflux (Briskin *et al*., 1985). The research for a similar system in sugarcane has led to numerous attempts to isolate and characterise proteins involved in transmembrane sucrose transport.



Analysis of gene function is of essential importance for the understanding of physiological processes. Expression of genes in heterologous organisms has allowed the isolation of many important genes (e.g. nutrient uptake and transport) and has contributed a lot to the functional analysis of gene products. For plant research yeast and *Xenopus leavis* oocytes have become the prevalent expression system. The ShSUT1 clone was obtained from Dr C Grof (CSIRO, Australia) to determine the functionality in *X. laevis* oocytes as well as to characterise the electrogenicity of the transporter using electrophysiology.. The functionality of ShSUT1 as a sucrose transporter was demonstrated in the yeast strain SEY 6210 (Rae et al., unpublished). The estimated Km of the ShSUT1 transporter for sucrose in yeast was 2mM.

Several sugar transporter proteins have been successfully expressed in both yeast and *Xenopus* oocytes although there are some proteins that are expressed in yeast but not in *Xenopus* (Dr C. Grof, personal communication). The *Xenopus* system is able to provide more information regarding the functionality, kinetics and electrogenicity of transporters. ^{14}C sucrose uptake studies with ShSUT1 mRNA injected oocytes revealed that ShSUT1 was functional in oocytes since ^{14}C sucrose was being taken up by injected oocytes and the water injected controls had no sucrose uptake (Chapter 3, section 3.4.5). Oocytes used for electrophysiological measurements revealed very low electrogenicity (Chapter 3, section 3.4.4) indicating that ShSUT1 transporter activity could be non-electrogenic and that sucrose uptake might be channel protein mediated.

The main disadvantages of oocytes are the seasonal variation in quality and alteration in the ability of individual cells to express a foreign message. Further problems are the transient expression period, which does not last longer than about two weeks, and the relatively small number of cells that can be handled in a single experiment. Several reasons are possible for failed expression of proteins or low expression levels as was the case in this study. The proteins might have been incorrectly targeted or the properties of the protein product might be incompatible with expression. Two *Arabidopsis* clones AKT1 and KAT1, have been isolated by yeast complementation, but only KAT1 has been subsequently expressed in oocytes (Sentenac *et al*., 1992). Both channels share extensive sequence homology, but only AKT1 contains hydrophilic carboxyl terminal domain homologous to the 33-residue ankyrin repeat sequence that is involved in protein interaction. This ankyrin repeat may block functional expression in oocytes. ShSUT1 sequence is similar to other sucrose transporters found in rice, maize and Arabidopsis but further sequence analysis needs to be done to determine if there is some domain that inhibits functional expression in oocytes. More than one protein or RNA species may be required for activity of transporters. This may be because the functional complex contains more than one protein subunit, or because a second protein mediates correct assembly as in the case of Na^+/K channels.

Southern blot analysis indicated that ScSUT1(p) is a low copy number gene (Chapter 3, section 3.4.2). This is similar to what was found for other sucrose transporters from species such as *Nicotiana tabacum* (Bürkle *et al*., 1998), *Solanum tuberosum* (Riesmeier *et al*., 1994, Kühn *et al*., 1997) and *Plantago major* (Gahrtz *et al*., 1994). For all of these species more than one gene were isolated (Lemoine, 2000) and it is thought that sucrose transporters are all part of a single multigene family as was found in rice (*Oryza sativa*) and *Arabidopsis thaliana* (Aoki *et al*., 2003). The fact that ScSUT(p) is a low copy number gene makes the potential for genetic manipulation less complicated than if it had multiple copies.

Northern blot analysis revealed that the ScSUT1(p) gene is expressed constitutively in sucrose accumulating and sucrose storing stem tissue, but was less abundant in immature tissue such as in internodes 2 and 3 and in lateral buds (Chapter 3, section 3.4.3). In contrast, ShSUT1 was only expressed in sucrose accumulating stem tissue and was not present in the sucrose storing tissue (Rae *et al*., *in press*). Sucrose transporters involved in phloem loading usually are high affinity transporters with a K_m lower than that found for ShSUT1. Both transporters are most abundant in mature leaves, which are involved in exporting sucrose (carbohydrates). The primary function of ScSUT1(p), is not phloem unloading but that the transporter may be involved in phloem loading, as it is abundant in mature source leaves. ShSUT1 may have a role in partitioning of sucrose between the vascular tissue and sites of storage in the parenchyma cells of sugarcane stem internodes as described by Rae *et al*., (*in press*).

The work in this project emphasises that sugar transport is a complex process and further studies are important to unravel this complex mechanism. Future work includes the isolation of the full-length sequence of ScSUT1(p) and this could be used for *in situ* hybridization as well as heterologous expression of ScSUT1(p) in yeast and *Xenopus* oocytes. Electrophysiological measurements indicated

that ShSUT1 may have very low electrogenic activity or be non-electrogenic using a channel or carrier-mediated sucrose uptake mechanism. ^{14}C sucrose uptake studies indicated that ShSUT1 is functional, as a sucrose transporter in *Xenopus* oocytes, but further investigation is needed for full kinetic characterization of ShSUT1 transporter. From both the molecular and physiological results there is strong evidence indicating a role for a sucrose transporter at low sucrose concentrations and a possible passive uptake/diffusion system at the higher sucrose concentrations.

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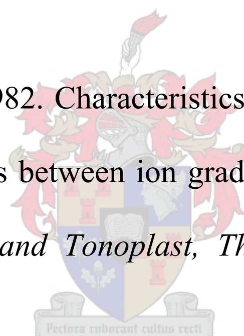

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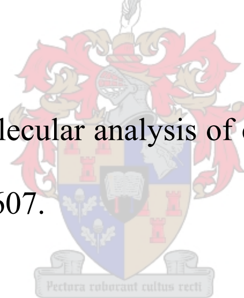

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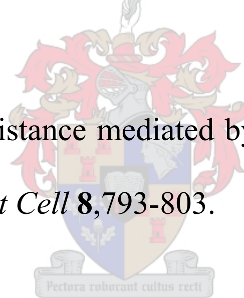

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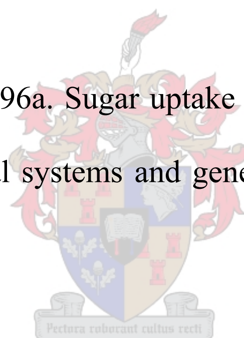

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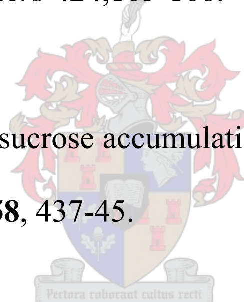

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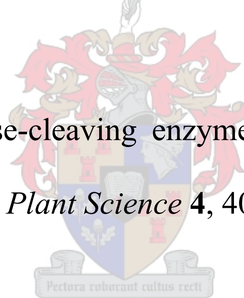

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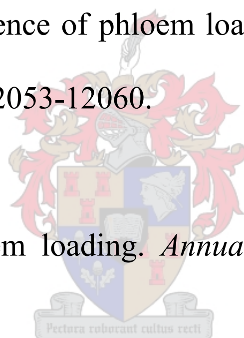

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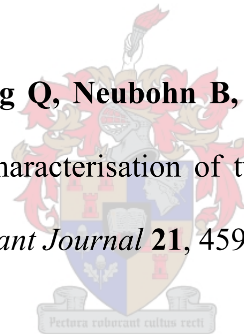

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