CHARACTERISATION OF SUCROSE SYNTHASE ACTIVITY IN THE SUGARCANE CULM

by

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Dissertation

Presented for the degree

Doctor of Philosophy

(Plant Biotechnology)

at the

University of Stellenbosch

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Institute for Plant Biotechnology

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April 2004

DECLARATION

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

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Signature Date

SUMMARY

This study had three main goals:

1. to investigate the occurrence on the protein level of sucrose synthase (SuSy) isoforms in sugarcane sink tissue,
2. to determine the kinetic properties of these isoforms,
3. to establish the tissue localisation of SuSy in the sugarcane culm

The results are summarised below:

Three SuSy isoforms were obtained from leaf roll tissue. The SuSyA and SuSyB isoforms differed in terms of charge characteristics, with SuSyA not binding to an anion exchange column that bound SuSyB and SuSyC under the same conditions. Both SuSyB and SuSyC isoforms were eluted at 180 mM KCl. The SuSyA and SuSyB isoforms were present during autumn, but during winter only the SuSyC isoform could be isolated. Even though they eluted at the same salt concentration, SuSyB and SuSyC were different isoforms, because they had different kinetic parameters, as well as different immunological properties. SuSyB and SuSyC could not have been mixtures of the same isoforms, since a polyclonal antiserum against SuSyB, which inactivates native SuSyB, did not inactivate SuSyC. All three isoforms had significantly different kinetic parameters, with the SuSyA isoform also having a much lower sucrose breakdown/synthesis ratio than the other two isoforms. Therefore, at least three SuSy isoforms occur in sugarcane leaf roll tissue on the protein level.

The SuSyC isoform was subsequently kinetically characterised in detail. Data showed that the enzyme employs an ordered ternary complex mechanism, with UDP binding first and UDP-glucose dissociating last. These experimentally obtained kinetic parameters were then used to extend a kinetic model of sucrose accumulation. Data show that when the experimentally determined SuSy kinetic

parameters were entered into the model, a 40 % increase in sucrose concentration and 7 times reduction in fructose concentration resulted. These data illustrate the pronounced physiological effects that may result from the presence of different SuSy isoforms.

SuSy protein localisation data, obtained by an immunohistochemical approach, indicated that SuSy protein was present in both storage parenchyma and vascular tissue of young, intermediate, and mature internodes. SuSy enzyme activity in different parts of the internodes was similar, except for internode 3, which had much higher activity in the bottom part of the internode, possibly because growth is faster here, hence a higher demand for sucrose cleavage exists here.

OPSOMMING

Hierdie studie het ten doel gehad:

1. om die teenwoordigheid van sukrose sintase (SuSy) isovorme in suikkerriet swelgweefsel te ondersoek
2. om die kinetiese eienskappe van hierdie isovorme te ondersoek
3. om die weefsellokalisering van SuSy in die suikerrietstingel te bepaal

Die resultate word hieronder opgesom:

Drie SuSy isovorme is gevind in blaarrol weefsel. Die SuSyA en SuSyB isovorme het verskil in terme van ladingseienskappe, met SuSyA wat nie aan ‘n anioonuitruilkolom gebind het nie waaraan SuSyB en SuSyC wel onder dieselfde kondisies gebind het. Beide SuSyB en SuSyC isovorme is geëlueer van die kolom teen 180 mM KCl. Die SuSyA en SuSyB isovorme was teenwoordig gedurende herfs, maar in die winter was slegs SuSyC teenwoordig. Ten spyte van die feit dat SuSyB en SuSyC teen dieselfde soutkonsentrasie geëlueer is, het hulle verskillende isovorme verteenwoordig, aangesien hulle kinetiese en immunologiese eienskappe verskil het. SuSyB en SuSyC kon nie mengsels van dieselfde isovorme gewees het nie, want ‘n poliklonale antiserum teen SuSyB, wat SuSyB geïnaktiveer het, het nie SuSyC geïnaktiveer nie. Al drie isovorme het betekenisvol verskil wat kinetiese eienskappe betref, met die SuSyA isovorm wat ook ‘n baie laer sukrose afbraak/sintese verhouding gehad het as die ander twee isovorme. Daar is dus ten minste drie SuSy isovorme teenwoordig op die proteïen vlak in suikerriet blaarrol weefsel.

Die in-detail kinetiese analise van die SuSyC isovorm het getoon dat die ensiem ‘n geordende drietallige kompleks meganisme het, met UDP wat eerste bind en UDP-glukose wat laaste dissosieer. Die eksperimenteel bepaalde kinetiese parameters is toe gebruik om ‘n kinetiese model van sukrose akkumulering uit te

brei. Data het getoon dat wanneer die generiese SuSy kinetiese parameters in die oorspronklike model vervang word met die eksperimenteel bepaalde waardes, die berekende sukrose konsentrasie met ongeveer 40 % toeneem, terwyl die fruktose konsentrasie ongeveer 7 keer afneem. Hierdie resultaat toon die groot fisiologiese effek wat die uitdrukking van verskillende SuSy isovorme op suikermetabolisme kan hê.

Die SuSy proteïen lokaliseringsdata, wat met ‘n immunohistochemiese benadering verkry is, het aangedui dat SuSy in beide bergingsparenchiemselle sowel as vaatweefsel teenwoordig is in jong, intermediêre en volwasse internodes. SuSy ensiemaktiwiteit in verskillende dele van die internodes was soortgelyk, behalwe in internode 3, wat baie hoër aktiwiteit gehad het in die onderste deel van die internode as bo, moontlik weens vinniger groei in hierdie deel van die internode, wat afhanklik is van afbraakprodukte van sukrose.

ACKNOWLEDGEMENTS

I hereby express my gratitude to the following persons and institutions:

Prof. F.C. Botha for his insight, guidance and support during the project and the preparation of the thesis.

Prof. J.M. Rohwer for helpful discussions and assistance with the preparation of the thesis.

Mr. J.H. Groenewald for helpful discussions.

The **University of Stellenbosch** for financial support.

The **Harry Crossley Foundation** for financial support.

The **National Research Foundation** for financial support.

The **South African Sugar Association** for financial support.

The **staff and students at the Institute for Plant Biotechnology** for their encouragement and support.

My **parents, family and friends** for their encouragement and support.

FOR MY PARENTS

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ABBREVIATIONS

AI	Acid invertase (EC 3.2.1.26)
NI	Neutral invertase (EC 3.2.1.26)
SPS	Sucrose phosphate synthase (EC 2.4.1.14)
SuSy	Sucrose synthase (EC 2.4.1.13)
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)
PEP	Phosphoenolpyruvate
CAM	Crassulacean acid metabolism
SMR	Sucrose metabolism related
GUS	β-glucuronidase
ENOD	Early nodulin
ORF	Open reading frame
DAF	Days after flowering
EST	Expressed sequence tag
DTT	Dithiothreitol
BSA	Bovine serum albumin
TBS	Tris-buffered saline
TBST	Tris-buffered saline transfer buffer
IGG	Immunoglobulin G
NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloro 3-indolyl phosphate
MCA	Metabolic control analysis
UDPGlc	Uridine 5’ diphosphoglucose
PEG	Polyethylene glycol
PFP	Pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90)
PBS	Phosphate-buffered saline
PPi	Pyrophosphate

CHAPTER ONE

PROJECT MOTIVATION

Sugarcane is a very important crop in many tropical and subtropical regions of the world and accounts for 60% of the world's sucrose production (Grivet and Arruda 2001). However, in sugarcane the biochemical processes that control sucrose accumulation itself are still poorly understood and are therefore the subject of intensive research in order to further improve yield. The enzymes associated with sucrose metabolism, such as sucrose synthase (EC 2.4.1.13, SuSy), sucrose-phosphate synthase (EC 2.4.1.14, SPS), neutral invertase (EC 3.2.1.26, NI) and acid invertases (EC 3.2.1.26, AI) have received appreciable attention over the years. The available information on sugarcane SuSy up to and including the last published study (Buczynski *et al.* 1993) is nonetheless incomplete, while several more recent developments in other species, some closely related to sugarcane, have necessitated renewed examination into sugarcane SuSy. These aspects are briefly discussed and their further study in sugarcane motivated below.

The kinetic properties of SuSy in sugarcane have only been superficially examined, with just K_m values reported. No information on other important kinetic parameters, such as substrate K_i values, or confirmation of the reaction mechanism is available. This information is important, since yield improvement strategies are based increasingly on results from kinetic models, for which extensive information about kinetic parameters is needed (Rohwer and Botha 2001). An important goal of this study was to extend knowledge in this area. Previously it was thought that most, if not all plants, contain only two SuSy isoforms (Chourey 1981; Gross and Pharr 1982; Marana *et al.* 1988). However, more than two have since been found in a variety of species (Barratt *et al.* 2001; Carlson *et al.* 2002; Yen *et al.* 1994; Komatsu *et al.* 2002). Although only two SuSy isoforms have thus far been recognised in sugarcane, there is a very high

likelihood of it containing more isoforms than other species, due to its extremely complex aneuploid, highly polyploid genome (Butterfield *et al.* 2001). Different isoforms likely have different physiological roles, so information on their number and the kinetic differences between them should provide insights into their function.

The elucidation of the organ, tissue, cell type or subcellular localisation of an enzyme is usually of great interest, because it provides important clues about its function. For example, SuSy was found to be partly plasma membrane associated in developing cotton fibres (Amor *et al.* 1995), which together with the fact that cellulose synthase is also membrane associated and uses UDP-glucose as substrate, gives the assertion that SuSy is involved in cellulose synthesis much more weight than if SuSy were only present in the cytosol. An important implication of possible membrane association in sugarcane is that overall SuSy activity could have been significantly underestimated in past studies, depending on experimental protocols followed. It therefore was one of the goals in the present study to determine if there is significant SuSy membrane association in the sugarcane culm. Our investigation showed that there is no significant membrane association in sugarcane culm tissue (Chapter 5).

The tissues in which SuSy is localised were identified by an immunohistochemical approach. In particular, the question whether SuSy is only associated with vascular bundles in mature internodes was of interest, since there are implications for sucrose yield improvement strategies (Chapter 5).

The overall working hypothesis for this study was that by improving the knowledge on enzyme kinetics, isoforms and localisation of SuSy, significant advancements to our understanding of the role of this enzyme in sugarcane will result. These findings may have significant commercial application.

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CHAPTER TWO

SUCROSE SYNTHASE – AN OVERVIEW

2.1 Introduction

The fact that Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is the world's most abundant enzyme (Mott 1997) attests to the success of photosynthesis and also reflects the dependence of almost all other life on this process. In green plants, photosynthesis can be divided into three main classes, the C_3, C_4 and CAM (Crassulacean Acid Metabolism) types. In C_3 photosynthesis, CO_2 (in the form of HCO_3^-) is incorporated directly in the synthesis of 3-carbon compounds in the Calvin cycle in mesophyll cells. In C_4 photosynthesis, CO_2 (again, in the form of HCO_3^-) is used to carboxylate phosphoenolpyruvate (PEP) by PEP carboxylase in mesophyll cells, followed by transport of the oxaloacetate to the bundle sheath cells where it is decarboxylated and the liberated CO_2 used by Rubisco in the Calvin cycle. This shuttling of CO_2 takes place in order to prevent or limit the energetically wasteful process of photorespiration, that occurs as a result of Rubisco's oxygenase activity under low CO_2 concentrations and high O_2 concentrations. Low (about 200 µbar) CO_2 concentrations in air with 20 mbar O_2 six to eight million years ago probably conferred a competitive advantage to C_4 plants (Sage and Monson 1999). C_4 photosynthesis has been a particularly successful strategy in tropical regions, where high temperatures and high illumination can increase photorespiration (Mathews and Van Holde 1990); e.g. this may occur through CO_2 depletion caused by stomatal closure under conditions of water stress. CAM plants are succulents that are subject to extreme water stress in their natural habitats. Their stomata are open at night and closed during the day in order to prevent excessive water loss. CO_2 acquisition takes place at night by incorporating CO_2 in C_4 acids, malate especially, similar to normal C_4 plants. During the day, when stomata are closed, the C_4 acids are decarboxylated and the CO_2 used in the Calvin cycle. CAM plants have therefore evolved the same

type of pre-Calvin cycle CO_2-storing reaction as normal C_4 plants, but for different reasons. Some of the world's most important crops, such as maize and sugarcane, are C_4 plants. C_4 plants are capable of remarkably high rates of carbon fixation under optimum conditions. Sugarcane is reported to fix CO_2 at rates as high as 2.8 mg per m^{-2} leaf area.s^{-1}, which can result in crop yields of about 150 tons per hectare per year (Moore and Maretzki 1996). Under favourable conditions, about 25% of the fresh weight of commercial sugarcane varieties can consist of sucrose (Moore and Maretzki 1996). *Saccharum spontaneum*, a wild relative of the *Saccharum officinarum* sugarcane hybrids used for cultivation, has photosynthetic rates 30% higher, but stores less than 2% sucrose on a fresh weight basis (Irvine 1975). Given these facts, it is not surprising that photosynthetic rate is not considered to be a limiting factor for sucrose accumulation in sugarcane (Moore and Maretzki 1996). Instead, regulation of sucrose accumulation is believed to occur at the translocation or sink level, or a combination of these.

Despite the different types of photosynthesis referred to above, the final product in each case is sucrose, and this is also the main or only transport carbohydrate in most plants. This sucrose can either be metabolised in sink tissues, or stored, but even if stored, carbohydrate is remobilised as sucrose again. This dual role of sucrose as a transport carbohydrate from source tissues, as well as from storage organs, introduces some complexity into the enzyme systems that have evolved around sucrose synthesis and breakdown. For example, the invertases are found in source and sink tissues, but sucrose synthase is associated more with non-photosynthetic sink tissues, with only residual phloem-associated activity present in mature maize source leaves (Nolte and Koch 1993). The presence of both sucrose-cleaving (invertase, sucrose synthase) and sucrose-synthesising enzymes (sucrose phosphate synthase, sucrose synthase) in the same compartment leads to cycles of sucrose synthesis and degradation. These "futile" cycles are reported in a variety of crops (Geigenberger and Stitt 1991; Whittaker and Botha 1997; Nguyen-Quoc and Foyer 2001) and are believed to contribute to

the ability of sucrose metabolism to respond to physiological changes, such as reduced phloem transport. When phloem transport is inhibited in *Ricinus communis* seedlings, sucrose is redirected towards starch synthesis, but concentrations of sugar and sugar phosphates, as well as respiration rate, stay relatively constant.

Enzymes involved in sucrose metabolism or translocation processes have been studied in a variety of crops to determine if there exist correlations between their activity and the ability of storage organs or tissues to act as a sucrose "sink" (Sung *et al.* 1989). One of these enzymes, sucrose synthase (SuSy, UDP-glucose: D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13), catalyses the reversible conversion of UDP-glucose and fructose to sucrose and UDP, with reported ΔG values ranging from -1.4 to -4.7 $kJ.mol^{-1}$ for the sucrose synthesis reaction (Geigenberger and Stitt 1993). However, substrate concentrations in most tissues where SuSy is found causes the enzyme to function in the sucrose breakdown direction (Xu *et al.* 1989; Amor *et al.* 1995; Kruger 1990). Since the discovery of the SuSy enzyme activity in wheat germ (Cardini *et al.* 1955) the enzyme has been quite extensively studied, which is not surprising given that sucrose is the major transported form of carbon in almost all plants, and central in carbon metabolism and partitioning.

The concept of "sink strength" refers to the ability of tissues or organs to import sucrose and is strongly correlated with the activity of SuSy in several crops, such as potato (*Solanum tuberosum*), lima bean (*Phaseolus lunatus*), cassava (*Manihot esculenta*), sweetgum (*Liquidambar styraciflua*) and pecan (*Carya illinoinensis*) (Sung *et al.* 1989). In fact, in all these crops, SuSy is by far the dominant sucrose cleaving activity in active sinks, with activity from about eight to ninety times higher than either neutral or acid invertase. In quiescent sinks, SuSy activity decreases dramatically; in potato tubers SuSy decreases to levels similar to those of the invertases, while the other crops also showed very large, but smaller decreases than potato, of about 5 to 70 times in SuSy activity. The

conclusion is that at least in these crops, SuSy is a strong indicator of sink strength and metabolic status of the sinks. On this evidence, SuSy also has potential for use as a "marker" for ripeness. Transgenic potato tubers with reduced SuSy activity have reduced starch content, which supports the contention that SuSy is important for sink strength in this crop (Zrenner *et al.* 1995). An important point is that if sink activity should fall significantly below the level of efficiently utilising or sequestering the sucrose supplied to it, this leads to an accumulation of sucrose in source leaves and an inhibition of source activity. This is illustrated by the expression of a yeast invertase in the cell wall of tobacco leaves, which leads to increased assimilate concentrations, inhibition of photosynthesis and blocking of phloem loading (Von Schwaewen *et al.* 1990).

In other crops, with tomato (*Lycopersicon esculentum*) a prime example, SuSy activity is only correlated with fruit growth rate and fruit set per plant (D'Aoust *et al.* 1999; Chengappa *et al.* 1999), not with ripening or storage carbohydrate accumulation as in potato. Once ripening is underway, SuSy activity is drastically reduced or absent in tomato fruit (Wang *et al.* 1994). Clearly, SuSy activity cannot be used as a blanket measure of sink strength in all plants.

Although most plants use sucrose as a carbon transport molecule, not many plants make use of sucrose as a storage carbohydrate, with sugarcane and sugar beet (*Beta vulgaris*) two important exceptions. Together they provide practically all the sucrose for the world market, with sugarcane accounting for about 60% (Grivet and Arruda 2001). The process of sucrose accumulation in sugarcane has been extensively studied, but despite this it is still poorly understood, with many fundamental questions remaining. For instance, contrary to expectation, sugarcane internodes undergo water loss as the osmotically active sucrose content[*] increases! Also, it is not known by what process sucrose is transferred to the vacuole in storage parenchyma cells. Evidence for a "group

[*] absolute content on a dry weight basis is intended, here and elsewhere in the thesis

translocator" (Thom and Maretzki 1985) has since been dismissed due to contaminated tonoplast membrane preparations (Maretzki and Thom 1988) and incomplete analysis of radiolabelled products (Preisser and Komor 1988). There exists some evidence for carrier-mediated or facilitated diffusion transfer of sucrose (Preisser and Komor 1991), which means that the sucrose concentration in the cytosol will have major control on its accumulation in the vacuole (Preisser *et al.* 1992).

In sugarcane, results of different studies that measure and correlate activities of enzymes of sucrose metabolism with sucrose content or accumulation rate often conflict. Sucrose-phosphate synthase (SPS) activity was not correlated with sucrose content in one study (Zhu *et al.* 1997), but showed strong positive correlation in another (Botha and Black 2000). SuSy was positively correlated with internode elongation rate, while acid invertase activity was positively correlated with elongation rate one year, but not the next (Lingle and Smith 1991). Some studies report negative correlation of neutral invertase with sucrose content (Rose and Botha 2000), but in others there is no correlation (Lingle and Smith 1991). The same is true for SuSy, with some studies showing negative correlation between SuSy activity and sucrose accumulation rate (Lingle and Smith 1991) and others showing no correlation (Botha and Black 2000). The patterns of SuSy activity in relation to sucrose content sometimes differ dramatically between studies: for example, in one study SuSy activity was more than twice as high in internode 6 than in internode 3 (Buczynski *et al.* 1993), while in another the relative activities between these internodes showed the reverse distribution (Lingle and Smith 1991). The difficulty lies in reconciling these differences with the different experimental protocols, growing conditions and cane varieties - clearly these aspects complicate comparison or integration of different studies.

It should be noted that sucrose accumulation in sugarcane is also strongly influenced by environmental and nutritional factors. Conditions that favour

vigorous growth, such as warm temperatures and abundant nitrogen and water supply, tend to produce cane with low sucrose content and juice purity (Clements *et al.* 1952; Thomas and Schmidt 1978). A study investigating the effects of temperature found that sucrose content in the stalk was lower in sugarcane grown at extreme high temperature (45 °C) than at optimal (27 °C) and low (15 °C) temperatures (Ebrahim *et al.* 1998). Both *in planta* and environmental factors thus determined the sucrose content of these plants.

This chapter seeks to explore the origin of SuSy and its role in higher plants, with emphasis on sink and carbon transport related topics.

2.2 Gene origin, structure and regulation of expression

2.2.1 Origin and evolution

One of the outstanding features of SuSy is that two or more isogenes occur in all plant systems studied thus far. This fact complicates the analysis of gene expression and adds complexity to the study of the association of this enzyme with various physiological processes, e.g. phloem transport (Nolte and Koch 1993; Geigenberger *et al.* 1993), nitrogen fixing (Gordon *et al.* 1999; Silvente *et al.* 2003) and cellulose and callose biosynthesis (Salnikov *et al.* 2001; Amor *et al.* 1995). Also, many of these processes will overlap or coincide temporally or spatially. The occurrence of multiple SuSy isogenes is not unique to plants; the cyanobacteria in the *Anabaena* (*Nostoc*) genus also contain multiple SuSys (Porchia *et al.* 1999) as well as invertases (Vargas *et al.* 2003), showing that these genes evolved before the existence of multicellular terrestrial plants, possibly through gene duplication events. The N-terminal of the prokaryotic SuSys is very different from that of higher plants, presumably because the N-terminal in plants is part of a regulatory domain lacking in the prokaryotic forms (Salerno and Curatti 2003). Therefore, plant SuSys apparently evolved regulatory capabilities useful or necessary in their new, more complex environment. What

makes the presence of SuSy in these prokaryotes interesting is that sucrose is also present in appreciable levels in chloroplasts of higher plants (Gerrits *et al.* 2001). If chloroplasts resulted from an endosymbiotic relationship with one of these prokaryotes, the presence of sucrose in chloroplasts today is hardly surprising. In fact, phylogenetic analyses of plant sucrose metabolism related (SMR) enzymes shows that they likely originated in prokaryotic ancestors (Salerno and Curatti 2003) and that plant sucrose metabolism itself was probably acquired at the time of the endosymbiotic origin of the chloroplast (Cumino *et al.* 2002). The SMR enzymes themselves may have originated from a common sucrose-phosphate-synthase (SPS)-like gene; SuSy and SPS share a similar glucosyltransferase domain (Cumino *et al.* 2002).

An interesting question is how the role of sucrose and SMR enzymes in modern plants evolved from the original roles of sucrose in the prokaryotic ancestors. Sucrose synthesis is suggested to have originated in the proteobacteria or a progenitor of the proteobacteria and cyanobacteria (Lunn 2002) as a response to osmotic stress and as a stabiliser of protein and membrane structures (Reed *et al.* 1986; Hagemann and Marin 1999). In the resurrection plant *Craterostigma plantagineum*, sucrose seems to play a clear role in desiccation tolerance, with low sucrose content during hydrated conditions and much higher levels under dehydrated conditions, and the opposite holding for octulose levels (Kleines *et al.* 1999). Hence, in this plant, sucrose seems to fulfil at least one of its primal functions, originating in a prokaryotic ancestor. The presence of SuSy in nitrogen-fixing cyanobacteria (Porchia *et al.* 1999) indicates that the association of SuSy with nitrogen fixing in plants may also have an ancient origin. Of course, sucrose has become the major source of long-distance transported carbon via the phloem in most plants; this function can be considered truly “new” relative to the prokaryotic ancestors. By extension, involvement of SMR enzymes in processes that are unique to higher plants, such as phloem transport, therefore represent a functional evolution from their original role. The functions of sucrose and SMRs therefore evolved along with plants themselves. The increased

complexity of eukaryotes on a genetic (the nucleus, chromosomes, meiosis), as well as whole-organism level (differentiated multicellular organs) has given impetus to this evolution of multiple isogenes and function. For example, the occurrence of polyploidism may have been one of the prime generators of multiple isoenzymes. Some crops that were thought to be diploids, such as maize, are now regarded as ancient polyploids that underwent extensive rearrangement and loss of genetic material following the origination of the ancestral polyploid (Gaut and Doebley 1997). *Arabidopsis thaliana*, as the simplest flowering plant, contains seven putative SuSy genes (Komatsu *et al.* 2002), although it is not known if all these are expressed. Most higher plants seem to contain more than the two known SuSy isoforms of the cyanobacteria (Wang *et al.* 1992; Barratt *et al.* 2001; Carlson *et al.* 2002). The fact that multiple SuSy genes were retained in, for example, maize, while other genetic material was lost after an initial polyploidisation event, indicates an advantage to the presence of these multiple isogenes. This is supported by the wide variety of processes SuSy is associated with and also the differential regulation between different classes of SuSy genes as manifested in e.g. tissue-specific expression of isoforms. There are several examples of SuSy isoforms that are specifically or predominantly expressed in particular tissues or organs (Yang and Russel 1990; Martinez, I *et al.* 1993; Sturm *et al.* 1999; Martinez, I *et al.* 1993; Yang and Russel 1990). On the other hand, some isoforms do not have a tissue-specific pattern of expression: for example, the newly discovered *Sus3* gene in maize is widely expressed (Carlson *et al.* 2002).

2.2.2 Gene structure

All known SuSy genes have some features in common: they encode very similar length mRNAs of about 2.7 kB that code for proteins of just over 800 amino acid residues, and the genes have from 13 to 16 exons. The maize *Sh1* gene on chromosome 9 was the first SuSy gene to have its structure determined fully (Werr 1985). This gene of 5.4 kB codes for an mRNA of 2.746 kB, which is

translated into a polypeptide of 802 amino acid residues with a predicted molecular mass of 91 731 Daltons. The gene contains 16 exons and 15 introns, with a long first intron of 1 028 bases. All introns comply with the GT-AG rule and the first 14 introns all contain a stop codon, so RNA must be spliced before the gene can be translated. The long leader intron is a feature of most SuSy genes (Werr 1985; Chopra *et al.* 1992; Yu *et al.* 1992; Shaw *et al.* 1994) and is of regulatory significance (see next section).

Four major SuSy gene classes based on the exon/intron structure have been suggested (Komatsu *et al.* 2002), but a somewhat more detailed phylogenetic tree has also been published (Barratt *et al.* 2001). Both these analyses suggest two major monocotyledonous groups, which diverged after the diversion of monocotyledonous and dicotyledonous plants. However, the maize *Sus3* gene shows higher homology to dicot than monocot SuSys (Carlson *et al.* 2002), possibly representing an ancestral form, so knowledge about the relationships among the various SuSy genes and also the relationships between monocot and dicot SuSys is accumulating and interpretations are changing constantly, which will be reflected in newer classifications. Unfortunately, predicting the number of SuSy genes in polyploids that resulted from early genome multiplication events cannot be inferred from the ancestral gene number, a general rule for all genes (Freeling 2001); the answers will have to be provided by whole genome sequencing.

The SuSy gene nomenclature is somewhat confusing, because of lack of consistency and renaming of genes once sequence homologies between genes from different species were established. A table indicating up-to-date SuSy gene and gene product names, as well as homologies, between three monocotyledonous crops is given below. Note the general convention that the gene name is in italics, while the gene product has the same name, but is written in regular type.

Table: Nomenclature and homologies of maize, rice and sugarcane SuSy genes. Gene name is followed by protein name, with former names in parenthesis. Columns 2 and 3 represent separate homologous groups. The maize *Sus3* gene is more similar to dicot than monocot SuSys and is not homologous to the rice *RSus3* gene.

Rice	*RSus1*, RSus1 (*RSs2*, RSs2)	*RSus2,* RSus2 (*RSs1*, RSs1*)*	*RSus3,* RSus3 (*RSs3*, RSs3)
Maize	*Sus1*, Sus1, SUS1* (*Sus1*, SS2)	*Sus2*, Sus2, *Sh1**, SH1* (*Sh1*, SS1)	*Sus3*, Sus3 (newly discovered gene)
Sugarcane		*Sus2*, Sus2 (*Sus1, SuSy-1*)	

* "Old style" names still in common use.

2.2.3 Gene expression and regulation

Expression of SuSy genes is sensitive to and determined by a variety of factors. This section will consider some general aspects of expression and regulation of SuSy genes, using specific examples from the literature as illustrations.

The leader intron, as well as gene flanking sequences, affect SuSy gene expression, as shown in potato (Fu *et al.* 1995a; Fu *et al.* 1995b). The effects of these sequences are influenced by the presence or absence of the other: e.g. removal of the leader intron and replacement of the *Sus3* 3' sequences with the nopaline synthase 3' sequences has no effect when 3.9 kB of 5' leader sequence is kept intact, but a construct containing only 320 base pairs of 5' sequence leads to five-fold reduction of GUS reporter gene expression in roots, but does not affect expression in other tissues. Removal of the leader intron in either of these constructs results in loss of GUS expression in vascular tissue of the anthers of transgenic tobacco plants, but induces strong expression in pollen. Native potato *Sus3* 3' flanking sequences have a negative effect on gene expression, but only in the absence of 5' sequences upstream from base –320 (Fu *et al.* 1995a). Removal of the leader intron from a construct containing potato *Sus4* native 5' and 3' sequences results in significant loss of sucrose inducibility (Fu *et al.* 1995b). Also, this construct results in eight times and four times lower GUS expression in tubers and roots respectively. Compared to the construct

containing the leader intron, tissue specificity is also affected, with expression in procambium tissue of roots, instead of the root cap and apical meristem (Fu *et al.* 1995b). A major difference between the potato *Sus3* and *Sus4* genes is that the *Sus4* gene is sucrose inducible and the *Sus3* gene is not. The necessity of the leader intron for both sucrose inducibility and high level expression of the *Sus4* gene is apparently due to this gene's different 5' and 3' flanking sequences. The leader intron of the *Sh1* gene in maize plays an important role in enhancement of gene expression (Clancy and Hannah 2002). Only a 145 base pair segment of the 1028 base pair intron is sufficient to enhance gene expression up to 50-fold, as indicated by a transient expression system using promoter-reporter gene fusions in cultured maize cells. This is in agreement with other reports that large parts of plant introns can be deleted without significantly affecting gene expression (Luehrsen and Walbot 1994; Rose and Beliakoff 2000). Interestingly, a T-rich 35 base pair region in the *Sh1* leader intron enhances reporter gene enzyme activity without significantly affecting transcript splicing, the first such report in plants. It is known that nuclear processes preceding transport of mature mRNA to the cytoplasm affect translation (Matsumota *et al.* 1998), this may be influenced by mRNA-binding proteins that remain bound after splicing (Le Hir *et al.* 2001).

In some crops, gene products from different SuSy gene classes seem to fulfil different functions (Chourey *et al.* 1998; Fu and Park 1995; Komatsu *et al.* 2002), but several SuSy genes, for example the *Sus3* gene in maize, and the rice *RSus1* and *RSus2* genes are expressed in a variety of tissues. It is more difficult here than in the case of potato, where the *Sus3* gene seems to provide the vascular function and the *Sus4* gene the sink function, to assign specific physiological roles. In legumes, there is at least one SuSy gene which is predominantly expressed in nodules (Hohnjec *et al.* 1999; Silvente *et al.* 2003). An interesting phenomenon in maize is that epistatic interaction occurs between the *Sh1* and *Sus1* genes; in wild-type plants, only the *Sus1* gene is expressed in the developing embryo, but in a *sus1* mutant the *Sh1* gene is also expressed in

the embryo, resulting in functional compensation (Chourey and Taliercio 1994). Hence, at least in this case, there seems to be an additional layer of regulation over SuSy gene expression, which can sense the absence of a functional SuSy and commence expression of a functional isozyme, even if it is not normally expressed in the affected tissue. This phenomenon also suggests another explanation for the presence of multiple isoforms: redundancy, to protect against the effect of damaging mutations, deletions and so forth. It certainly provides a possible reason for the existence of isozymes that are apparently very similar biochemically. Another interesting phenomenon related to expression patterns of SuSy genes is that the *Sh1* gene in maize is transcribed very actively during periods of anaerobic stress, and high levels of mRNA accumulate. This mRNA is not translated, but is bound to ribosomes, suggesting that this is to prevent transcription of other genes during this time by “occupying” most of the ribosomes (Taliercio and Chourey 1989). It is tempting to suggest that the lack of translation of these transcripts may be controlled by specific mRNA-binding proteins (Le Hir *et al.* 2001) - the effect of the T-rich 35 base pair sequence in the *Sh1* leader intron (referred to above) on reporter gene enzyme activity is consistent with this idea. The disparity between *Sh1* transcript levels and enzyme activity under anoxia should discourage studies that only rely on one type of data (such as mRNA levels) to measure gene expression. From the examples given above it is clear that SuSy genes have, through the combined, but not necessarily exclusive, interaction of 5’ and 3’ flanking sequences and the leader intron, evolved an extensive array of regulatory capabilities. Some of the regulation of SuSy gene expression takes place on a post-transcriptional level and may also function to block expression of other genes under certain conditions, but the mechanisms are still to be elucidated.

One of the unknown factors in the regulation of SuSy gene expression is the question whether 14-3-3 proteins play any role (Comparot *et al.* 2003). Spinach SPS enzyme is shown to be inhibited directly by 14-3-3 proteins (Toroser *et al.* 1998), but 14-3-3 proteins also interact with transcription factors (De Vetten *et al.*

1992; Igarashi *et al.* 2001), so there is an effect on the transcriptional level as well. In plants, 14-3-3 proteins interact with enzymes of nitrogen assimilation, such as nitrate reductase (Bachmann *et al.* 1996) and glutamine synthase (Moorhead *et al.* 1999) and therefore may play a role in coordinating sucrose and nitrogen metabolism. In addition, it needs to be mentioned that SuSy in nodules of soybean binds two small peptides of 12 and 22 amino acid residues, encoded by the ENOD40 gene which contains two overlapping ORFs (Rohrig *et al.* 2002). It is not yet known if these peptides also bind other enzymes or transcription factors, like the 14-3-3 proteins.

SuSy genes respond to sugar levels or changes in osmotic potential. The transcription of the *Sh1* and *Sus1* SuSy genes in maize root tips responds differentially to glucose and sucrose, with the *Sh1* gene repressed by both sugars and the *Sus1* gene induced (Koch *et al.* 1992). Fructose strongly induces the *Sus1* gene, but has no effect on the *Sh1* gene. Mannitol or non-metabolisable sugars do not change gene expression, showing that the genes respond to the *sugars*, and not to changes in osmotic potential. Native protein gel blots show the levels of SuSy isozymes to correspond to the changes in their respective transcript levels under these conditions, unlike under anoxic stress, when *Sh1* transcripts accumulate, but not protein (Taliercio and Chourey 1989). The effect of sugars on the expression of the maize SuSy isoforms is also manifested in a change in enzyme localisation, with SuSy evenly distributed in roots incubated in high sugar, but sugar-starved roots showed preferential localisation in peripheral tissues, particularly the epidermis, as well as vascular tissues. In contrast to the maize *Sh1* and *Sus1* genes, the *Arabidopsis Sus1* gene is also regulated by changes in osmotic potential, not only by the sugars themselves (Dejardin *et al.* 1999). It is suggested that at least two pathways for regulating *Arabidopsis Sus1* exist; a hexokinase-dependent pathway at low sugar levels, and an osmotic potential-sensitive pathway at higher sugar levels (Ciereszko and Kleczkowski 2002). The results from this later study also suggest that the effect of sucrose (at

least at low concentrations) is mediated through glucose via hexokinase after cleavage of sucrose.

Sh1 and *Sus1* genes do not only respond differentially to sugar levels, but also to hypoxia and anoxia. *Sh1* is strongly induced by anoxia, but not hypoxia, while the opposite is true for *Sus1* (Zeng *et al.* 1998). SuSy is found to contribute greatly to root tip viability during anoxic conditions, as shown by experiments with maize single (*sh1Sus1*) and double (*sh1sus1*) mutants, where root tip viability is positively correlated with the number of functional SuSy genes (Ricard *et al.* 1998). SuSy and invertases show opposite responses during low oxygen stress, with invertases downregulated, and SuSy expression mostly similar or higher than pre-stress conditions (Zeng *et al.* 1999). SuSy also responds to wounding (Salanoubat and Belliard 1989) and cold stress (Crespi *et al.* 1991), with expression down- and upregulated respectively.

From the examples given here it is evident that SuSy gene expression, as well as post-transcriptional regulation, are influenced and determined by a variety of environmental and physiological factors. SuSy is also subject to a variety of potential regulatory measures on the protein level; these will be referred to in the next section.

2.3 Physical/biochemical properties and fine regulation of enzyme activity

SuSy polypeptides generally have a molecular mass of about 90-94 kDa, although both higher (100 kDa) (do Nascimento *et al.* 2000) and lower (80 kDa) (Sebkova *et al.* 1995) values are reported. SuSy enzymes almost always exist as tetrameric molecules, e.g. from plants, (Delmer 1972; Graham and Johnson 1978; Yen *et al.* 1994; Sebkova *et al.* 1995; Barratt *et al.* 2001; Klotz *et al.* 2003) and cyanobacteria (Porchia *et al.* 1999), but under certain conditions, especially lack of Mg^{2+}, higher order multimers can form (Su and Preiss 1978). The

tetrameric form exhibits the highest specific activity. Heterotetrameric SuSys are present in several plants e.g. maize (Chourey *et al.* 1986), sugarbeet (Klotz *et al.* 2003) and rice (Huang and Wang 1998). In maize, root extracts contain various combinations of SS1 and SS2 heterotetramers, but endosperm extracts contain only SS1 and SS2 homotetramers. In rice seedlings, only heterotetramers were isolated. Sugarbeet root contains a SuSy isoform consisting of two 84 kDa and two 86 kDa subunits.

SuSys are generally inhibited in the sucrose cleavage direction by both Ca^{2+} and Mg^{2+} ions; for rice SuSys this inhibition is fairly mild (less than 20 % inhibition at 10 mM (Huang and Wang 1998)), but pear fruit SuSys are more sensitive, with an average of about 40 % inhibition at only 5 mM (Tanase and Yamaki 2000). However, in the sucrose synthesis direction, Mg^{2+} ions have a stimulatory effect with activation ranging from about 40 to 60 % in the rice and pear SuSys respectively, also at 5 mM concentration. The effect of Ca^{2+} ions is similar to that of Mg^{2+}. Like many enzymes, pear fruit SuSys are strongly inhibited by Cu^{2+}, Zn^{2+} and Hg^{+} with 80 % or more inhibition at 1 mM concentration.

SuSy enzymes are usually not absolutely specific for a particular nucleoside-diphosphate. Invariably, UDP is the most efficient substrate, but others, ADP and TDP in particular, can also be utilised. For rice SuSy isozymes, TDP is about 30-95 % as efficient as UDP, while for ADP it is about 15-55 %, with CDP, GDP and IDP giving various levels of activity among the isozymes of up to about 30 % of the activity with UDP (Yen *et al.* 1994). The ability to use different nucleoside-diphosphates, good stability, and the fact that SuSy is not strongly inhibited by Mn^{2+}, which some other enzymes use as a cofactor, increases the attractiveness of using SuSy in commercial synthesis of nucleoside diphosphates, as well as compounds that are synthesised downstream from these in the same process (Elling and Kula 1995).

For a given SuSy, the pH optimum of the sucrose cleavage reaction is lower than for the synthesis reaction. Otherwise, pH optima vary between species: pH optima for the cleavage reaction vary from about 6.0 in sugarbeet (Klotz *et al.* 2003) to 7.5 in pear fruit and mung bean (Tanase and Yamaki 2000; Delmer 1972), with optima of close to 7.0 most common, while for the synthesis reaction pH optima vary from about 7.0 in sugarbeet (Klotz *et al.* 2003) to 9.0 for a cucumber fruit SuSy (Gross and Pharr 1982). SuSy activity in the sucrose cleavage direction is confined to a narrower pH range than sucrose synthesis activity. Sucrose synthesis activity at 50 % or greater than that at optimum pH is retained at pH levels as high as 10.5 in sugarbeet SuSy, while breakdown activity for most SuSys ceases completely at pH 9.0. Within a species, pronounced differences can occur in the behaviour of different SuSy isozymes with regard to pH; for example, sugarbeet isozymes differ in pH optima and activity range in both sucrose breakdown and synthesis directions, while the behaviour of sugarcane (Buczynski *et al.* 1993) and cucumber fruit isozymes is much more similar at the different pH values.

Amino acid analyses show that maize SuSy isozymes have a high hydrophobic amino acid residue content at roughly a third of the total residues (Su and Preiss 1978; Echt and Chourey 1985). All three known maize SuSy isozymes contain at least one potential transmembrane domain (Carlson and Chourey 1996; Carlson *et al.* 2002) and the SS1 and SS2 isoforms have been shown to associate with the plasma membrane (Carlson and Chourey 1996). The phosphorylation state of SuSy apparently regulates the distribution of SuSy between the cytosol and the plasma membrane, with dephosphorylation favouring association with the plasmalemma (Winter *et al.* 1997). Dephosphorylation is shown to enhance binding of hydrophobic probes to SuSy, which probably means that more hydrophobic residues are exposed to the solvent in the dephosphorylated state, favouring membrane association. The membrane-associated form may be involved in cellulose and callose synthesis (Amor *et al.* 1995). Other known SuSy interactions not involving metabolites include binding to G- and F-actin (Winter *et*

al. 1998); also, soybean nodule SuSy binds two small peptides encoded by the ENOD40 gene (Rohrig *et al.* 2002), but the physiological significance of these interactions is not yet known.

The kinetic parameters of SuSy differ widely between enzymes from different sources and different isozymes; however, some general trends are evident: for a given SuSy the K_m value for sucrose is always highest, followed by that for fructose. Substrate K_m values for UDP and UDP-glucose may either be the lowest or second-lowest of the four substrates. Approximate ranges for the different K_m values are, for sucrose: 32-87 mM (Tanase and Yamaki 2000; Sebkova *et al.* 1995), for fructose: 1.1-20.9 mM (Klotz *et al.* 2003; Barratt *et al.* 2001), for UDP-glucose: 0.03-1.3 mM and for UDP: 0.02-0.39 mM (Buczynski *et al.* 1993; Sebkova *et al.* 1995). Random (Delmer 1972) and substituted (ping-pong) (Sung and Su 1973) reaction mechanisms have been reported for SuSy but most studies favour an ordered mechanism (Wolosiuk and Pontis 1974; Doehlert 1987). SuSy isoforms show differences in their sensitivity to inhibition. For example, the pea Sus1 isoform is very sensitive to substrate inhibition by fructose, while the Sus2 and Sus3 isoforms are not (Barratt *et al.* 2001). Generally, SuSys are subject to product inhibition by fructose (Sebkova *et al.* 1995; Wolosiuk and Pontis 1974), although the reported inhibition types differ, with non-competitive and competitive inhibition described, respectively, in these two studies. Glucose is an uncompetitive inhibitor with regard to sucrose (Sebkova *et al.* 1995; Doehlert 1987). Like fructose, UDP-glucose is also a product inhibitor (Wolosiuk and Pontis 1974). The significance *in vivo* of the inhibition qualities noted above have not yet been comprehensively studied. Generally, SuSy follows Michaelis-Menten kinetics, but deviations from this can occur (Su and Preiss 1978), which may be due to several multimeric SuSy forms encountered in that study with degree of polymerisation higher than four.

Several of the physical and biochemical properties described above may have significant implications for fine regulation of enzyme activity. *In vitro*,

phosphorylation increases the sucrose breakdown activity of both maize and *Vigna radiata* (mung bean) SuSy (Huber *et al.* 1996; Nakai *et al.* 1998), so in addition to regulation of partitioning of SuSy between the cytosol and plasmalemma, phosphorylation may serve to regulate the ratio between sucrose breakdown and synthesis activities. Phosphorylation of soybean nodule SuSy occurs through a Ca^{2+}-dependent protein kinase (Zhang and Chollet 1997), which is shown to be required for starch accumulation in rice grains (Asano *et al.* 2002). Rice plants deficient in the SuSy kinase produce watery seeds that accumulate sucrose instead of starch, which agrees with the observations that phosphorylation preferentially stimulates the sucrose cleavage reaction.

The purpose of the binding of two small peptides to SuSy in soybean nodules is suggested to be regulation of sucrose use in nodules (Rohrig *et al.* 2002), but this has not yet been confirmed. Changes in intracellular pH could play a role in the regulation of the activity of the sugarbeet SuSy isozymes; in the direction of sucrose synthesis the SuSy1 isoform in particular exhibits twice the activity of the SuSy2 isoform (Klotz *et al.* 2003). SuSy is subject to both substrate and product inhibition by fructose, but the sensitivity differs substantially between isoforms, as in the case of pea Sus1, which is very sensitive to fructose substrate inhibition compared to the Sus2 and Sus3 forms (Barratt *et al.* 2001). Glucose competes with fructose as a “substrate” inhibitor (Doehlert 1987) and is proposed to inhibit sucrose synthesis by SuSy during starch breakdown in germinating maize kernels (Echeverria and Humphreys 1985). However, the fact that maize SuSy is shown to bind to actin (Winter *et al.* 1998) and is thus immobilised, probably means that the local reactant concentrations often differ dramatically from experimentally determined “average” metabolite concentrations. This means that *in vitro* inhibition data may not always be applicable to the situation *in vivo*.

2.4 SuSy in higher plants

This section will consider SuSy in the context of whole-plant development, physiology and the influence of environmental conditions.

2.4.1 SuSy expression related to physiological stage/condition

Sucrose is the final product of photosynthesis and is the dominant form in which assimilated carbon is transported in plants, except those that also use carbohydrates such as raffinose and stachyose for this purpose (Zimmerman and Ziegler 1975). Once sucrose arrives at a carbon sink organ such as a young growing leaf, roots, or a storage organ, it is usually cleaved and used to fuel respiration and biosynthetic processes, or the carbon is stored in some form. Few plants store carbon as sucrose in high concentrations in storage organs, with sugarcane and sugarbeet two major exceptions. Starch, fructans, lipids and storage proteins, such as patatin in potato tubers, are usually synthesised from the imported sucrose. In young and growing organs the imported sucrose will be used not for storage, but primarily for growth and expansion processes. Although the end use of the imported sucrose differs between storage organs and growing tissues, they all share the requirement for the cleavage of this sucrose for it to be further utilised. This cleavage can occur either via invertases (cytosolic neutral invertase, cell wall bound acid invertase, vacuolar acid invertase) or SuSy. The activity of both SuSy and invertases vary with plant and organ development, but SuSy is often by far the dominant or exclusive sucrose cleavage activity in various plants' storage sinks (Sung *et al.* 1989) or actively growing tissue, such as very young soybean leaves (Schmalstig and Hitz 1987). There is strong evidence that a membrane-bound form of SuSy provides UDP-glucose for cellulose synthesis (Amor *et al.* 1995) and this may be of special importance in young, growing tissue. The fact that in the last-mentioned study SuSy was localised not only to the plasmalemma, but specifically to sites of cellulose

synthesis, suggests that the products of sucrose cleavage by SuSy in the *cytosol* are not used for cellulose synthesis, but for respiration etc.

SuSy is known to be involved in the development of seeds; for example, SuSy activity significantly affects starch synthesis in maize kernels. *sh1* mutants with reduced SuSy activity in kernels are starch-deficient and exhibit a characteristic shrunken seed phenotype (Chourey and Nelson 1976). Subsequently, *sh1sus1* double mutants were obtained which had further reduced starch content, at about 50 % of wild-type levels (Chourey *et al.* 1998). A finding from the latter study is that the *Sus1* gene is actually more important than the *Sh1* gene for starch formation and that the shrunken phenotype in *Sh1* mutants is due to impaired cellulose synthesis. Thus, it is concluded that the *Sh1* gene is more important for normal cellulose synthesis, rather than starch synthesis, while the *Sus1* gene is more important for starch synthesis. Despite the fact that the double mutants have barely 0.5 % of wild-type SuSy activity, they are perfectly viable plants under normal growing conditions, but their adaptability during adverse conditions is impaired (see section: *SuSy expression during stress conditions*). The residual enzyme activity present in the mutants is probably due to a recently discovered third SuSy isoform (Carlson *et al.* 2002) rather than leakiness of the *sus1* mutation. From the results of these studies it seems as if SuSy in wild-type maize plants is present in levels far exceeding that required for normal plant development and growth under favourable conditions. Given that epistatic interaction occurs between the *Sh1* and *Sus1* genes in a *sus1* mutant – normally only the *Sus1* gene is expressed in developing embryos, but in *sus1* mutants, the *Sh1* gene is expressed here – indicates an apparent preference or need for SuSy expression specifically in this tissue. That the recently discovered *Sus3* isoform is also strongly expressed in embryos of *sh1sus1* double mutants supports this (Carlson *et al.* 2002).

SuSy is subject to both transcriptional and posttranscriptional regulation in developing seeds of *Vicia faba* (Heim *et al.* 1993). SuSy mRNA is present in

cotyledons at 15 days after flowering (DAF), but SuSy enzyme activity is only detectable at 20 DAF. SuSy mRNA levels in *V. faba* cotyledons are positively correlated with the sucrose concentration and these changes are not due to osmotic effects. High SuSy activity in *V. faba* cotyledons is correlated with high rates of starch synthesis, so SuSy is probably involved in providing precursors for starch synthesis, as in potato tubers (Zrenner *et al.* 1995). Rise in starch content and cotyledon growth occur together in *V. faba* seeds, so SuSy could also contribute precursors for cellulose synthesis. Sucrose levels in the seed coat follow the opposite temporal pattern compared to cotyledons, with sucrose levels peaking at about 7 DAF compared to about 25 DAF in cotyledons. SuSy mRNA levels are also positively correlated with sucrose levels in seed coat; hence, gene regulation is apparently similar between these different seed tissues, although temporal expression patterns differ.

There is very little information in the literature on the role, if any, of SuSy during seed germination, except a report which concludes that SuSy in the maize scutellum is most probably inhibited by glucose entering from the adjacent endosperm during germination (Echeverria and Humphreys 1985) and so does not contribute to sucrose synthesis at this time. In contrast, in dormant artichoke (*Helianthus tuberosus*) tubers, sucrose is almost exclusively (~ 95-97%) synthesised by SuSy during mobilisation of fructans (Noël and Pontis 2000).

Overall, SuSy activity is highest in non-photosynthetic and sink tissues; indeed, SuSy activity is a good measure of sink strength in several plants (Sung *et al.* 1989). SuSy is present at high levels in immature, developing maize leaves, but in fully autotrophic leaves it is only just detectable, following the opposite pattern to SPS activity (Nguyen-Quoc *et al.* 1990). The residual SuSy activity in maize leaves after the sucrose import-export transition is localised in the phloem (Nolte and Koch 1993) and most probably consists of the SS1 isoform (Yang and Russel 1990), while the SS2 isoform is almost exclusively present in heterotrophic leaves with high SuSy activity (Nguyen-Quoc *et al.* 1990).

Experiments with tomato and carrot plants where SuSy activity was decreased by transforming plants with SuSy genes that are aligned in the antisense orientation, give results that can be compared with the outcome of double *sh1sus1* mutations in maize, in the sense that the transformants are viable plants. However, in contrast to maize double mutants, the antisense tomato and carrot plants do have a visible phenotype. Transformed carrot plants are much smaller than control plants and also have higher levels of sucrose in the tap root, but lower levels of UDP-glucose, fructose, glucose, cellulose and starch (Tang and Sturm 1999), which indicates that sucrose cleavage by SuSy is important for growth. Tomato plants which have up to 99 % reduced SuSy activity specifically in fruit, have similar starch and sugar levels to control plants (Chengappa *et al.* 1999). However, drastic (98 %) reduction of SuSy activity reduces sucrose import capacity of very young (7-day old) tomato fruit and the transformants have significantly less fruit (up to 60 % less) per plant at maturity (D'Aoust *et al.* 1999). SuSy thus appears to participate in the regulation of tomato fruit setting at an early stage of fruit development.

2.4.2 SuSy involvement in specific physiological processes

The fact that sucrose is the major form of transported carbon in most plants, and therefore the source of carbon for all of metabolism in non-photosynthetic tissues, necessarily means that enzymes of sucrose metabolism take on a central role. Despite the apparent generality of this role (for example providing substrate for glycolysis), strong evidence shows that SuSy is specifically involved in a variety of other physiological processes. This section will present a few of these processes, which also impact directly on carbon partitioning and availability.

The association of SuSy with vascular bundles is noted in several studies (Hawker and Hatch 1965; Yang and Russel 1990; Tomlinson *et al.* 1991).

Specifically, SuSy is localised in the companion cells of vascular bundles (Nolte and Koch 1993; Rouhier and Usuda 2001), which display characteristics of cells with increased respiratory rate, such as very high density of mitochondria (Warmbrodt *et al.* 1989). On this evidence, SuSy may function to fuel respiration to satisfy the high ATP demand because of the plasma membrane H^+/ATPase (Nolte and Koch 1993), which is needed to maintain an H^+ gradient for sucrose/H^+ symport. Interestingly, in young, heterotrophic maize leaves, SuSy is specifically excluded from vascular tissue (Hanggi and Fleming 2001), while in mature, autotrophic maize leaves, the SuSy activity that remains after the sucrose import-export transition is associated with the vascular bundles (Nolte and Koch 1993), supporting a role for SuSy in phloem loading in maize leaves. Very recent work in *Coleus blumei* shows that because of symplastic continuity between companion cells and sieve elements, a variety of low molecular weight compounds can enter the sieve elements, but long-distance phloem transport favours stachyose, raffinose and sucrose, through specific retention and retrieval mechanisms (Ayre *et al.* 2003). Supporting this model is that sucrose/H^+ symporters are present along the length of the phloem to retrieve leaked sucrose (Van Bel 1993). Significantly, SuSy is not only localised at the sites of phloem loading and unloading, but also in phloem that functions in long-distance transport, such as mature citrus leaf midrib (Nolte and Koch 1993). This points to involvement in the retrieval of leaked sucrose, with the same function as in regions of phloem loading – providing substrate for respiration to supply the ATP needed to maintain an H^+ gradient for the sucrose/H^+ symporters. Further evidence for the functioning of SuSy in phloem is that metabolite levels in phloem sap from castor bean indicate the SuSy reaction is close to equilibrium (Geigenberger *et al.* 1993). Also, the preference for sucrose cleavage by SuSy, instead of invertase, under low oxygen conditions (Zeng *et al.* 1999) is consistent with the presence of SuSy in the vascular tissue, which has very low oxygen content compared to other tissues (about 7 % versus up to 15 % in the rest of *Ricinus communis* stems) (Van Dongen *et al.* 2003).

Several lines of evidence connect SuSy with synthesis of polysaccharides. About half of total SuSy protein is tightly associated with the plasma membrane in developing cotton fibres. Also, SuSy can be immunolocalised to cellulose microfibrilles after plasmolysis, which could indicate a complex between SuSy and cellulose synthase (Amor *et al.* 1995). In the latter study, permeabilised cotton fibre cells synthesised both cellulose and callose using carbon from sucrose. Another study shows that SuSy protein is absent in ovules of a cotton fibreless seed mutant on the day of anthesis, but abundantly present in initiating fibre cells of wild-type ovules at the same stage (Ruan and Chourey 1998). In maize, SuSy is present in the Golgi apparatus, which is the site of synthesis of mixed linkage (1→3), (1→4) β-D-glucan, and is suggested to fulfil the same function as in cotton seeds – supplying substrate to the synthase complex (Buckeridge 1999).

SuSy is shown to be required for normal storage carbohydrate accumulation in a number of plants. Maize kernels of an *sh1sus1* double mutant contain only about half the normal starch levels (Chourey *et al.* 1998), while antisense inhibition of SuSy in potato tubers leads to significant reductions in both starch and storage proteins, such as patatin, as well as decreases in tuber dry weight (Zrenner *et al.* 1995). In these tubers, 40-fold increases in invertase activities did not compensate for the loss of SuSy activity, showing that sucrose cleavage specifically by SuSy is needed. SuSy activity is proposed to be a measure of sink strength in several plants, including potato, cassava and sweetgum (Sung *et al.* 1989). The SuSy activity in the storage organs of these crops correlate positively with the periods of highest rates of sucrose accumulation. In sugarbeet, a specific isoform of SuSy is induced at the onset of root maturation and sucrose accumulation (Klotz *et al.* 2003). Sugarcane represents an “intermediate case” as far as the relation between SuSy activity and storage carbohydrate content is concerned, since substantial levels of SuSy activity remain in mature, sucrose-storing internodes, but the highest activity is usually found in one of the younger internodes with lower sucrose content (Zhu *et al.* 1997; Botha and Black 2000).

Its activity is, however, not positively correlated with sucrose content. At the other end of the scale, maturing tomato fruit contain no SuSy activity (Wang *et al.* 1994), so SuSy is obviously not a determinant of sink strength in all cases.

2.4.3 SuSy expression during stress conditions

In addition to regulation by carbohydrates or changes in water stress, SuSy genes also respond to stress conditions such as anoxia, low temperature and wounding.

Maize *sh1sus1* double mutants have no visible phenotype, except the shrunken seed morphology, and the plants grow normally under aerobic conditions (Chourey *et al.* 1998). However, *sh1Sus1* single mutant and *sh1sus1* double mutant maize seedlings show a 20 % and 70 % reduction in root tip viability under low oxygen stress conditions (Ricard *et al.* 1998). Interestingly, both acid and neutral invertase levels in the mutant seedlings are at levels comparable or higher than SuSy at all times. Invertases are downregulated under low oxygen, favouring sucrose breakdown via SuSy (Zeng *et al.* 1999). The results of these studies show that SuSy, and not invertases, will contribute to enhanced survival under flooding conditions, which always impose varying degrees of hypoxia or anoxia on roots.

In the desiccation-tolerant resurrection plant, *Craterostigma plantagineum*, two SuSy genes are present that respond differentially to hydration levels (Kleines *et al.* 1999). During periods of dehydration, both the CpSS1 and CpSS2 isoforms accumulate on the mRNA and protein levels in leaves, and decrease during rehydration. In roots, the pattern is similar, except that the downregulation of the CpSS1 gene on the mRNA level is quicker. Dehydration is associated with a rapid decrease in the levels of the C-8 sugar 2-octulose, and increase in sucrose levels, possibly to keep osmotic potential as steady as possible. It is not known whether SuSy participates directly in sucrose synthesis, or if phloem unloading of

sucrose is increased - SuSy is mostly upregulated in phloem tissue during dehydration.

Other stresses that influence SuSy are wounding, which results in decreases in mRNA levels in potato (Salanoubat and Belliard 1989) and sugarbeet (Hesse and Willmitzer 1996) and cold stress, which leads to an increase in SuSy enzyme (Crespi *et al.* 1991; Sasaki *et al.* 2001). In the latter case, both SuSy and SPS are upregulated and sucrose, glucose and fructose levels increase, but decrease to normal levels after return to normal temperature. It is unknown what the relative contribution of SuSy to sucrose breakdown or synthesis is under these conditions.

2.5 Concluding remarks

The overview presented in the preceding sections makes it clear that although SuSy can ultimately only break down or synthesise sucrose, the enzyme is directly involved in a variety of important physiological processes, reflecting the central role and importance of sucrose in plant metabolism. It is also clear that the role of SuSy and SuSy isoforms differ between species, reflecting environmental and evolutionary differences. The way SuSy is involved in the unique water-stress tolerance process of the resurrection plant is a good example of how plant metabolism can adapt to serve survival needs. The existence and maintenance, over evolutionary time, of two pathways of sucrose synthesis (SuSy and SPS), as well as two pathways of sucrose breakdown (SuSy and invertases) in photosynthetic organisms from cyanobacteria to higher plants, provides flexibility in metabolism to cope with different environmental, developmental and physiological conditions, for example the differential responses of invertases and SuSy to low oxygen stress. Current knowledge on SuSy is limited on questions regarding the roles of multiple enzyme isoforms.

Given the large number of putative SuSy genes in *Arabidopsis thaliana*, and whole-genome sequencing efforts on major crops such as rice and maize, it looks certain that new SuSy genes will be discovered in several, if not all, important crop plants. In the case of sugarcane only one full-length sequence, that for SuSy-1, the equivalent to maize *Sh1*, is known (Lingle and Dyer 2001), but at least two isoforms are reported in the literature based on results with monoclonal antibodies against maize SuSy isoforms (Buczynski *et al.* 1993). However, three rice isoforms have been cloned, while several different putative SuSy expressed sequence tags in sugarcane show only limited homology to known SuSy genes (Carson and Botha 2002). All this points to only one conclusion: that more SuSy genes are present in sugarcane than those that are currently known. Study of these SuSy genes and the isozymes encoded by them may provide insight into the process of sucrose accumulation in sugarcane, which is still poorly understood. Information on different SuSy isozymes in sugarcane could contribute to knowledge on the specific roles of these different isozymes and hence address the question of why there are multiple isoforms of SuSy in general. When specific SuSy gene sequences are known, studies can be designed to investigate the roles of these genes, using molecular biology techniques such as antisense methods that have been referred to in previous sections. However, knowledge on the enzyme kinetics and localisation of SuSy isoforms and integration of this into metabolic models will also enhance our understanding. The latter approach was taken in this project and results will be discussed in the chapters to follow.

2.6 Aim and outline of following chapters

Chapter 3: The occurrence of SuSy isoforms in leaf roll tissue was investigated. Since four putative SuSy ESTs have been reported from this tissue, the likelihood of finding different isoforms here seemed very high.

Chapter 4: One of the SuSy isoforms present in leaf roll tissue was characterised kinetically in detail. This was to obtain kinetic parameters, unavailable for sugarcane SuSy, for use in a kinetic model of sucrose accumulation. This lead to interesting findings on the metabolic effects of different SuSy isoforms.

Chapter 5: The tissue localisation and expression of SuSy in the culm was investigated. Particularly, the question whether SuSy is present in storage parenchyma cells of mature internodes, where it would have a direct effect on sucrose metabolism, was answered.

Chapter 6: Conclusions and recommendations for further research on sugarcane SuSy are presented.

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CHAPTER THREE

PURIFICATION AND CHARACTERISATION OF THE SUCROSE SYNTHASE IN SUGARCANE

3.1 Abstract

Three sucrose synthase forms were isolated from sugarcane leaf roll tissue. During anion exchange chromatography one peak of activity (SuSyA) eluted during the wash step and the other peak (SuSyB) during the salt gradient phase at 180 mM KCl concentration. A third form of activity (SuSyC) that co-eluted at 180 mM KCl was also present in the leaf roll. Substrate K_m values, as well as sucrose breakdown/synthesis ratios, differed between these forms. K_m values (mM) were, for sucrose, 41.8 ± 3.4, 109 ± 23 and 35.9 ± 2.2; for UDP, 1.07 ± 0.08, 0.21 ± 0.04 and 0.02 ± 0.002; for fructose, 6.62 ± 1.55, 11.7 ± 2.5 and 6.49 ± 0.60 and for UDP-glucose 3.59 ± 0.37, 0.53 ± 0.14 and 0.24 ± 0.03 for SuSyA, SuSyB and SuSyC respectively. Sucrose breakdown/synthesis ratios at saturating substrate concentrations were 0.079, 0.38 and 0.49 respectively. The ratio of peak areas of peak one (low breakdown/synthesis ratio) to peak two (high breakdown/synthesis ratio) in sucrose accumulating tissue (internode 9) was 0.88 and in non-accumulating (leaf roll) tissue it was 14.5 at the same time of year. The molecular mass of the denatured subunits of all three forms was 94 kDa by SDS-PAGE. A polyclonal antiserum raised against SuSyB cross-reacted with all three forms on an immunoblot, but only SuSyA and SuSyB were immunoinactivated by this serum.

3.2 Introduction

Sucrose synthase (SuSy, UDP-glucose: D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13) is a central enzyme of sucrose metabolism and partitioning (Koch *et al.* 1996; Schrader and Sauter 2002; Zrenner *et al.* 1995; Chourey *et al.* 1998; N'tchobo *et al.* 1999; Sturm *et al.* 1999). Given that sucrose is the main transport carbohydrate in almost all plants, the enzyme is implicated in a wide variety of processes, e.g. cellulose synthesis, phloem transport, storage carbohydrate accumulation and stress response mechanisms (Amor *et al.* 1995; Nolte and Koch 1993; Asano *et al.* 2002; Dejardin *et al.* 1999). Although the enzyme catalyses a freely reversible reaction, with reported ΔG values ranging from -1.4 to -4.7 $kJ.mol^{-1}$ for the sucrose synthesis reaction (Geigenberger and Stitt 1993), the main physiological function is thought to be the cleavage of sucrose in sink organs (Hawker 1985), which would be greatly facilitated by very high sucrose concentrations in the phloem and in areas of phloem unloading (Kruger 1990). In *Ricinus communis* seedlings, sucrose concentrations of up to about 250 mM have been measured in phloem (Verscht *et al.* 1998). A role for SuSy in phloem transport has been suggested in several studies (Martin *et al.* 1993; Nolte and Koch 1993; Yang and Russel 1990). High SuSy activity has also been implicated in sink strength (Zrenner *et al.* 1995; Sung *et al.* 1989).

Early work on maize (Chourey 1981; McCormick *et al.* 1982) indicated two non-allelic genes for SuSy, but recently a third form was discovered (Carlson *et al.* 2002). Three forms have been cloned from *Pisum sativum* and expressed in a bacterial system (Barratt *et al.* 2001). At least three SuSy genes, which show differences in expression between tissues, are present in the rice genome (Wang *et al.* 1999; Wang *et al.* 1992). Interestingly, the isoforms occurring in the phloem, roots and leaves are different, with *RSus2* expressed in leaf phloem and *RSus1* in root phloem. On the amino acid level, it is apparent that there is less homology between different SuSys within a species than there is between corresponding SuSys of different species. For example, there is 75% homology between the maize SS1 (from *Sh1* gene)

and SS2 (from *Sus1* gene) forms, but 90% homology between the rice RSus1 and maize SS2 proteins. The SS1 from sugarcane is 97% identical on amino acid level to maize SS1 (Lingle and Dyer 2001). It is also evident that certain regions in all SuSy forms are highly conserved, even identical between species. For example a stretch of 55 residues from residue 643 in various rice, maize, wheat and potato SuSys. The highest sequence diversity is found at the N and C termini (Wang *et al.* 1992).

The maize SS1 and SS2 forms are not resolvable on a MonoQ® anion exchange column (Nguyen-Quoc *et al.* 1990), but a similar approach in rice resolves four SuSy isozymes from milky-stage seeds (Yen *et al.* 1994). In an alternative approach three pea isozymes were cloned and expressed (Barratt *et al.* 2001). Currently it is accepted that at least two SuSy isoforms are present in both monocotyledonous (Echt and Chourey 1985) and dicotyledonous plants (Sturm *et al.* 1999; Barratt *et al.* 2001). The pH optima and substrate affinities of the isoforms within and between species are mostly quite similar (Buczynski *et al.* 1993; Nguyen-Quoc *et al.* 1990). However, expression patterns of the isoforms differ (Sturm *et al.* 1999; Huang *et al.* 1996). The available kinetic data for the two known sugarcane SuSys are limited, but they have similar substrate K_m values to the maize SuSys (Buczynski *et al.* 1993). There are differences in the sucrose breakdown/synthesis ratios of SuSy preparations from different tissues (Buczynski *et al.* 1993). This would imply that different ratios of isoforms must be present and that these must have different breakdown/synthesis ratios.

Very high SuSy activity is present in sink tissues of sugarcane, varying from about 450 nmol.min^{-1}.mg protein^{-1} in the apex to 1 600 nmol.min^{-1}.mg protein^{-1} in internode 14 (Buczynski *et al.* 1993). The SuSy activity in the leaf roll tissue is also very high; about 1 400 nmol.min^{-1}.mg protein^{-1} (Buczynski *et al.* 1993). Interestingly these sink tissues include both sucrose accumulating (culm) and non-accumulating tissue such as the leaf roll and roots. Recently it was shown that multiple different SuSy genes are expressed in sugarcane, including some purported SuSy ESTs from a leaf roll cDNA library that show only low

homology to known SuSy sequences (Carson and Botha 2002; Lingle and Dyer 2001).

Maize anti-SS1 and SS2 monoclonal antibodies have been used to separate two forms of SuSy in sugarcane (Buczynski *et al.* 1993). Four sucrose synthase activities were separated by anion exchange chromatography in rice, using milky-stage seeds, and these showed different preferences for nucleotides and had differing sucrose breakdown/synthesis ratios (Yen *et al.* 1994). Three full-length SuSy sequences were isolated from rice using both genomic and cDNA-based approaches (Huang *et al.* 1996). Gene expression studies have indicated three SuSy isoforms in maize (Carlson *et al.* 2002), pea (Barratt *et al.* 2001) and citrus fruit (Komatsu *et al.* 2002). The *Arabidopsis thaliana* genome contains seven putative SuSy genes, some of which are very similar to those of other plants, but others fall into a distinct group in a phylogenetic tree (Komatsu *et al.* 2002). It is not yet known if all these genes are expressed. Given these developments, we attempted to identify different SuSy proteins in sugarcane sink tissue and establish whether these differ kinetically.

Here we report on the properties of three SuSy isoforms present in sugarcane. Depending on the physiological status of the tissue, the ratio between these activities varies. The activities are kinetically different, resulting in different breakdown/synthesis ratios.

3.3 Materials and methods

3.3.1 Materials

Sugarcane (*Saccharum officinarum*) variety N19 plants field grown at the University of Stellenbosch experimental farm were used. Internode one was taken as the internode attached to the leaf with the first exposed dewlap.

Tris buffer, DTT and coupling enzymes were obtained from Roche (Grenzacherstrasse 124, CH-4070, Basel, Switzerland), except UDP-glucose pyrophosphorylase which was from Sigma (3050 Spruce St., St. Louis, MO 63103, USA). Merck (Frankfurter Strasse 250, 64293, Darmstadt, Germany) provided the other chemicals.

3.3.2 Enzyme purification and chromatography

Tissue was ground to powder in liquid nitrogen and extracted in a 1:2 (m/v) ratio of 300 mM Tris-HCl (pH 7.5) buffer containing 10 % (v/v) glycerol, 2 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA and Roche Complete™ protease inhibitor. The homogenate was filtered through a double-layered nylon cloth, centrifuged at 10 000 g for 10 min, and the pellets discarded. The proteins in the supernatant were precipitated by 80 % saturation with ammonium sulphate and recovered by centrifugation at 10 000g for 10 min. The pellets were resuspended in 100 mM Tris-HCl (pH 7.5) buffer containing 2 mM $MgCl_2$, 2 mM DTT and 2 mM EDTA (Buffer A). The protein extract was then desalted by passage through a Pharmacia PD-10 (Sephadex G25) column and the eluant was diluted two times with buffer A. The desalted extract was applied to a 5 ml Amersham/Pharmacia Hi-trap Q anion exchange column that had previously been equilibrated with buffer A. The column was eluted with a linear KCl gradient at a flow speed of 1 ml/min and fractions containing 20 % or more of maximum activity were pooled. Active fractions from the column were dialysed against buffer A.

Affinity chromatography was performed using a 2 ml bed volume of UDP-glucuronic acid agarose (Sigma). Sample was circulated through the column for at least five column volumes at 0.5 ml.min^{-1}, followed by washing with five column volumes buffer A and elution with buffer A plus 10 mM or 100 mM UDP-glucose. Active fractions from the column were dialysed against buffer A.

Gel permeation chromatography was performed with an Amersham/Pharmacia Superose 6™ column with a 24 ml bed volume at a flow speed of 0.2 ml/min with 100 mM Tris-HCl (pH 7.5) buffer containing 2 mM $MgCl_2$, 2 mM EDTA and 100 mM KCl. Ferritin, BSA, ovalbumin, chymotrypsinogen A and ribonuclease A were used as molecular mass standards.

3.3.3 Enzyme assays

Tris-HCl buffer was used for enzyme assays, because of its very strong inhibition of sugarcane invertase (Vorster and Botha 1998).

Activity in the sucrose synthesis direction was measured in 100 mM Tris-HCl (pH 7.5) buffer (Zeng *et al.* 1998). The sucrose formed was measured by the anthrone binding method (Van Handel 1968).

Activity in the sucrose breakdown direction was measured in an assay containing 100 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, 2 mM NAD^+, 1 mM pyrophosphate and appropriate concentrations of sucrose and UDP. UDP-glucose pyrophosphorylase, phosphoglucomutase and *Leuconostoc* glucose-6-phosphate dehydrogenase were added to a final activity of 4 U.ml^{-1}. NADH production was monitored at 340 nm.

Maximal rates, as used for calculation of the sucrose breakdown/synthesis ratio, were determined with 320 mM sucrose and 1.5 mM UDP in the sucrose breakdown direction. For the sucrose synthesis reaction, 10 mM of both fructose and UDP-glucose were used. 1.5 mM UDP was used since both the SuSyA and SuSyB isoforms displayed substrate inhibition at higher

concentrations. For the same reason, 10 mM fructose was utilised. These UDP and fructose concentrations were chosen such that no substrate inhibition was apparent, same for the highest concentrations used for the K_m determinations.

3.3.4 Electrophoresis

SDS-PAGE was performed at room temperature in a Bio-Rad Mini-PROTEAN II electrophoresis cell. The separating gel contained 7.5 % polyacrylamide, the stack gel 4 %, with a 37.5:1 acrylamide/bisacrylamide ratio.

Native PAGE was performed similarly at 4 °C, but the gel and buffers did not contain SDS.

3.3.5 Preparation of antigen, immunoinactivation and immunoblotting

SuSy antigen was prepared by anion exchange chromatography followed by affinity chromatography and native gel electrophoresis. The part of the gel containing SuSy activity was excised, crushed in liquid nitrogen and the resulting powder extracted with water. After centrifugation the supernatant was used to immunise a rabbit.

Immunoinactivation incubation mixtures contained 0.1% (m/v) BSA. Appropriate mixtures contained 1 % (m/v) *Staphylococcus aureus* cell suspension (Protein A) in 20 mM Tris-HCl (pH 7.5) Buffered Saline (TBS), with or without day 0 or day 39 serum. Incubations without Protein A contained an equal volume TBS. The total volume of every immunoinactivation mixture was 150 μl. All components except Protein A were added, the contents mixed and the tubes incubated at 4 °C for 45 min. Protein A was then added to the appropriate tubes, followed by a further incubation at 4 °C for 30 min. After centrifugation at 13 000g for 5 min the supernatants were assayed for SuSy in the sucrose breakdown direction with the UDP-glucose pyrophosphorylase, phosphoglucomutase and *Leuconostoc* glucose-6-phosphate dehydrogenase coupled assay.

Immunoblotting was performed after SDS gel electrophoresis and transfer to a nitrocellulose membrane (Hybond™-C Extra, Amersham Biosciences) using a Bio-Rad Transblot™ SD semi-dry transfer cell and 48 mM Tris-HCl (pH 8.6) with 39 mM glycine, 20 % (v/v) methanol and 0.0375 % (m/v) SDS. The membrane was blocked for 2 h at room temperature with gentle agitation with a TBST buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1 % (v/v) Tween 20) containing 3 % (m/v) BSA. A 2 000 times dilution of anti-SuSy serum in TBST buffer was used to probe the membrane for 1 h. After rinsing and washing three times for 15 min with TBST buffer, an alkaline-phosphatase conjugated goat anti-rabbit IGG antibody (Roche) was added to 2 000 times dilution and the membrane incubated for 1 h. The membrane was washed as before and developed with a solution consisting of an NBT/BCIP tablet (Roche) dissolved in deionised water. Development was stopped with running tap water.

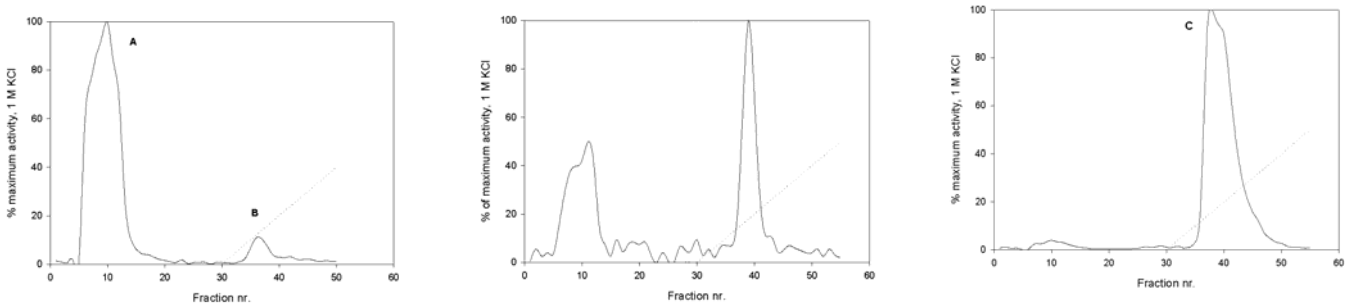
3.3.6 Protein determinations

Protein concentrations were determined with mouse IGG as standard (Bradford 1976).

3.3.7 Determination of kinetic parameters

Substrate K_m values were calculated by non-linear fit to the Michaelis-Menten equation using Grafit™ version 4 for Windows™ (http://www.erithacus.com/). Initial estimates were calculated automatically by the program based on linear regression of rearranged data. Simple weighting was used for all data points. Breakdown/synthesis ratios were calculated at saturating substrate concentrations (see enzyme assays).

3.4 Results



A B C

Fig. 1 Separation of SuSy activity during chromatography on an anion exchange column using leaf roll (panel A) and internode 9 tissue (panel B) during autumn. Panel C shows a typical chromatogram obtained during winter from leaf roll tissue. The dotted line indicates the salt gradient as a percentage of 1 M KCl.

Table Substrate K_m values (mM, ± SE) and sucrose breakdown/synthesis ratios of different SuSy activities. The standard error represents the fitting error of the different data sets to the Michaelis-Menten equation.

SuSy form	Substrate K_m value (mM)				V_f/V_r
	Sucrose	UDP	UDP-glucose	Fructose	
SuSyA	41.8 ± 3.4	1.07 ± 0.08	3.59 ± 0.37	6.62 ± 1.55	0.079
SuSyB	109 ± 23	0.21 ± 0.04	0.53 ± 0.14	11.7 ± 2.5	0.38
SuSyC	35.9 ± 2.3	0.02 ± 0.002	0.235 ± 0.025	6.49 ± 0.60	0.49

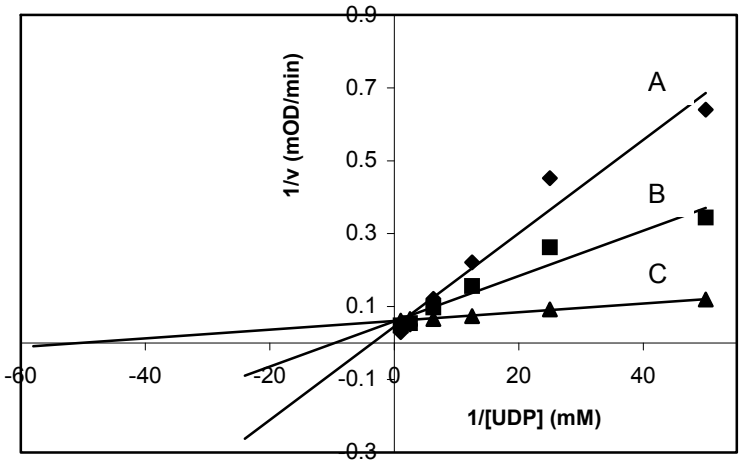


Fig. 2 Lineweaver-Burk plot of 1/v against 1/S for SuSys A, B and C with UDP as the variable substrate. Sucrose was kept constant at 320 mM. K_m values were not determined from the Lineweaver-Burk plots, but from non-linear fit of the data to the Michaelis-Menten equation (see Materials & Methods).

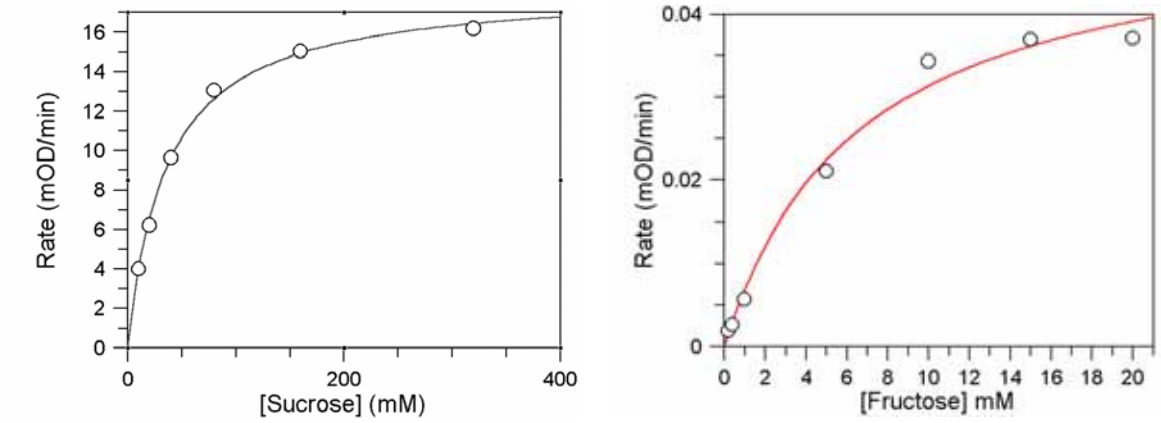


Fig 3. Saturation curves for SuSyC with sucrose as variable substrate and SuSyA with fructose as the variable substrate. Sugarcane SuSy follows Michaelis-Menten kinetics with all Hill coefficients close to 1.

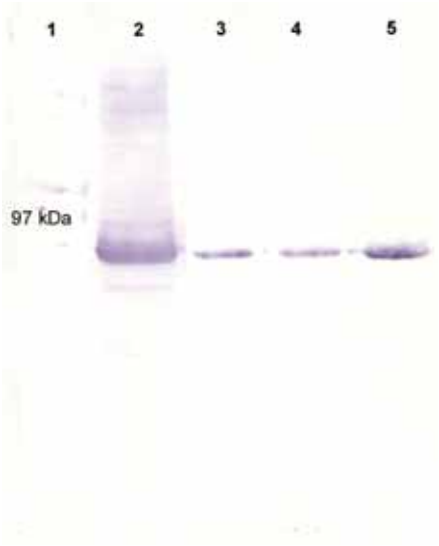


Fig. 4 Immunoblot of sugarcane SuSy. A crude extract (20 μg protein) from leaf roll (lane 2) and 2 μg each of partially purified SuSyA (lane 3), SuSyB (lane 4) and SuSyC (lane 5) was blotted to a nitrocellulose filter which was probed with a 1:2000 dilution of a serum against sugarcane SuSyB.

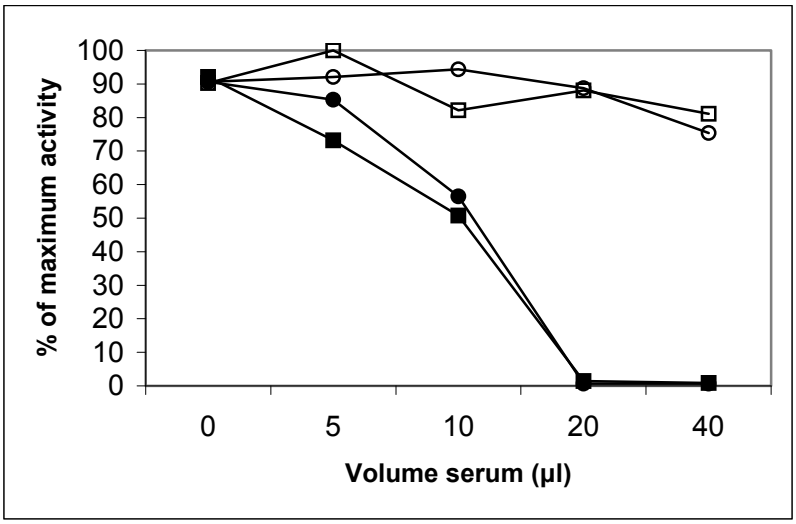


Fig. 5 Immunoinactivation and immunoremoval of SuSy in crude extract from leaf roll that contained both SuSyA and SuSyB forms. Open symbols indicate pre-immune serum and filled symbols SuSy antiserum. Circles indicate samples with added Protein A, squares samples without.

3.4.1 Separation of isoforms

As noted in the Introduction, more that two SuSy isoforms are present in several species. At the time of the last published study into sugarcane SuSy (1993), two isoforms were separated using an antibody affinity column approach, but the separation of, for example, rice isozymes with an anion exchange column led us to try this method to see if there are possibly more than two SuSy isoforms in sugarcane sink tissues. Differences between these isoforms may provide information about the physiological roles of the isoforms. As can be seen from Fig. 1 (panels A and B), two SuSy isoforms (SuSyA and SuSyB) were separated from crude extracts of sugarcane sink tissues – both leaf roll and internode 9 tissue – during autumn. One isoform eluted during the column wash step, and the other at 180 mM KCl concentration. Almost no activity eluted during the wash step when a crude extract from winter-collected leaf roll tissue was loaded on the column (panel C), with only one major activity eluting at 180 mM KCl concentration. In leaf roll tissue the ratio of peak one to peak two was 14.5 and in internode 9 0.88, which may indicate differences in the function of SuSy isozymes between non-accumulating (leaf roll) and sucrose accumulating tissues (internode 9). Results from the anion exchange chromatography suggested at least two

different SuSy isoforms in sugarcane sink tissues. Although SuSyB and SuSyC eluted at the same salt concentration, these peaks were treated as different forms for the kinetic analyses.

3.4.2 Kinetic differences between isoforms

Data from the kinetic analyses (Table and Figs. 2 & 3) indicated that the three SuSys are different isoforms, with significant differences in substrate K_m values and sucrose breakdown synthesis ratios. The kinetic properties of SuSyA and SuSyB, expressed in the leaf roll of the autumn collected material, differed substantially. SuSyA had almost three times higher affinity for sucrose than SuSyB, while the latter had a much higher affinity for UDP than SuSyA. Despite their similar behaviour during anion exchange chromatography, SuSyB and SuSyC represent different isoforms of the enzyme, based on differences in their kinetic parameters, e.g. SuSyC had about ten times higher affinity for UDP (see Fig. 2). Sugarcane SuSy exhibited Michaelis-Menten kinetics, and Hill coefficients were all close to 1 (data not shown), irrespective of the variable substrate, which means that sugarcane SuSy does not display cooperative binding like some other multimeric enzymes. Based on the results it is evident that at least three different forms of SuSy are expressed in this tissue.

A striking feature of the kinetic properties of the isoforms (especially SuSyA versus SuSyB and C) is the difference in the ratio between the maximum catalytic activities in the sucrose synthesis and breakdown reactions. SuSyA was obviously more efficient in sucrose synthesis than the other two isoforms.

3.4.3 Physical properties of isoforms

The difference in the affinities of the SuSyA and SuSyB isoforms for UDP-glucose was verified during subsequent further purification using a UDP-glucuronic acid affinity column. SuSyA could be eluted with a 10 mM UDP-glucose solution, but not SuSyB. This is consistent with the fact that SuSyB

has a much lower K_m value for UDP-glucose than SuSyA, and hence binds with higher affinity to the column.

A polyclonal antiserum raised against purified sugarcane SuSyB cross-reacted with the denatured form of all three SuSys (Fig. 4). The antiserum also efficiently immunoinactivated total SuSy activity in crude extracts (Fig. 5) as well as individually collected peaks of SuSyA and SuSyB activity after anion exchange chromatography. Although recognised on an immunoblot, native SuSyC was not immunoinactivated or recognised by the serum like SuSyA and SuSyB, since addition of Protein A followed by centrifugation did not remove activity (results not shown). Even though they eluted at the same 180 mM KCl concentration from the anion exchange column, SuSyB and SuSyC are different isoforms, based on their different kinetic properties and the fact that the antiserum against SuSyB does not recognise native SuSyC, but does recognise native SuSyB. Possibly, the epitopes recognised by the antiserum may be shielded in the tetrameric native SuSyC, but are accessible on the monomers bound to the nitrocellulose membrane in the immunoblotting procedure.

The protein blot data show that all three the SuSy isoforms contain an approximately 94 kDa subunit. Native molecular weight analyses have indicated that the enzymes are tetramers with a molecular weight of approximately 380 kDa (data not shown). These results are similar to those obtained for other SuSys, which are also tetramers *in vivo*, containing subunits that typically have a molecular weight of approximately 90 kDa.

3.5 Discussion

Although previous work on sugarcane has implicated that there are two different isoforms of SuSy present, these could not be separated with anion exchange chromatography. In this study an extract from the mature, sucrose storing, internode 9 gave two peaks on an anion exchange column (Fig. 1, panel B). The results suggest that there are at least three different forms of

SuSy in sugarcane. Two of these forms could be separated by anion exchange chromatography. However, separation of SuSyB and SuSyC could not be achieved. Although SuSyB and SuSyC eluted at the same salt concentration from the anion exchange column, they are different forms. If they were mixtures of the same isoforms in different ratios, then all the activity in both native SuSyB and SuSyC preparations would have been immunoinactivated by the polyclonal antiserum raised against SuSyB. Instead, only SuSyB was inactivated, but both forms were recognised on an immunoblot. This indicates that the two forms differ in their native structures, with SuSyC having no epitopes recognised by the antiserum.

The presence of at least three isoforms of SuSy in sugarcane is in agreement with the finding that three SuSy genes are present in the rice genome (Huang *et al.* 1996). The results of a study in pea suggests that different SuSy isoforms channel carbon towards different uses in the cell, e.g. cellulose and starch (Barratt *et al.* 2001). The sugarcane SS1 sequence shares only 41-45% homology with four sugarcane ESTs putatively identified as SuSy (Carson and Botha 2002). With seven putative SuSy genes present in *Arabidopsis thaliana* (Komatsu *et al.* 2002), there is a high likelihood that other plants, especially sugarcane with its highly complex aneuploid, double hexaploid genome, could contain more SuSy genes than have been found up to now.

The two peaks of SuSy activity isolated during autumn from sugarcane leaf roll tissue contrast with results reported in the literature for both sugarcane and maize SuSy, where just one peak of activity was recovered from the same type of anion exchange column (Nguyen-Quoc *et al.* 1990; Buczynski *et al.* 1993), although four isozymes were separated by anion exchange chromatography in rice (Yen *et al.* 1994). SDS-PAGE and immunoblotting with polyclonal antiserum indicate that all three SuSy polypeptides have the same molecular mass. The calculated molecular mass for sugarcane SS1 from the cDNA sequence is 91 602 Da (Lingle and Dyer 2001). In this study, the molecular mass for all three SuSys as determined by SDS-PAGE, was about 94 kDa. It has been shown for various SuSys that the native form consists of

a homotetrameric molecule (Delmer 1972; Porchia *et al.* 1999; Echt and Chourey 1985; Yen *et al.* 1994; Sebkova *et al.* 1995). Results obtained for sugarcane SuSy in this study agree with these findings as well as the molecular weight determined for the coding region of the sugarcane SS1 gene.

The observation that SuSy contributes to sucrose synthesis in young sugarcane tissue (Botha and Black 2000) is consistent with the presence in leaf roll of SuSyA with its low breakdown/synthesis ratio. Probably the most striking distinction between these SuSys is this difference in their respective breakdown/synthesis ratios. In rice, one SuSy isozyme was found to have a much different breakdown/synthesis ratio from the other three forms isolated (Yen *et al.* 1994). In sugarcane, SuSyB and SuSyC have a much higher breakdown/synthesis ratio compared to SuSyA, so they seem to be more biased towards sucrose breakdown than SuSyA. The ratio between peaks 1 and 2 differed significantly between leaf roll and internode 9 tissue during the same season (autumn). The ratio of peak 1 to peak 2 in internode 9 was substantially lower than in leaf roll tissue. This is consistent with the fact that the total sucrose breakdown/synthesis ratio increases with increasing internode maturity (see Chapter 5). Hence, it appears that total SuSy activity is biased more towards sucrose breakdown in internode nine than in leaf roll. This is supported by evidence from a radiolabel study, which found that SuSy is almost exclusively involved in sucrose breakdown in mature internodal tissue, but contributes to sucrose synthesis in young internodes (Botha and Black 2000). Different physiological requirements in sucrose metabolism probably dictate expression of different SuSy isoforms between tissues. The cleavage of sucrose by SuSy in sink organs is very important for the import and accumulation of storage carbohydrate, such as starch, to high levels (Zrenner *et al.* 1995). Phloem unloading in mature sugarcane culm tissue occurs symplastically, and the maintenance of a sucrose concentration gradient may function in addition to bulk flow in these tissues (Komor 2000). This agrees with the observed bias of SuSy towards sucrose breakdown in mature tissue. UDP-glucose produced by SuSy can then serve as substrate, together with fructose-6-phosphate, for resynthesis of sucrose. The continual

cleavage and resynthesis of sucrose in culm tissue is well known (Whittaker and Botha 1997).

Comparing the substrate K_m values obtained in this study with those previously reported for sugarcane and maize SuSy (Buczynski *et al.* 1993; Nguyen-Quoc *et al.* 1990) reveals no obvious clues as to the identity of these forms. SuSyC does show strong similarity with K_m values reported for sugarcane SS1, except for the fructose value. Certainly the K_m value for sucrose of SuSyB and the K_m values for UDP for SuSyA and SuSyB are much higher than those that have been reported for maize and sugarcane SuSy, although similarly high values have been reported for carrot SuSy (Sebkova *et al.* 1995).

Immunoblot analysis with monoclonal antibodies against maize SuSy indicates the presence of both SS1 and SS2 in sugarcane leaf roll tissue (Buczynski *et al.* 1993), but the SS1 isoform is the only form expressed here according to Northern blot analysis using RNA probes from the sugarcane *SS1* and maize *Sus1* (coding for the SS2 enzyme) genes (Lingle and Dyer 2001). It would appear therefore that these isoforms are not always expressed simultaneously. The identity of the three distinct SuSy activities that were found in leaf roll tissue during this study is still unclear. Native gel electrophoresis showed two major bands of SuSy activity, but it is unclear if these represent different gene products, or different post-translationally modified states of the same polypeptide. The N-terminal of these SuSy forms was found to be blocked, so peptide sequencing was unsuccessful. If these SuSys are the same polypeptide, then only a post-translational modification(s) can account for the observed differences in elution behaviour and kinetic properties. There are no indications in the literature that SuSy is a glyco or lipoprotein, but phosphorylation of both SuSy isoforms in maize has been demonstrated (Huber *et al.* 1996). However, only a single phosphorylation site was suggested for both isoforms, therefore if only SS1 is present, differences in phosphorylation state alone cannot explain three SuSy forms. The affinity of maize SuSy for UDP and sucrose was increased by phosphorylation, with appreciably lower K_m values for these substrates, while no significant effect

was apparent on the K_m values for fructose and UDP-glucose (Huber *et al.* 1996). It would therefore seem that phosphorylation selectively activates the cleavage reaction. There are no such clear distinctions in terms of K_m values between the SuSys reported here. Overall, the results of this study show notable similarity to a study where different rice SuSy activities were separated on an anion exchange column and the enzymes were also N-terminal blocked (Yen *et al.* 1994). An interesting phenomenon worth noting was an apparent seasonality to the expression of SuSyA, since it only appeared in the autumn months, while being absent, or nearly so, the rest of the year.

The results obtained in this study point to at least three forms of sucrose synthase in sugarcane, which fall into high and low sucrose breakdown/synthesis ratio groups. These differences in breakdown/synthesis ratios likely reflect different physiological roles *in vivo*.

3.6 Reference List

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CHAPTER FOUR

A KINETIC STUDY OF SUCROSE SYNTHASE IN SUGARCANE

4.1 Abstract

We characterised kinetically a SuSy activity (SuSyC, see Chapter 3) partially purified from sugarcane variety N19 (*Saccharum* spp. hybrid) leaf roll tissue. Primary plot analysis and product inhibition studies showed that a compulsory order ternary complex mechanism is followed, with UDP binding first and UDP-glucose dissociating last from the enzyme. Product inhibition studies showed that UDP-glucose is a competitive inhibitor with respect to UDP and a mixed inhibitor with respect to sucrose. Fructose is a mixed inhibitor with regard to both sucrose and UDP. Kinetic constants are as follows: K_m values (mM, ± SE) were, for sucrose, 35.9 $\pm$ 2.2; for UDP, 0.02 $\pm$ 0.002; for UDP-glucose, 0.235 $\pm$ 0.025 and for fructose, 6.49 $\pm$ 0.60. K_i^S values were, for sucrose, 227 mM, for UDP, 0.086 mM, for UDP-glucose, 0.104, for fructose, 2.23 mM. Product inhibition constants for UDP-glucose were w.r.t. sucrose, K_i, 0.18 mM, K_i', 0.19 mM, w.r.t. UDP, K_i, 0.12 mM. For fructose, product inhibition constants were w.r.t. to sucrose, K_i, 1.8 mM, K_i', 0.65 mM, w.r.t. UDP, K_i, 4.1 mM, K_i', 3.9 mM. Replacing estimated kinetic parameters of SuSy in a kinetic model of sucrose accumulation with experimentally determined parameters of the SuSyC isoform had significant effects on model outputs, with a 40 % increase in sucrose concentration and 7 times reduction in fructose the most notable. Doubling and halving of SuSy activity reduced and increased the steady state fructose concentration by about 43 and 137 % respectively.

4.2 Introduction

The kinetic parameters of enzymes provide important information about their interactions with substrates, products and effectors. Typically, substrate K_m values are interpreted to give an indication of the affinity of enzymes for their substrates, and conclusions about enzymes' physiological roles are often based on these values. However, the kinetic parameters of individual enzymes do not by themselves provide much insight into the behaviour of an intact, functioning metabolic pathway. Cellular network models, such as applied in the approach of computational systems biology, extend the usefulness of kinetic data on individual enzymes immensely and can have both explanatory and predictive value.

Several papers that give an overview of different approaches for studying and modelling metabolism, such as metabolic flux analysis, metabolic control analysis (MCA) and positional isotopic labelling combined with NMR or MS, have recently been published (Giersch 2000; Wiechert 2001; Morgan and Rhodes 2002). Of these approaches, MCA (Kacser and Burns 1973; Heinrich and Rapoport 1974) is particularly useful in studies of metabolic pathways, since it quantifies the degree of control of individual reaction steps on the steady-state pathway flux or metabolite concentrations. Hence, MCA can be a great help in determining potential target steps for metabolic engineering, because the reactions in the pathway that have the most potential of modifying a target flux or metabolite concentration can be identified. For example, MCA has been used to study the control of different steps on mitochondrial respiration (Groen *et al.* 1982), and successfully predicted that overexpression of NADH oxidase is more successful than acetolactate synthase overexpression for increasing production of diacetyl by *Lactococcus lactis* (Hoefnagel *et al.* 2002). In plants, MCA was used to estimate the flux control coefficient of phosphoglucoisomerase on sucrose and starch production using *Clarkia xantiana* mutants with decreased levels of this

enzyme (Kruckeberg *et al.* 1989). MCA has been discussed in the context of plant metabolism (Ap Rees and Hill 1994) and further examples of its application are given therein, as well as practical advice on isolation and assay of plant enzymes and extraction of metabolites. It should be mentioned that plants pose particular challenges as far as analysis of their metabolism by MCA (or other methods for that matter) is concerned: the degree of compartmentalisation of metabolism is extremely high, and isolation of active enzymes can be a challenge, owing to various factors such as proteases, interfering compounds, high acidity and so forth. Apart from these considerations, the lack of uniform data sets for use in the construction of kinetic models can be a hindrance. Addressing this point, techniques to measure considerable numbers of metabolites simultaneously are now available and will contribute greatly to analyses of metabolism and our understanding thereof (Stitt and Fernie 2003).

A kinetic model describing sucrose accumulation in sugarcane was recently published (Rohwer and Botha 2001). This model was used to calculate the control coefficients of enzymes in the sucrose synthesis pathway for sucrose futile cycling (cleavage and resynthesis of sucrose), with a view to determining which reactions control this energetically wasteful process. Like any kinetic model, it requires the rate equations of all reactions in the pathway and therefore the kinetic parameters of every enzyme. Typically the rate equations require more information than simply K_m values for the substrates, which are the only kinetic parameters reported in most studies not focusing exclusively on kinetics. For sugarcane SuSy, substrate K_m values have been reported (Buczynski *et al.* 1993), but not other important parameters, such as substrate K_i values, or confirmation of the reaction mechanism, which are also needed for kinetic modelling.

The objective of this study was to obtain more extensive data on the kinetic parameters of sugarcane SuSy, that can be used to enhance modelling of

sucrose accumulation and also improve our understanding of sugarcane SuSy and its influence on sucrose accumulation.

4.3 Materials and methods

4.3.1 Materials

Sugarcane (*Saccharum* spp. hybrids) variety N19 plants field grown at the University of Stellenbosch experimental farm were used. Internode one was taken as the internode attached to the leaf with the first exposed dewlap (Van Dillewijn 1952).

Tris buffer, DTT and coupling enzymes were obtained from Roche (Grenzacherstrasse 124, CH-4070, Basel, Switzerland), except UDP-glucose pyrophosphorylase which was from Sigma (3050 Spruce St., St. Louis, MO 63103, USA). Merck (Frankfurter Strasse 250, 64293, Darmstadt, Germany) provided the other chemicals.

4.3.2 Enzyme purification and chromatography

Leaf roll tissue was ground to powder in liquid nitrogen and extracted in a 1:2 (m/V) ratio of 300 mM Tris-HCl (pH 7.5) buffer containing 10 % (V/V) glycerol, 2 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA and Roche Complete™ protease inhibitor. The homogenate was filtered through a double-layered nylon cloth, centrifuged at 10 000 g for 10 min, and the pellets discarded. The proteins in the supernatant were precipitated by 80 % saturation with ammonium sulphate and recovered by centrifugation at 10 000g for 10 min. The pellets were resuspended in 100 mM Tris-HCl (pH 7.5) buffer containing 2 mM $MgCl_2$, 2 mM DTT and 2 mM EDTA (Buffer A). The protein extract was then desalted by passage through a Pharmacia PD-10 (Sephadex G25) column and the eluant was diluted two times with buffer A. The desalted extract was applied to a 5 ml Amersham/Pharmacia

Hi-trap Q anion exchange column that had previously been equilibrated with buffer A. The column was eluted with a linear KCl gradient at a flow speed of 1 ml/min and fractions containing 20 % or more of maximum activity were pooled. Active fractions from the column were dialysed against buffer A.

The partially purified extract was tested for the potential presence of the interfering activities invertase, UDPGlc dehydrogenase, fructokinase and sucrose phosphate synthase. Results showed that under the conditions used for the SuSy assays (pH 7 for the sucrose breakdown assay or pH 7.3 for the synthesis reaction, 100 mM Tris buffer) there were no significant levels of these interfering activities present, with only invertase barely detectable at less than 0.5 % of SuSy activity.

4.3.3 SuSy assays

Activity in the sucrose synthesis direction was measured in 100 mM Tris-HCl (pH 7.3) buffer. The assay contained 15 mM $MgCl_2$, 0.2 mM NADH, 1 mM PEP, and appropriate concentrations of UDP-glucose and fructose. Pyruvate kinase and lactate dehydrogenase were each added to a final activity of 4 $U.ml^{-1}$. NADH oxidation was monitored at 340 nm.

Activity in the sucrose breakdown direction was routinely measured in an assay containing 100 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, 2 mM NAD^+, 1 mM pyrophosphate and appropriate concentrations of sucrose and UDP. UDP-glucose pyrophosphorylase (UDPGlcPP), phosphoglucomutase (PGM) and *Leuconostoc* glucose-6-phosphate dehydrogenase (G6PDH) were each added to a final activity of 4 $U.ml^{-1}$. NADH production was monitored at 340 nm.

For the UDP-glucose product inhibition study, activity was measured in an assay containing 100 mM Tris-HCl (pH 7.0), 2 mM NAD^+, 2 mM $MgCl_2$ and 1 mM ATP.

4 $U.ml^{-1}$ hexokinase, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were added and NADH production monitored at 340 nm.

4.3.4 Determination of kinetic parameters and modelling

Substrate K_m values were calculated by non-linear fit to the Michaelis-Menten equation using Grafit™ version 4 for Windows™ (http://www.erithacus.com/). Initial estimates were calculated automatically by the program based on linear regression of rearranged data. Simple weighting was used for all data points.

Kinetic parameters other than the substrate K_m values were taken as the median values calculated from the experimental data. To calculate the product inhibition constants, kinetic experiments were performed at the product inhibitor and substrate concentrations as indicated in Figures 2 & 3.

The program WinScamp v1.2 (Sauro 1993) was used for kinetic modelling, using a published model of sucrose accumulation (Rohwer and Botha 2001).

4.4 Results

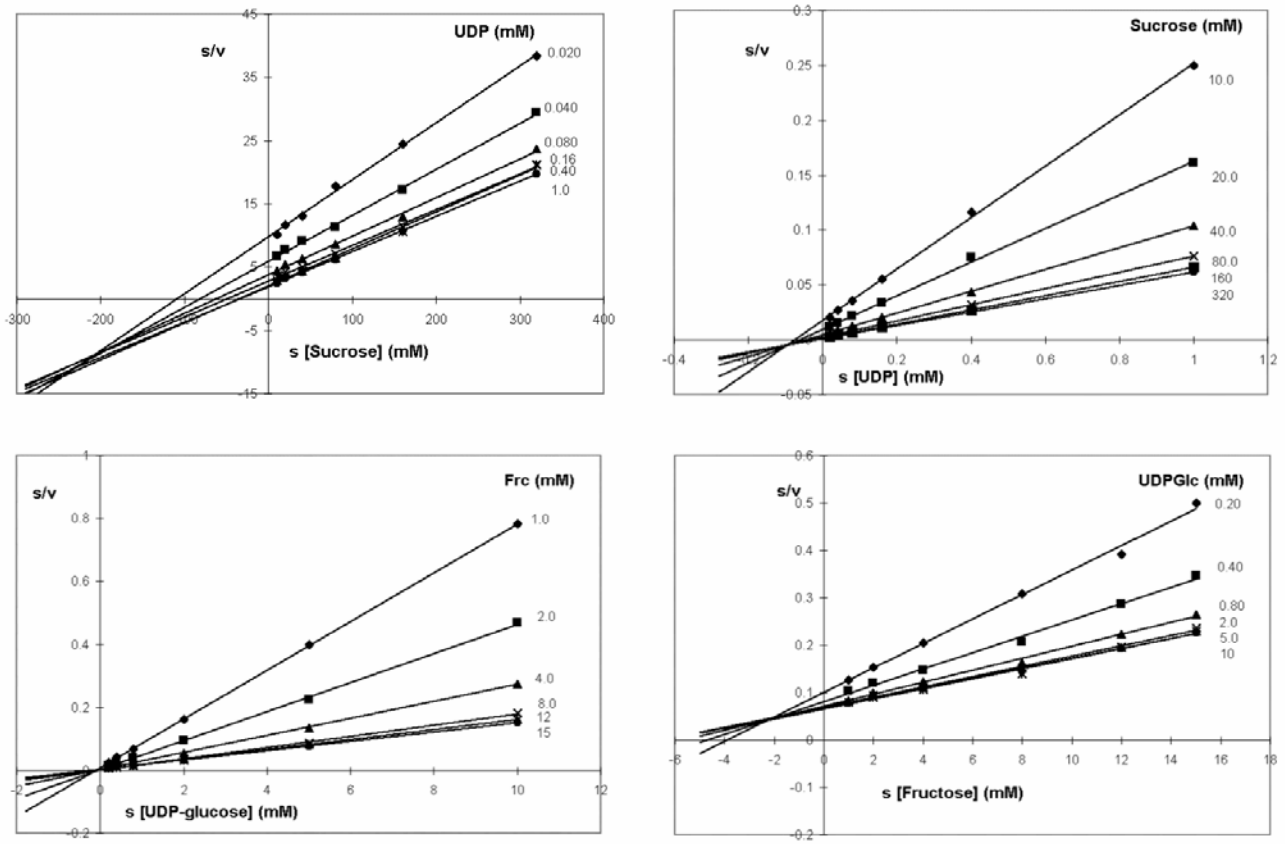

Fig. 1 Primary (Hanes-Woolf) plots for the substrates of SuSy at zero initial product concentrations: A, sucrose at varying concentrations of UDP; B, UDP at varying concentrations of sucrose; C, UDP-glucose at varying concentrations of fructose; D, fructose at varying concentrations of UDP-glucose. Lines reflect K_m and V_{max} values that were derived from non-linear fit (n=6) to the Michaelis-Menten equation as described in Materials and Methods. Kinetic assays were performed as described in Materials and Methods.

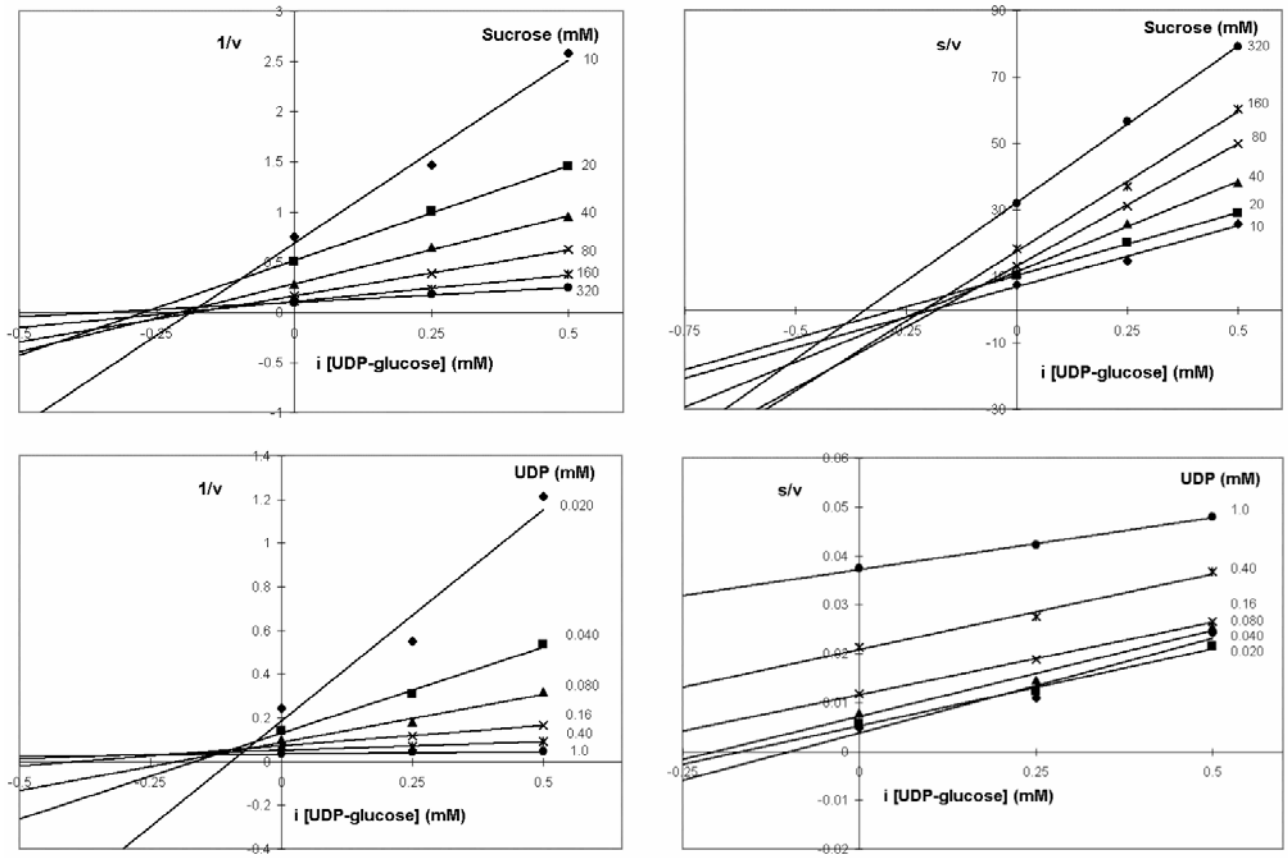

Fig. 2 UDP-glucose product inhibition. Dixon (A, C) and Cornish-Bowden plots (B, D) with sucrose (A, B) and UDP (C, D) as the variable substrates. For A and B, UDP was kept constant at 0.020 mM, while for C and D sucrose was kept constant at 40 mM.

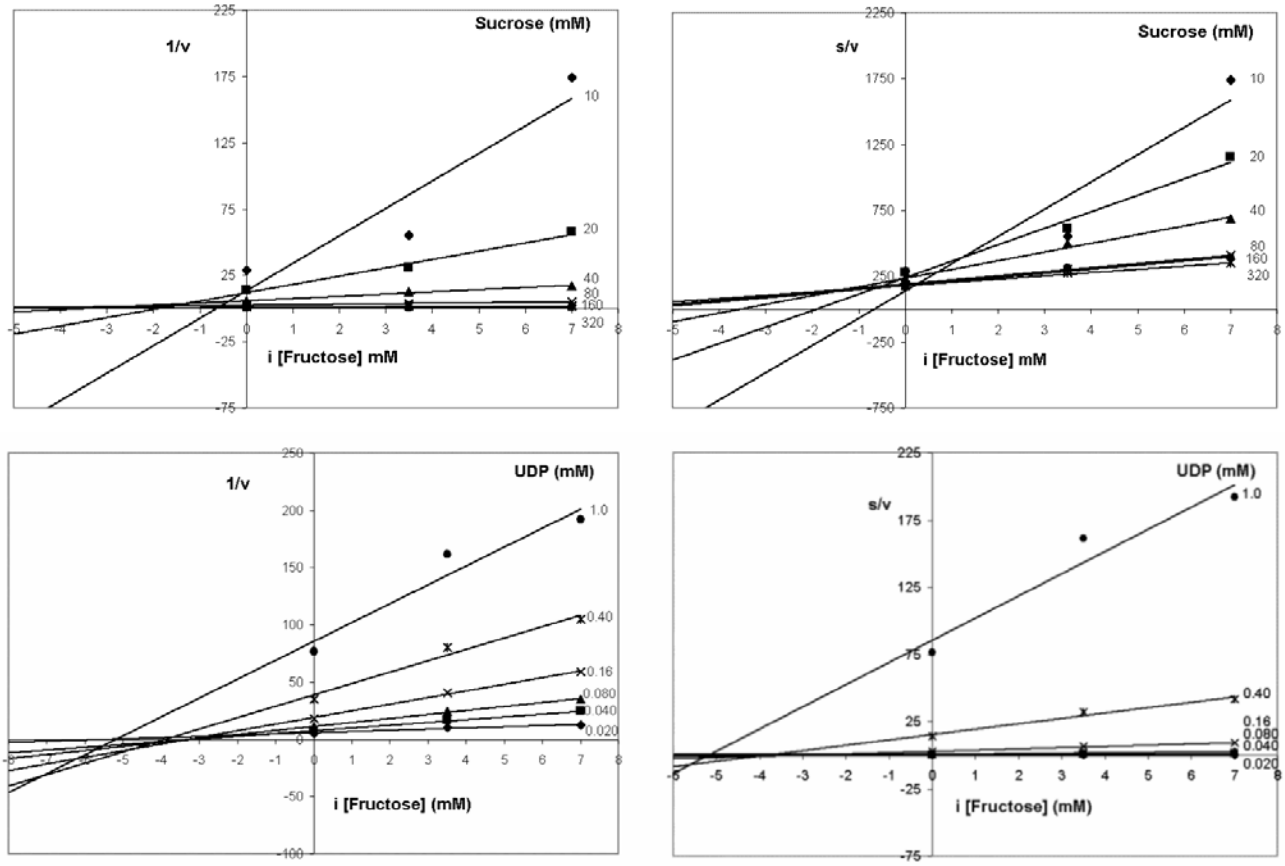

Fig. 3 Fructose product inhibition. Dixon (A, C) and Cornish-Bowden plots (B, D) with sucrose (A, B) and UDP (C, D) as the variable substrates. For A and B, UDP was kept constant at 0.020 mM, while for C and D sucrose was kept constant at 40 mM.

Table: Inhibition types and kinetic parameters for SuSyC.

	Sucrose	**UDP**	**UDP-Glucose**	**Fructose**
K_i^S	227	0.086	0.104	2.23
K_m	35.9 ± 2.3	0.02 ± 0.002	0.235 ± 0.025	6.49 ± 0.60
Inhibition constants	**UDP-Glucose w.r.t. UDP (competitive)**	**UDP-Glucose w.r.t. sucrose (mixed)**	**Fructose w.r.t. UDP (mixed)**	**Fructose w.r.t. sucrose (mixed)**
K_i	0.12	0.18	4.1	1.8
K_i'	-	0.19	3.9	0.65

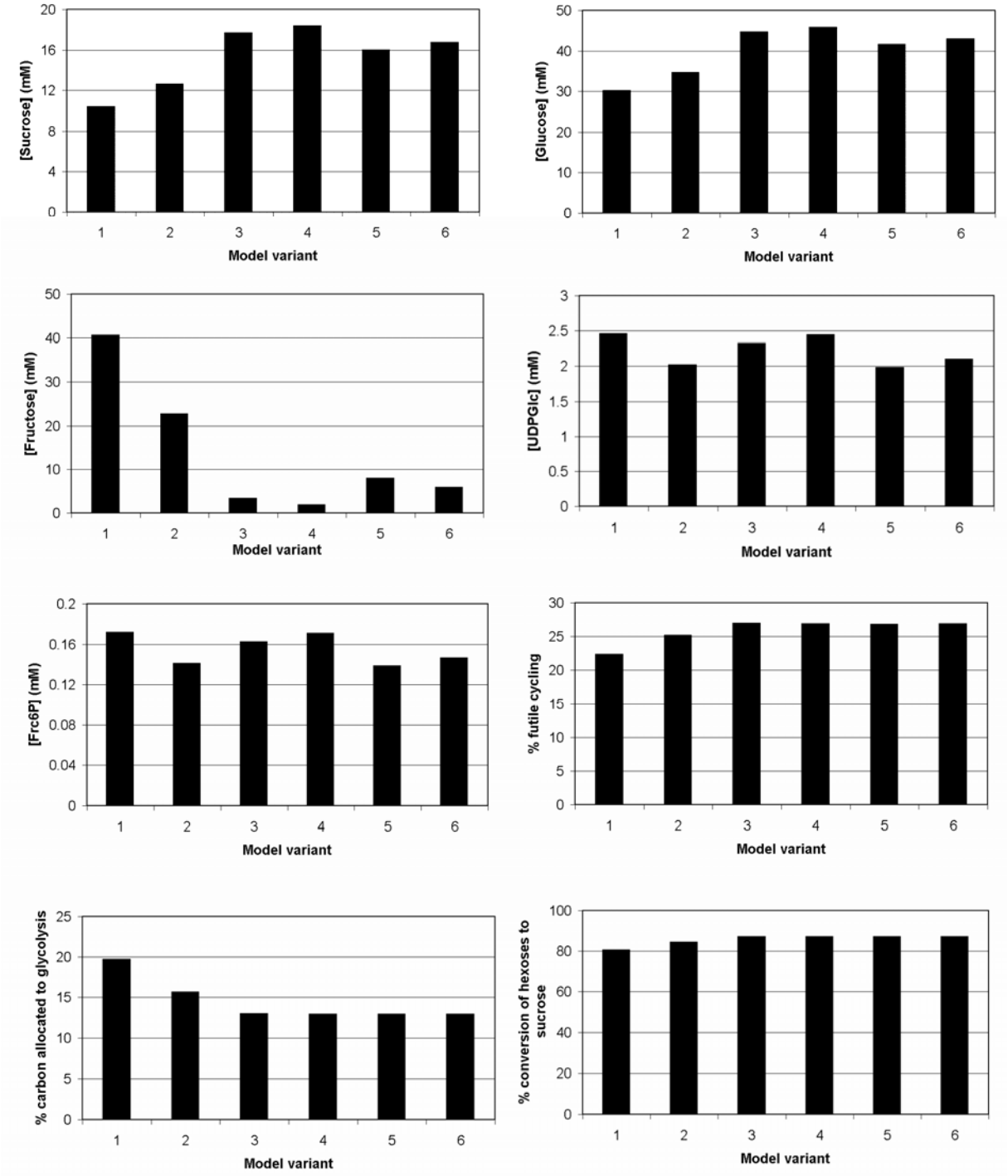

Fig. 4: WinSCAMP kinetic model variable outputs: A, Sucrose concentration; B Glucose concentration; C, Fructose concentration; D, UDP-glucose concentration; E, Fructose-6-phosphate concentration; F, % futile cycling; G, % carbon allocated to glycolysis; H, % hexoses converted to sucrose. Model variants are as follows: 1, original published model; 2, model with K_{eq} and K_i values corrected (see Results section); 3, model with SuSyC parameters; 4, SuSyC parameters with doubled activity; 5, SuSyC parameters with halved activity; 6, model containing two SuSy isoforms, one with generic parameters and the other with experimentally determined parameters; total SuSy breakdown activity was the same as in model variants 1, 2, and 3 above.

The purpose of the kinetic experiments reported in this chapter was to establish the reaction mechanism of sugarcane SuSy and also determine kinetic parameters needed for metabolic modelling. As far as the SuSy reaction mechanism is concerned, there are conflicting reports in the literature, and some of these results do not agree with the theoretically predicted properties of the proposed reaction mechanisms (see Discussion). Hence, there was a need to establish these properties of sugarcane SuSy.

4.4.1 Primary (Hanes-Woolf) plot analysis

Primary plot analysis is used to obtain information on the reaction mechanism of an enzyme – in combination with product inhibition studies, the complete mechanism can be established. Primary plots (Fig. 1) for all substrates gave straight lines with intersection points to the left of the a/v vs. a axis, which indicates a ternary complex mechanism (for a substituted (ping-pong) mechanism the intersection points are on the axis). The substrate K_i values obtained from the intersection points of the lines are indicated in the table. Sugarcane SuSy exhibited Michaelis-Menten kinetics, and Hill coefficients were all close to 1 (data not shown), irrespective of the variable substrate, which means that sugarcane SuSy does not display cooperative binding like some other multimeric enzymes.

To distinguish between a random order and ordered ternary complex mechanism, it is necessary to perform product inhibition experiments, because the primary plots for these two mechanisms have the same attributes and can therefore not be used to discriminate between the two.

4.4.2 Product inhibition studies

Inhibition types and inhibition constants derived from Dixon and Cornish-Bowden plots for UDP-glucose (Fig. 2) and fructose product inhibition (Fig. 3) are shown in the table. Competitive inhibition is characterised by a series of parallel lines in

the Cornish-Bowden plot, while the Dixon plot shows the lines intersecting to the left of the y-axis. Mixed inhibition shows the lines intersecting to the left of the y-axis in both plots. The inhibition patterns indicate an ordered mechanism with UDP binding first and UDP-glucose dissociating last. Product inhibition patterns for both fructose and UDP-glucose agreed fully with the predicted patterns for an ordered ternary complex mechanism (Segel 1975), with UDP-glucose a competitive inhibitor with regard to UDP and a mixed inhibitor with regard to sucrose. Fructose was a mixed inhibitor with regard to both UDP and sucrose.

The ordered ternary complex mechanism, with UDP binding first and UDP-glucose dissociating last, agrees with that proposed for *Helianthus tuberosus* SuSy (Wolosiuk and Pontis 1974) and validates the assumption made in a kinetic model of sucrose accumulation (Rohwer and Botha 2001), although the substrate K_i values obtained experimentally differ substantially from those used in the model. The data obtained from the kinetic experiments were then incorporated in the model of sucrose accumulation, to investigate the effect of changes in SuSy kinetic parameters on the output variables.

4.4.3 Modelling

Kinetic parameters obtained experimentally were used to query a kinetic model of sucrose accumulation (Rohwer and Botha 2001). This model, constructed using the program “WinSCAMP” (Sauro 1993), consists of 11 reactions that are directly or indirectly involved in sucrose metabolism. Enzymes with sucrose as substrate or product are included explicitly, while others, specifically glycolysis and the enzymes phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and UDP-glucose pyrophosphorylase (UGPase) are included as a single “drain” reaction and a so-called “forcing function” respectively. The forcing function assumes that the reactions catalysed by PGI, PGM and UGPase are close to equilibrium *in vivo*, which is supported by metabolite measurements in most tissues. The reactions are entered as rate equations in the model, which means

that all the relevant kinetic parameters are needed for each enzyme. Because of the paucity of kinetic information on sugarcane enzymes most of these parameters were estimated. Enzyme levels were taken mostly from the literature on sugarcane, others were estimated. The model solves the differential equations describing the synthesis and degradation of each metabolite in order to calculate the steady-state levels. The model “behaves” like a sugarcane storage parenchyma cell, in that it accumulates sucrose, with other metabolite levels fairly close to experimentally measured values.

Variable outputs from the model are shown in Fig. 4. Outputs from the original model are shown as the first bar in every panel. For all the other model variants, the equilibrium constant for the SuSy reaction was changed to 0.50 (the published model used an equilibrium constant of 5 in the sucrose breakdown direction (Kruger 1997), but this is incorrect; reported values range from 0.15 – 0.56 (Geigenberger and Stitt 1993)). Also, the SuSy parameters which were input in the original model did not obey the two Haldane relationships, which relate the K_{eq} to the V_f/V_r ratio, K_m and K_i values (Segel 1975). The two equations are given below:

$$K_{eq} = V_f/V_r \; \Delta \; (K_iQEK_mP/K_iAEK_mB) \qquad (1)$$

$$K_{eq} = (V_f/V_r)^2 \; \Delta \; (K_iPEK_mQ/K_iBEK_mA) \qquad (2)$$

Where A is UDP; B, sucrose; P, fructose; Q, UDP-glucose; V_f and V_r refer to maximal reaction rates in the sucrose breakdown and synthesis directions, respectively.

For the corrected model (model variant 2 in Fig. 4) all kinetic parameters were kept the same as the values used in the published model, except the K_i value for UDP (K_iA) was changed from 0.3 mM to 0.108 mM, and the K_i value for fructose (K_iP) was changed from 4 mM to 3.92 mM in order to obey the two Haldane

relationships. In order to ensure compliance with these thermodynamic relationships, the K_i values used for the models incorporating the SuSyC parameters were also modified from the experimental values. These modified values were, (in mM), 0.24, 0.0426, 27.6 and 156 for UDP-glucose, UDP, fructose and sucrose respectively, with K_m values used in the models as shown in the table. Note that these modified K_i values are all in the same range as the experimentally determined values, except for the fructose value.

The output variables differed between models containing two different SuSy isoforms. Sucrose, glucose, Fru-6P and UDP-glucose concentrations were all higher in model variant nr. 3 than in nr. 2. Fructose was the variable most affected by changes in the SuSy isoform in the model or changes in SuSy activity (see Discussion). Sucrose content was positively correlated with SuSy activity, but these changes were quite small compared to the changes in enzyme activity, at about a 4 % increase and 9 % decrease in sucrose for a doubling and halving of activity respectively. Sucrose futile cycling was marginally higher (~1.7 %) in the models containing the SuSyC isoform, compared to the model (variant 2) with the “generic” SuSy.

4.5 Discussion

It is interesting to compare the results obtained in this study with those for maize (Nguyen-Quoc *et al.* 1990) and *Helianthus tuberosus* SuSy (Wolosiuk and Pontis 1974). UDP-glucose is a competitive inhibitor with regard to UDP, and fructose a competitive inhibitor with regard to sucrose, according to both these studies. These results, however, conflict with the predicted patterns of product inhibition for an ordered ternary mechanism (Segel 1975); instead, they agree with the expected patterns for a substituted (ping-pong) mechanism. A random mechanism was proposed for SuSy from *Phaseolus aureus* (Delmer 1972), but this finding was later challenged (Wolosiuk and Pontis 1974). The results of the study on sugarcane SuSy indicated that it follows an ordered ternary mechanism,

with no evidence to suggest otherwise. The apparent conflict between the product inhibition patterns obtained in the studies on maize and *Helianthus* SuSy on the one hand and sugarcane SuSy on the other is puzzling and merits further investigation.

The kinetic data obtained in this study was used to query a model of sucrose accumulation (Rohwer and Botha 2001). It was found that substituting the (mostly) estimated kinetic parameters of SuSy in the original model with the experimentally determined parameters of the SuSyC isoform had a marked effect on most variables output by the model. The 40 % increase in sucrose concentration and almost 7 times reduction in fructose concentration were the most notable. Evidently, changes in kinetic parameters of enzymes involved in sucrose metabolism can have large effects on metabolite concentrations and expression of multiple enzyme isoforms may therefore play an important role in the regulation of metabolism.

Changes in SuSy activity also impacted the model variables. The biggest changes were in fructose concentration, which decreased by 43 % when activity was doubled, and increased by 137 % when activity was halved. Incorporation of the SuSyC isoform in the model dramatically reduced the steady-state concentration of fructose compared to the model with estimated SuSy parameters, from 22.6 to 3.3 mM. This may seem alarming when compared to experimentally reported values of about 30 mM for fructose in internode 5 (Whittaker and Botha 1997), but it has to be kept in mind that these experimental values assume equal distribution of fructose between the cytosol and vacuole. Up to 99 % of glucose and fructose in this tissue may actually be present in the vacuole (Vorster and Botha 1999), and hence the low value for cytosolic fructose obtained with the modified model may well be correct. On the other hand, one would expect the glucose and fructose values to be more or less equal, but this is not so in the modified model. Only metabolite measurement methods which can

distinguish between the cytosolic and vacuolar compartments can resolve this issue.

Next, the model was expanded so that in addition to the SuSy isoform with generic kinetic parameters, it included a second SuSy isoform, with experimentally determined kinetic parameters. Total SuSy breakdown activity was kept the same as in the models with only one SuSy isoform. Modelling results with this version were very similar to the model containing only the SuSyC isoform, except for the fructose concentration, which was 74 % higher. This change in the fructose concentration indicates that expressing different enzyme isoforms simultaneously may add to the regulatory capabilities that plants have over their metabolism, in addition to expressing isoforms in spatially and temporally separate ways.

Reducing SuSy activity tenfold results in the fructose concentration increasing about nineteen-fold and halving of sucrose concentration (data not shown). This is consistent with experimental data that show that SuSy participates in sucrose synthesis in younger internodes (Botha and Black 2000). It would be insightful to modify the model for a mature internode, and then see what effects changes in SuSy activity have. It would be best to establish enzyme activity levels for all the enzymes incorporated in the model simultaneously with a single enzyme extract, in order to avoid the fragmented and approximate data set used for the current model.

The utility of modelling sucrose metabolism was illustrated in this work – the results obtained could not easily have been predicted by other means. Computational systems biology approaches can therefore play a very useful role in studying processes that impact on sucrose accumulation, such as futile cycling. Futile cycling is an energetically wasteful process, since for sucrose to be resynthesised the hexoses have to be phosphorylated again at the expense of ATP, and therefore reduction of this process in sucrose accumulating tissue is an

important goal. The modelling results indicate that, at least in a fairly young internode, sucrose futile cycling is not much affected by specific SuSy isoforms. This may not be the case in a mature internode; therefore mature tissue should also be modelled in order to answer this question.

In conclusion, kinetic modelling can be used not only to predict the effects of variation in the activity or kinetic parameters of enzymes catalysing different reactions, but can also yield information about the metabolic effects of the presence of more than one isoenzyme, such as SuSy isoforms in sugarcane. This makes possible much more informed decisions on manipulation strategies for yield improvement in any system that can be modelled this way. Obtaining the reaction mechanisms and kinetic parameters of all enzymes involved in such a system is an essential component of this approach.

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CHAPTER FIVE

EXPRESSION AND LOCALISATION OF SUCROSE SYNTHASE IN THE SUGARCANE CULM

5.1 Abstract

We investigated the expression and localisation of sucrose synthase (SuSy, EC 2.4.1.13) in young (internode three) to mature (internode nine) internodes of sugarcane (*Saccharum* species hybrids) variety N19. Enzyme activity in the top and bottom, as well as the peripheral and core parts of the internodes suggested that SuSy is present ubiquitously but that levels can differ significantly in different parts of the internodes and with maturity. This was also confirmed by immunohistochemistry, which showed that both vascular and storage parenchyma tissues contain SuSy in young and mature internodes. The ratio of sucrose breakdown to synthesis activity increased approximately 12-fold from an average of 0.12 in internode three to 1.4 in internode nine. This indicates that different forms of SuSy are present in young and mature internodes, or that the ratios of different isoforms differ between young and mature internodes. Immunoblotting showed that at least one form of SuSy present in young tissue was absent or present below detection limits in mature culm tissue.

5.2 Introduction

Sucrose synthase (SuSy, UDP-glucose: D-fructose 2 α-D-glucosyltransferase, EC 2.4.1.13), catalysing the reversible conversion of sucrose and UDP to UDP-glucose and fructose, is a central enzyme of carbohydrate metabolism in all plant species. SuSy is implicated in a wide variety of processes, which include nitrogen fixation (Gordon *et al.* 1999), starch synthesis (Ricard *et al.* 1998; Chourey *et al.* 1998), cellulose biosynthesis (Amor *et al.* 1995) phloem transport (Martin *et al.* 1993; Nolte and Koch 1993; Geigenberger *et al.* 1993) and the ability of storage organs to act as carbon sinks (Zrenner *et al.* 1995; Nolte and Koch 1993; Huber and Akazawa 1986). Of particular interest here is the fact that that in almost all plants sucrose is the main form of translocated carbon, and in addition it is also the main storage carbohydrate in some plants, for example the tap root of sugar beet and the mature internodes of sugarcane (*Saccharum spp.* hybrids).

Enzymes of sucrose metabolism are of particular interest in sugarcane since sucrose is the main storage carbohydrate and sugarcane accounts for about 60% of the world's sucrose production (Grivet and Arruda 2001). In contrast to tomato fruit, where SuSy activity is drastically reduced in mature fruit, mature sugarcane internodes still contain appreciable amounts of activity (Botha and Black 2000). SuSy is associated with vascular bundles (Nolte and Koch 1993; Yang and Russel 1990; Tomlinson *et al.* 1991), and there has been some speculation in the literature on whether the SuSy activity in mature sugarcane tissues is associated exclusively with vascular bundles (Buczynski *et al.* 1993). It was noted that without tissue printing or staining data one cannot assume that SuSy is only present in vascular bundles in mature tissue. Information on the localisation of SuSy is important for the study of sucrose accumulation, and for programs for its improvement. Metabolic models of sucrose accumulation (Rohwer and Botha 2001) need to take into account enzyme localisation in order to have additional utility. It also needs to be pointed out that localisation and expression of enzymes often depend on the developmental stage of plants and

their organs. For example, SuSy is phloem-associated in mature maize leaves (Nolte and Koch 1993) but in young leaves this is not the case (Hanggi and Fleming 2001). Hence, both structural and kinetic data need to be incorporated into models, which need to be carefully defined in order to approximate the system to be modelled as accurately as possible.

Evidence exists that a membrane-associated form of SuSy could be involved in the biosynthesis of cellulose and callose (Amor *et al.* 1995). In sugarcane this has not yet been investigated. This aspect is important, because if the purpose of plasma membrane associated SuSy is to provide the UDP-glucose precursor for the synthesis of glucans (Amor *et al.* 1995), then only part (maybe very little or nothing) of this UDP-glucose would be released into the cytosolic compartment, as the glucan synthases are membrane associated enzymes (Delmer 1999). In fact, it is possible that SuSy and cellulose synthase may interact, resulting in metabolite channelling (Ovádi 1991). This hypothesis is supported by the fact that cellulose synthase contains cytosolic domains that have high homology to domains in animal proteins that are known to be involved in protein-protein interaction (Delmer 1999). The implications are clear: assuming that all measured SuSy activity is cytosolic potentially overestimates the activity of SuSy in this compartment. Therefore, knowing the partitioning of SuSy between membrane fractions and the cytosol will be useful for metabolic modelling, as well as for providing clues about its physiological roles in specific tissues.

The tissue localisation of SuSy protein has only been studied in a limited number of species. Isoforms of SuSy were preferentially expressed in specific tissues, e.g. rice (*Oryza sativa*) RSus3 was immunolocalised predominantly in endosperm cells and therefore is thought to provide precursors for starch synthesis (Wang *et al.* 1999). The RSus1 and RSus2 isoforms were more widely expressed and were found in both leaves and roots. The RSus1 isoform was localised in mesophyll cells in leaves, but in roots it occurred in the phloem,

indicating differences in SuSy gene regulation between these organs (Wang *et al.* 1999).

As SuSy occurs in a variety of organs, tissues and subcellular locations, clarifying functional relationships is difficult. This is especially true where there is a lack of tissue or organ specificity between isoforms, as in the case of RSus1 and RSus2 in rice. Nevertheless, localisation data and experiments on specific plant organs and SuSy isoforms have provided insight into SuSy functions, e.g. the abovementioned membrane association study, while antisense inhibition of a tuber-specific SuSy showed that SuSy activity is highly correlated with the sink strength of potato (*Solanum tuberosum*) tubers, with reduced tuber size and starch content in the antisense plants (Zrenner *et al.* 1995). In tomato (*Lycopersicon esculentum*) fruit, SuSy activity was found to be high during the phase of rapid fruit growth, but during the sugar accumulation and ripening stage SuSy activity was much lower (N'tchobo *et al.* 1999). Significantly, in tomato fruit the mechanism of phloem unloading switches from symplastic in young, fast growing fruit to apoplastic in maturing, sugar accumulating fruit (N'tchobo *et al.* 1999; Ruan and Patrick 1995). Therefore, the SuSy present in cells surrounding vascular bundles in young tomato fruit (Wang *et al.* 1994) may be involved in symplastic phloem unloading. The value of both enzyme activity and localisation data to elucidate enzyme function is clear. There are currently no immunolocalisation data in the literature for sugarcane SuSy.

The aim of this study was to investigate the localisation and expression of SuSy enzyme in young to mature sugarcane internodal tissue. The results show that SuSy is present in both vascular and storage parenchyma tissue in young and mature internodes. An increase in sucrose breakdown/synthesis ratio occurs with increasing internode maturity. No membrane association in mature internodal tissue was evident.

5.3 Materials and methods

5.3.1 Materials

Sugarcane (*Saccharum* spp. hybrids) variety N19 plants field grown at the University of Stellenbosch experimental farm were used. Internode one was taken as the internode attached to the leaf with the first exposed dewlap (Van Dillewijn 1952).

Tris buffer, DTT, coupling enzymes and alkaline phosphatase-conjugated goat anti-rabbit antibody were obtained from Roche (Grenzacherstrasse 124, CH-4070, Basel, Switzerland), except UDP-glucose pyrophosphorylase which was from Sigma (3050 Spruce St., St. Louis, MO 63103, USA). Merck (Frankfurter Strasse 250, 64293, Darmstadt, Germany) provided the other chemicals.

5.3.2 Tissue preparation

Core and peripheral parts of internodes were obtained by punching out progressively larger diameter cylinders with a cork borer in three steps, starting from the centre. The tissue from step two was discarded. Cork borer sizes were chosen so that almost the whole internode was used, up to about 3 mm from the edges.

5.3.3 Protein extraction

Tissue was ground to powder in liquid nitrogen and extracted in a 1:2 (m/V) ratio of 300 mM Tris-HCl (pH 7.5) buffer containing 10 % (V/V) glycerol, 1 % (V/V) β-mercapto ethanol, 2 mM $MgCl_2$, 2 mM EDTA and Roche Complete™ protease inhibitor at the recommended concentration. The homogenate was filtered through a double-layered nylon cloth, centrifuged at 10 000 g for 10 min, and the pellets discarded. The proteins in the supernatant were precipitated by adding 25 % (m/V) PEG 6 000 and recovered by centrifugation at 10 000 g for 10 min. The

pellets were resuspended in a small volume of 100 mM Tris-HCl (pH 7.5) buffer containing 2 mM $MgCl_2$, 2 mM DTT and 2 mM EDTA (buffer A). Glycerol was added to 20 % (V/V) and the samples were then rapidly frozen by submersion in liquid nitrogen, followed by storage at –80 °C. Enzyme assays using these samples were conducted within one month from the protein extraction. Enzyme samples treated in this way lost about 2.5 % enzyme activity over a one-month period under these storage conditions.

For isolation of membrane-bound SuSy the supernatant from the first centrifugation step was centrifuged at 10 000 g for 10 min, the supernatant transferred to clean tubes, and the centrifugation repeated. This supernatant was ultracentrifuged for 60 min at 100 000 g in order to obtain the microsomal fraction. The pellet was resuspended in a small volume of buffer A containing 5 % (v/v) Triton X100™. Samples were frozen in liquid nitrogen and stored at –80 ºC. Contamination from cytosolic proteins in the microsomal fraction was assessed by assaying for pyrophosphate-dependent phosphofructokinase (PFP), which only occurs in the cytosol. The real cytosolic and microsomal SuSy activities were calculated as follows: $SuSy_{cytosolic\ (real)} = SuSy_{cytosolic\ (measured)} \times (x + y)/x$ and $SuSy_{membrane\ (real)} = SuSy_{membrane\ (measured)} - SuSy_{cytosolic\ (measured)} \times (y/x)$, where x = PFP activity in cytosolic fraction and y = PFP activity in microsomal fraction. Average contamination of microsomal fractions with cytosolic PFP was about 1.5 %.

5.3.4 Enzyme assays

Activity in the sucrose synthesis direction was measured in 100 mM Tris-HCl (pH 7.5) buffer (Zeng *et al.* 1998). The sucrose formed was measured by the anthrone binding method (Van Handel 1968).

Activity in the sucrose breakdown direction was measured in an assay containing 100 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, 2 mM NAD^+, 1 mM pyrophosphate and appropriate concentrations of sucrose and UDP. UDP-glucose

pyrophosphorylase, phosphoglucomutase and *Leuconostoc* glucose-6-phosphate dehydrogenase were added to a final activity of 4 $U.ml^{-1}$. NADH production was monitored at 340 nm.
Sucrose breakdown/synthesis ratios were determined from reactions with 320 mM sucrose and 1.5 mM UDP for the sucrose breakdown reaction, and 10 mM UDP-glucose and fructose in the sucrose synthesis reaction, at zero initial product concentrations in each case.

PFP activity in the direction of fructose-1,6-bisphosphate synthesis was assayed in buffer containing 50 mM Tris-HCl (pH 7.2), 2 mM $MgCl_2$, 0.1 mM NADH, 5 mM fructose-6-phosphate, 10 µM fructose-2,6-bisphosphate, 1 U aldolase, 1 U glycerol-3-phosphate dehydrogenase and 10 U triose-phosphate isomerase per reaction. Pyrophosphate was used to initiate the reaction. Reactions were carried out in a 96-well microtitre plate and NAD^+ formation was monitored at 340 nm in a Bio-Tek Instruments PowerWave X spectrophotometer.

5.3.5 Electrophoresis

SDS-PAGE was performed at room temperature in a Bio-Rad Mini-PROTEAN II™ electrophoresis cell. The separating gel contained 7.5 % polyacrylamide; the stack gel 4 %, with a 37.5:1 acrylamide/bisacrylamide ratio (Laemmli 1970). Native PAGE was performed similarly at 4 °C, but the gel and buffers did not contain SDS.

5.3.6 Preparation of antigen, immunoblotting and immunoinactivation

Leaf roll tissue was ground to powder in liquid nitrogen and extracted, filtered and centrifuged as for the protein extraction. The proteins in the supernatant were precipitated by 80 % saturation with ammonium sulphate and recovered by centrifugation at 10 000g for 10 min. The pellets were resuspended in 100 mM Tris-HCl (pH 7.5) buffer containing 2 mM $MgCl_2$, 2 mM DTT and 2 mM EDTA (Buffer A). The protein extract was then desalted by passage through a

Pharmacia PD-10 (Sephadex G25) column and the eluant was diluted two times with buffer A. The desalted extract was applied to a 5 ml Amersham/Pharmacia Hi-trap Q anion exchange column that had previously been equilibrated with buffer A. The column was eluted at 4 °C with a linear KCl gradient at a flow speed of 1 ml/min and fractions containing 20 % or more of maximum activity were pooled. Active fractions from the column were dialysed against buffer A.

Affinity chromatography was also performed at 4 °C using a 2 ml bed volume of UDP-glucuronic acid agarose (Sigma). Sample was circulated through the column for at least five column volumes at 0.5 $ml.min^{-1}$, followed by washing with five column volumes buffer A and elution with buffer A plus 100 mM UDP-glucose. Active fractions from the column were dialysed against buffer A.

The dialysed active fractions from the affinity column were used for native gel electrophoresis. The part containing SuSy activity (of the two major bands containing SuSy activity, the one with the higher electrophoretic mobility was used) was excised, crushed in liquid nitrogen and the resulting powder extracted with water. After centrifugation the supernatant was used to immunise a rabbit.

Immunoblotting was performed after SDS gel electrophoresis and transfer to a nitrocellulose membrane (Hybond™-C Extra, Amersham Biosciences) using a Bio-Rad Transblot™ SD semi-dry transfer cell and transfer buffer (48 mM Tris-HCl (pH 8.6), 39 mM glycine, 20 % (V/V) methanol, 0.0375 % (m/V) SDS). The membrane was blocked for 2 h at room temperature with gentle agitation with a TBST buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1 % (V/V) Tween 20) containing 3 % (m/V) BSA. Anti-SuSy antiserum was diluted 1:2 000 in TBST buffer with 3 % (m/V) BSA and used to probe the membrane for 1 h. After rinsing and washing three times for 15 min with TBST buffer, a 2 000 times diluted alkaline-phosphatase conjugated goat anti-rabbit IGG antibody was added and the membrane incubated for 1 h. The membrane was washed as before and

developed with a solution consisting of a Roche NBT/BCIP tablet dissolved in deionised water. Development was stopped with running tap water.

Immunoinactivation incubation mixtures contained 0.1% (m/V) BSA, in 20 mM Tris-HCl (pH 7.5) buffered saline (TBS), with day 0 or day 39 serum in a total volume of 50 µl. Different serum volumes were compensated for with TBS. After addition of crude extract, the contents were mixed and the tubes incubated at 4 °C for 45 min. After centrifugation at 13 000g for 5 min the supernatants were assayed for SuSy in the sucrose breakdown direction with the UDP-glucose pyrophosphorylase, phosphoglucomutase and *Leuconostoc* glucose-6-phosphate dehydrogenase coupled assay.

5.3.7 Immunohistochemistry

Cylindrical pieces of tissue were bored out of internodes with a cork borer. The cylinders were bisected lengthways and left overnight at 4 °C in fixing solution, consisting of PBS buffer with 2 % (m/V) paraformaldehyde and 2 mM DTT. The next day sections about 1 mm thick were cut by hand from the tissue with a blade. The sections were rinsed in PBS buffer and then washed for 15 min at room temperature with gentle agitation. Sections were then blocked in 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 15 mg/ml gelatine and 10 mg/ml BSA for 2 h at 37 °C with gentle agitation. The block buffer was replaced with block buffer containing anti-SuSy antiserum and pre-immune serum at 1000 and 3000 times dilutions, and sections incubated for 1 h as before. Sections were rinsed and then washed three times for 15 min in PBS buffer containing 0.5 µl Tween.20 ml^{-1}. Block buffer with 2 000 times diluted alkaline-phosphatase conjugated goat anti-rabbit IGG antibody was added and the sections incubated for 1 h as before. Sections were washed as before. For detection, an NBT/BCIP tablet (Roche) dissolved in deionised water containing 10 % (m/V) polyvinylalcohol was used. Colour development was monitored at 5 min intervals until satisfactory (about 1 h). Sections were washed carefully under running tap

water until all visible traces of detection solution was gone. Sections were then stored in tap water containing 0.1 M EDTA to prevent further staining.

The sections were studied with a Nikon Eclipse E400 microscope and photographed with a Nikon Coolpix 990 digital camera.

5.3.8 Protein determinations

Protein concentrations were determined using mouse immunoglobulin G as a protein standard (Bradford 1976).

5.4 Results

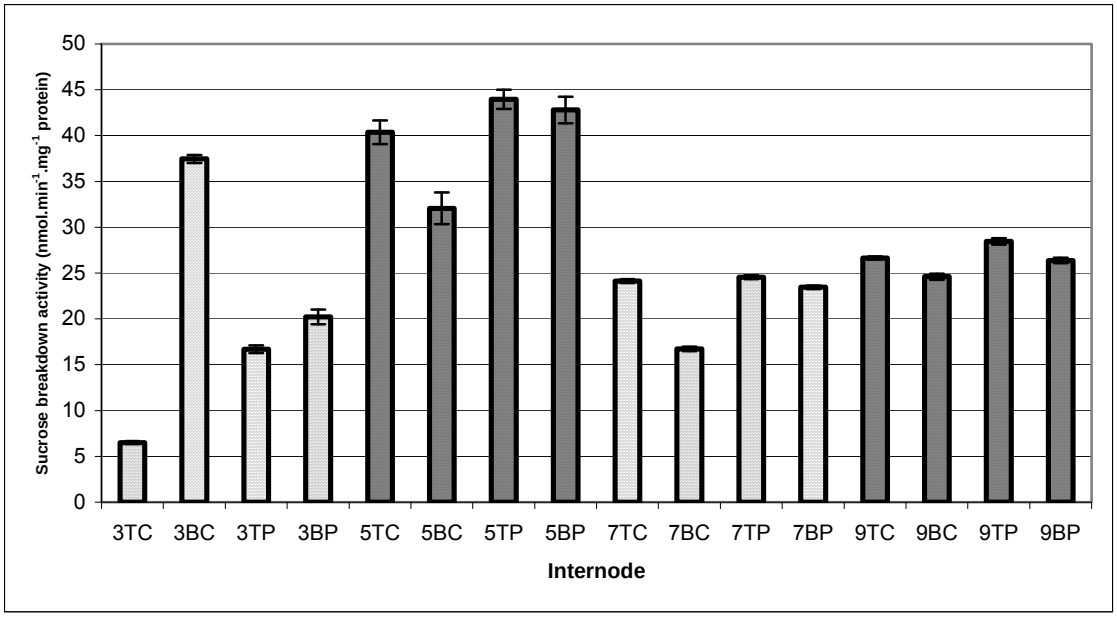

Fig. 1 SuSy activity ±SE (n=3) in the sucrose breakdown direction in different parts of young to mature internodes. TC: top core, BC: bottom core, TP: top periphery, BP: bottom periphery. Higher numbers indicate more mature internodes.

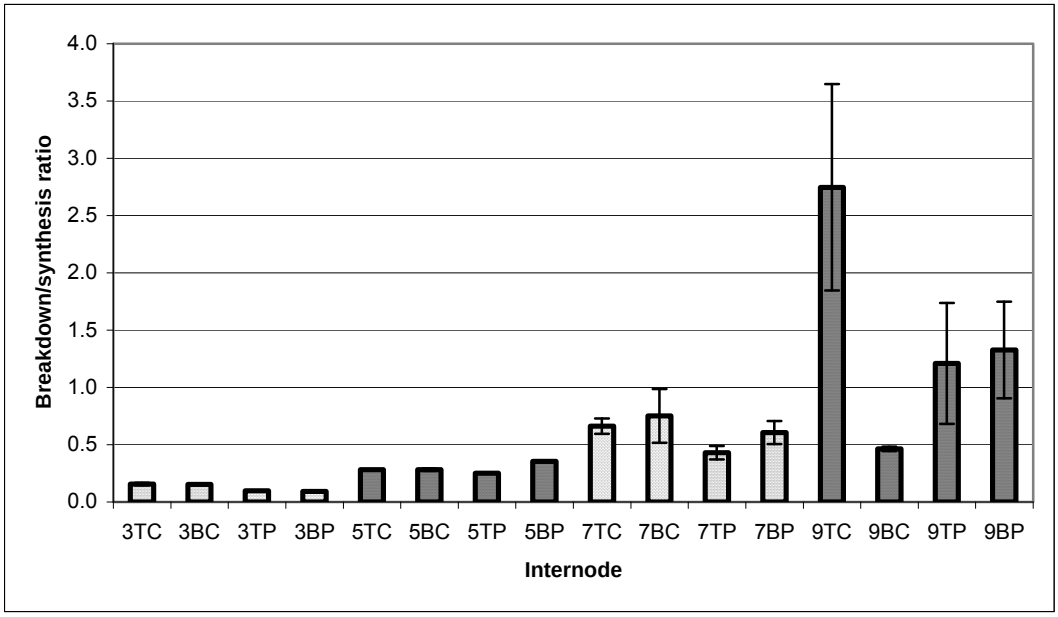

Fig. 2 Breakdown/synthesis ratios ±SE (n=3) in different parts of young to mature internodes. TC: top core, BC: bottom core, TP: top periphery, BP: bottom periphery. Higher numbers indicate more mature internodes.

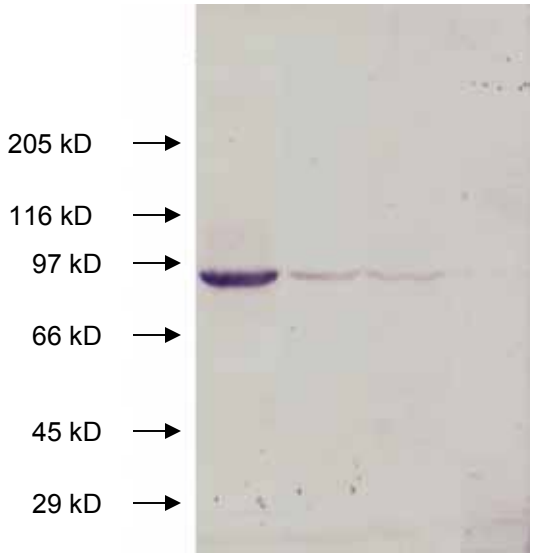

Fig. 3 Immunoblot of crude protein extracts from internodes three to nine. 10 µg protein per lane. Lane 1, int. 3; lane 2, int. 5; lane 3, int. 7; lane 4, int. 9.

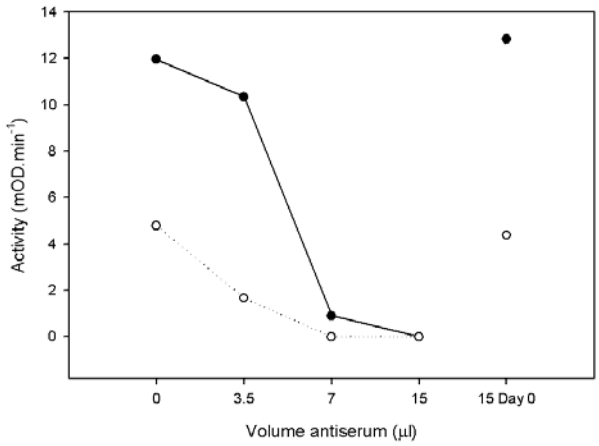

Fig. 4 Immunoinactivation of SuSy in crude extract from internode 3 (filled circles) and internode 9 (open circles).

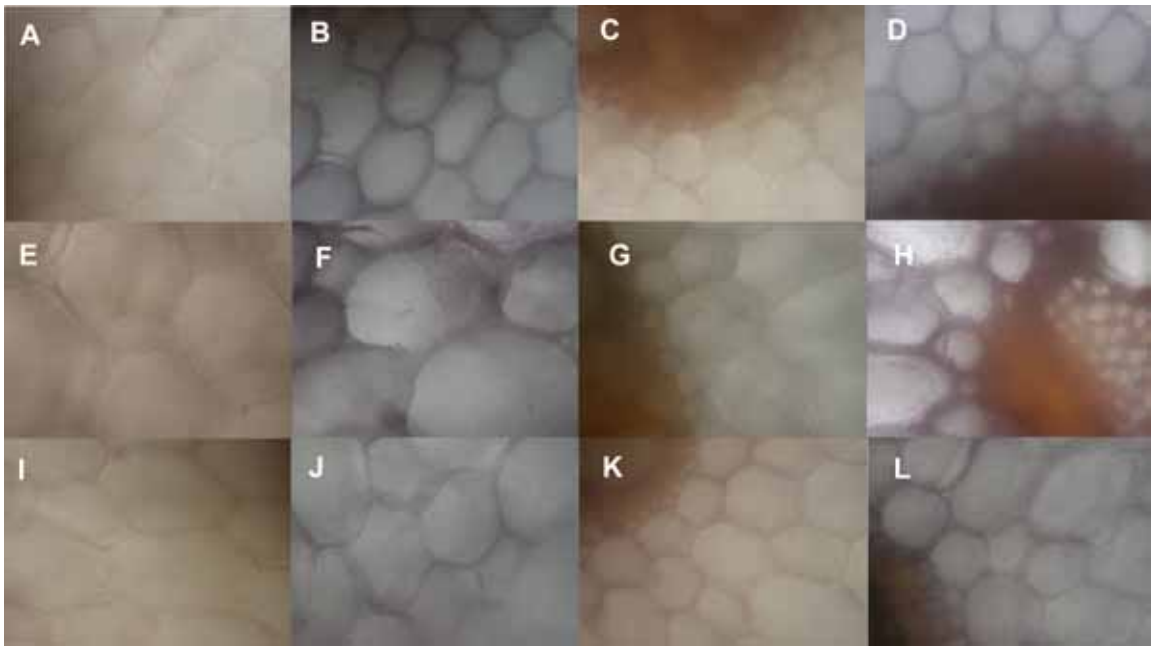

Fig. 5 Internode immunohistochemistry sections incubated with 1000 times diluted pre-immune serum (A, E, I, C, G, K) and anti-SuSy serum (B, F, J, D, H, L) at 400 times magnification. A-D: internode 3, E-H: internode 5, I-L: internode 9

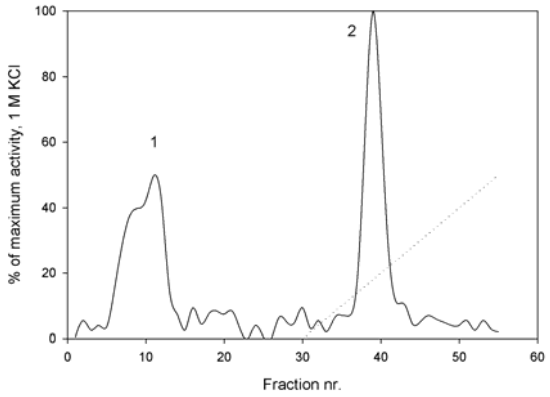

Fig. 6 Separation of two SuSy isoforms during chromatography on an anion exchange column using internode 9 tissue.

The experiments described in this section had as their main aim the generation of data on sugarcane SuSy localisation, by measuring SuSy activity in different parts of internodes, as well as by employing a histochemical technique to assess the tissue localisation of SuSy protein. It was also established that different SuSy isoforms were present in internodes differing in maturity, and that multiple isoforms were present in mature tissue – as was found in leaf roll tissue (Chapter 3).

5.4.1 SuSy activity in different regions of internodes

The level of SuSy activity in the different regions within each internode was similar from internode five to nine. Internode 5 had the highest overall activity at an average of 40 nmol.min^{-1}, while the average activity in internodes 7 and 9 was 22 nmol.min^{-1} and 26 nmol.min^{-1} respectively. Internode 3 exhibited the biggest differences between different regions, with the lowest activity in the top core region at 6.5 nmol.min^{-1}, and the highest activity in the bottom core region, at 37 nmol.min^{-1} (Fig. 1). These activities are somewhat lower than have previously been reported (Botha and Black 2000), but the trend is similar in that activity decreases somewhat in the older internodes.

5.4.2 Breakdown/synthesis ratios

The ratio of maximum sucrose breakdown to synthesis activity increased with internode maturity, from internode 3 to internode 9 (Fig. 2). There is a positive correlation between sucrose breakdown/synthesis ratio and sucrose content, with previous experiments that have shown sucrose levels in internode 9, at about 50 % of dry mass, to be roughly five times higher than in internode 3 (Botha and Black 2000). The increase in sucrose/breakdown synthesis ratio with maturity is a surprising fact: intuitively one might have expected a decrease in the sucrose/breakdown synthesis ratio.

5.4.3 Immunological analyses

All SuSy activity in crude extracts from both the young internode 3, and the mature internode 9, was immunoinactivated by a polyclonal antiserum, while pre-immune serum had no effect (Fig. 4). This proves the specificity of the antiserum for SuSy and, together with the fact that only one band of the correct size is recognised on an immunoblot, indicates the serum's suitability for immunohistochemistry work.

An immunoblot with crude extracts from internodes 3 to 9 (Fig. 3) using the polyclonal antiserum detected a polypeptide of about 94 kDa, which is in approximate agreement with the calculated molecular mass of sugarcane SuSy (Lingle and Dyer 2001). From the immunoblot it is evident that the antiserum discriminates between the denatured SuSy polypeptides from the different internodes. This is shown by the lack of signal for internode 9, even though enzyme activity was similar to internode 7 (Fig. 1). The SuSy from internode 3 was best detected, while internodes 5 and 7 gave similar signals.

5.4.4 SuSy localisation

SuSy protein was present in vascular and storage parenchyma tissue in young, intermediate and mature internodes (Fig. 5). For accurate localisation on the cellular level, a more suitable sectioning method needs to be developed – however, the results obtained in this study were significant in that it showed SuSy localisation in storage parenchyma tissue in mature internodes (see Discussion).

5.5 Discussion

The results obtained in this study indicate that SuSy is present in all regions of young and mature sugarcane internodes. Activity determinations on core and peripheral regions of internodes clearly indicated that the enzyme activity is not only present in vascular or storage parenchyma tissue. If there had been preferential or exclusive vascular or storage parenchyma localisation of SuSy, one would have expected significant differences in activity between peripheral and core regions of internodes because of the distribution of vascular bundles, the occurrence of which declines toward the centre of internodes. It has to be mentioned that the vascular bundles on the extreme periphery (outer 3 mm) of sugarcane internodes sometimes do not contain phloem (Jacobsen *et al.* 1992), but this part of the internodes was discarded in this study. All vascular bundles in tissue sections stained positive for SuSy. The biggest difference in activity was found between the top and bottom core parts of internode three, where activity in the bottom was about six times higher than in the top part. It is proposed that the lower sucrose content in the bottom parts of sugarcane internodes (most evident in very young internodes) indicates a metabolically more active environment in terms of respiratory and growth processes (Rose and Botha 2000). Growth and elongation is shown to occur mostly in the bottom parts of internodes (Jacobsen *et al.* 1992). This is consistent with a higher demand for hexoses and precursors for cellulose synthesis in the bottom part of the internode, which could both be provided by SuSy. Sucrose content in the bottom of internode three is about half that in the top section, with this difference in sucrose content between different

parts of the same internode the highest among the young, maturing, and mature internodes tested (Rose and Botha 2000).

The relatively high SuSy activity found in the mature internode 9 contrasts with crops such as tomato, where SuSy activity is much reduced during the later stages of fruit maturation and ripening (N'tchobo *et al.* 1999). In potato (Zrenner *et al.* 1995) and citrus fruit (Komatsu *et al.* 2002), SuSy activity stays high and is associated with maturation and accumulation of starch in potato and sucrose and reducing sugars in citrus. A critical distinction between the tomato and sugarcane systems is that phloem unloading in tomato fruit is believed to be apoplastic, based on rapid hydrolysis of sucrose during unloading and loss of symplastic connections through plasmodesmata in mature fruit (Ruan and Patrick 1995). In this mode of phloem unloading, sucrose is unloaded into the apoplastic space where it is cleaved by invertases and the resulting hexoses are taken up by hexose transporters. Phloem unloading in sugarcane is believed to be symplastic (i.e. through plasmodesmatal connections), because the apoplastic spaces in the vascular bundles and storage parenchyma are not connected and the bundle sheath cells contain numerous plasmodesmata (Komor 2000). Hence, the anatomical features of the sieve element-companion cell complex in mature sugarcane internodes contrast strongly with those of tomato fruit. The trend in SuSy activity is also different, with activity strongly downregulated in maturing tomato fruit, but still appreciable in mature sugarcane internodes. The reason for this could well be that SuSy functions to maintain a sucrose concentration gradient through sucrose breakdown during symplastic phloem unloading in sugarcane, while for apoplastic unloading, as in tomato fruit, SuSy is not needed. The promotion of phloem unloading in this way is likely to function in addition to other mechanisms such as bulk flow.

The increase in the SuSy sucrose breakdown/synthesis ratio as internodes mature certainly points to sucrose breakdown as perhaps the only function of SuSy in mature tissues. A study using [U-^{14}C]-glucose showed that in mature

internodes, sucrose synthesis is exclusively through SPS (sucrose-phosphate synthase), since labelling in the glucose and fructose moieties was equal, while in younger internodes both SuSy and SPS were implicated in sucrose synthesis, because of higher labelling in glucose (Botha and Black 2000). These findings, combined with our data, indicate that sucrose breakdown is also the function of SuSy in mature storage parenchyma tissue. The existence of a “futile” cycle of sucrose breakdown and synthesis is well known (Sacher *et al.* 1963; Batta and Singh 1986) and SuSy will be a major contributor to sucrose breakdown in this cycle. These “futile” cycles of sucrose synthesis and degradation are believed to allow sucrose metabolism to respond more rapidly to physiological changes without major changes in sucrose or metabolite concentrations (Geigenberger and Stitt 1991). Cycling between triose-phosphates and hexoses also occurs in sugarcane, and the flux through this cycle decreases with internode maturity, indicating that these “futile” cycles could have a regulatory function (Bindon and Botha 2002).

The signal decrease with increasing internode maturity, observed on an immunoblot using polyclonal antiserum raised against a form of SuSy purified from leaf roll tissue is interesting, and parallels the increase in sucrose breakdown/synthesis ratios (see also Chapter 3) with increasing internode maturity. By internode nine, there was no signal on the blot (Fig. 3), despite appreciable SuSy activity. Thus, there is discrimination by the antiserum between isoforms from the different internodes. The antiserum did, however, immunoinactivate all SuSy activity in internodes, whether young or mature (Fig.4), and was therefore used for immunohistochemistry. The results clearly indicate differential expression of SuSy isoforms with substantially differing breakdown/synthesis ratios between young and mature tissues, with the SuSy in mature internodes apparently more biased towards sucrose breakdown than the SuSy in younger internodes. This increased propensity towards sucrose breakdown in mature internodes is partly explicable in terms of SuSy assisting in phloem unloading, but seems contradictory in mature storage parenchyma cells,

which accumulate sucrose. A partial explanation could be that under certain conditions sucrose breakdown in mature storage parenchyma follows the “alternative” route (Huber and Akazawa 1986). It was suggested that sucrose is broken down by SuSy and the UDP-glucose is converted to UTP and G1P by UDP-glucose pyrophosphorylase. The UTP is then used by fructokinase to phosphorylate fructose, which in turn provides UDP for SuSy. In potato, SuSy and fructokinase are simultaneously upregulated at the onset of tuberisation (Appeldoorn *et al.* 2002). It has been shown that SuSy/invertase ratios increase (invertase levels are reduced, SuSy levels remain constant or increase) under anoxic stress (Ricard *et al.* 1998; Zeng *et al.* 1999) and that the speed of response is important. This is consistent with the proposed alternative sucrose breakdown route, because one less ATP is consumed in this route for breakdown of sucrose. Ultimately though, this “alternative” pathway needs a source of PPi, and so it appears as though this pathway is used as a temporary strategy to conserve ATP until oxygen is restored to normal levels. The response to hypoxia and anoxia does point to an invertase-mediated and a SuSy-mediated path of sucrose breakdown, but whether the same responses occur in sugarcane internodes is not yet known. Obviously some of the incoming sucrose in storage parenchyma cells will need to be cleaved to be used in glycolysis and for precursor molecules, so a “housekeeping” function for SuSy (and neutral invertase) in sugarcane in this regard is a given.

An exciting possibility, which has not yet been addressed in sugarcane, is whether different SuSy isoforms function in vascular and storage tissue. In potato, the Sus3 isoform was expressed mainly in stems and roots and so appears to provide a vascular function, while the Sus4 isoform was expressed chiefly in the storage and vascular tissue of tubers (Fu and Park 1995). In other words, there seems to be a distinction between *sink* and vascular function in potato rather than vascular and storage. No conclusions whether different isoforms of SuSy operate in sugarcane vascular and storage parenchyma tissue can be drawn from this study. However, both native gel electrophoresis and

anion exchange chromatography (Fig. 6) show that at least two forms occur in mature culm tissue. Therefore the theoretical possibility for isoform-specific sink and vascular functions exists and should be further investigated, particularly with possible improvements to sucrose accumulation in commercial sugarcane varieties in mind. N-terminal sequencing of sugarcane SuSy isoforms proved unfeasible, because the proteins were blocked at the N-terminal. Up to now it has been assumed that only the SS1 isoform occurs in mature culm tissue (Buczynski *et al.* 1993), but this assumption needs revision in light of the above and also findings in other crops that show expression of more than two SuSy genes, e.g. (Carlson *et al.* 2002; Wang *et al.* 1992; Komatsu *et al.* 2002).

Investigating the expression and localisation of SuSy in sugarcane internodes differing in maturity has particular relevance for efforts to quantify the contribution of specific enzymes to sucrose accumulation. A kinetic model of sucrose accumulation has been published (Rohwer and Botha 2001). For obvious reasons, actual localisation of enzymes considered in such a model should be confirmed for the particular tissue modelled. Furthermore, the differential expression of kinetically different isoforms between tissues, such as that observed in sugarcane internodes of differing maturity, needs to be integrated with the localisation of these isoforms. This study is a first step in that regard, and shows: (a) that the assumption made in the abovementioned model that SuSy is present in storage parenchyma tissue of internode 5 is correct, and (b) that a model describing sucrose accumulation in the more mature internode 9, where both sucrose content and sucrose accumulation rate are at near maximum levels, must also take into account the presence of SuSy in storage parenchyma cells. Apart from their localisation, the distinct kinetic parameters of different SuSy isoforms present in internodes of differing maturity will also impact differently on factors important for sucrose accumulation, such as the degree of sucrose breakdown and resynthesis (futile cycling) and the net sucrose accumulation rate. In this regard, kinetic models that can calculate the coefficients of metabolic control analysis (MCA) (Kacser and Burns 1973;

Heinrich and Rapoport 1974) and also the direction of reversible enzyme reactions, such as that catalysed by SuSy, are very useful. MCA can be used to quantify the contribution of individual reaction steps to the pathway flux or steady state metabolite concentrations, as in pioneering experiments to determine the contribution of individual enzymes to mitochondrial respiration (Groen *et al.* 1982) and has been reviewed from a plant metabolism perspective (Ap Rees and Hill 1994). The enzymes (or enzyme isoforms) that have the highest control coefficients for futile cycling, for example, would therefore be good candidates for manipulation in order to increase sucrose content. Hence, it can be seen that localisation, identification and characterisation of all SuSy isoforms in sucrose accumulating tissue would enhance the accuracy of metabolic models and therefore contribute to strategies for increasing sucrose content.

A preliminary investigation showed that the level of SuSy activity in the microsomal fraction of crude extracts from culm tissue was similar to that of the cytosolic marker enzyme pyrophosphate-dependent phosphofructokinase (results not shown). We conclude that membrane-associated SuSy does not constitute a significant portion, if at all, of overall SuSy activity in the sugarcane culm and can therefore be disregarded as far as this tissue is concerned.

In conclusion, we have shown that SuSy is present in both vascular bundles and storage parenchyma of young and mature internodal tissue. Although localisation was similar between these tissues, the increase in the sucrose breakdown/synthesis ratio from young to mature tissue indicates a change in the expression of SuSy isoforms between young and mature tissues. With the exception of internode 3, SuSy activity was similar in different parts of internodes. At least one isoform present in young tissue is absent in mature tissue, but more than one isoform is present in mature tissue. No significant membrane association was evident in internodal tissue. The question whether different isoforms are present in vascular and storage tissue could potentially be

addressed using monoclonal antibodies for immunohistochemistry, or with *in situ* hybridisation with very specific probes.

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CHAPTER SIX

CONCLUSIONS

This investigation succeeded in meeting all the goals that were set in the introductory chapter. Significant contributions were made to our knowledge of SuSy isoforms in sugarcane and the kinetic differences between them. In addition, insight was obtained into the impact of individual SuSy isoforms on sucrose accumulation, by way of kinetic modelling. The study of SuSy localisation elucidated the expression of SuSy in different parts of internodes differing in maturity. Therefore, this study contributes appreciably to our understanding of the regulation of carbohydrate metabolism in sugarcane and the role played by SuSy in this process.

6.1 SuSy isoforms

At least three SuSy isoforms are expressed on the protein level in sugarcane sink (leaf roll) tissue (Chapter 3). These isoforms differ significantly in their kinetic properties: K_m values (mM) were, for sucrose, 41.8 ± 3.4, 109 ± 23 and 35.9 ± 2.2; for UDP, 1.07 ± 0.08, 0.21 ± 0.04 and 0.02 ± 0.002; for fructose, 6.62 ± 1.55, 11.7 ± 2.5 and 6.49 ± 0.60 and for UDP-glucose 3.59 ± 0.37, 0.53 ± 0.14 and 0.24 ± 0.03 for SuSyA, SuSyB and SuSyC respectively. The SuSyC isoform is also immunologically distinct from the SuSyA and SuSyB isoforms, since, unlike SuSyA and SuSyB, it is not immunoinactivated by a polyclonal antiserum against SuSyB. Major changes in expression of SuSy isoforms occur along the culm with change in maturity, as evidenced by an increase in sucrose breakdown/synthesis ratio and a decrease in the signal on an immunoblot (using a polyclonal antiserum against SuSyB) with increasing internode maturity. The level of SuSy proteins that are recognised by the antiserum decreases with culm maturity, to the point where it falls below immunological detection limits for internode 9 (Chapter 5).

In a study using monoclonal antibodies against the maize SS1 and SS2 isoforms, which also specifically recognise the corresponding sugarcane isoforms, it is shown that the SS2 isoform is not present in mature internodes (Buczynski *et al.* 1993). The SuSyC isoform described in this study was also absent in mature tissue. This isoform had a much lower sucrose breakdown/synthesis ratio (0.49) than the isoforms present in mature tissue (average breakdown/synthesis ratio of 1.2), indicating preferential expression of isoforms with high breakdown/synthesis ratios in mature tissue. The purified sugarcane SuSy proteins were N-terminal blocked, so apart from the fact that they are different forms, their identity was not established.

Previously, it was thought that only the SS1 isoform occurs in mature internodes (Buczynski *et al.* 1993), but data from anion exchange chromatography and native gel electrophoresis conducted in this project indicated the presence of more that one SuSy isoform in mature internodes (Chapter 3).

When the multiplicity of SuSy isoforms in other crops is considered, the presence of more isoforms than the two currently recognised SuSys in sugarcane is expected. What is less clear, are the reasons for the presence of these multiple isoforms.

6.2 The relevance of enzyme kinetics

A central goal of this project was to obtain more extensive kinetic data. With this in mind, the kinetic properties of SuSyC were studied in detail (Chapter 4). These kinetic properties were then used to query a kinetic model of sucrose accumulation (Rohwer and Botha 2001). Entering the kinetic parameters for SuSyC into the model led to a 40 % increase in sucrose and 7 times reduction in fructose concentration compared to the corrected original model. This illustrates the dramatic physiological effects that changes in enzyme kinetic parameters –

expression of different enzyme isoforms - can have. Sucrose levels were positively correlated with SuSy activity when kinetic parameters of either SuSyC or the generic SuSy were entered into the model. It has to be kept in mind that the model represents an internode 5, which is still immature, based on both sucrose content and accumulation rate (Whittaker and Botha 1997). After feeding [U-^{14}C]-glucose to internode 5 tissue disks, there is more label in the glucose moiety of sucrose, which indicates that SuSy participates in sucrose synthesis in this tissue (Botha and Black 2000); therefore an increase in sucrose content when increasing SuSy activity in the model makes sense. In mature tissue, the ratio between the labelled hexoses in sucrose is unity, indicating synthesis exclusively by SPS. The decreased participation of SuSy in sucrose synthesis with increased maturity indicated by the labelling experiments is consistent with the increased SuSy sucrose breakdown/synthesis ratio as internodes mature (Chapter 5).

It is much more difficult to explain or relate SuSy kinetic parameters and the modelling results to patterns of carbon partitioning than to trends in sucrose synthesis and content. As the sugarcane culm matures, less carbon is partitioned into fibre, protein and total respiration, with increased incorporation into sucrose (Whittaker and Botha 1997; Bindon and Botha 2002). Maturation of the culm is also associated with a decrease in cycling (“futile cycling”) between sucrose and hexoses, judging by a decrease in label returned in glucose when feeding labelled fructose to internodes 2 and 7 (Whittaker and Botha 1997). However, CO_2 production is similar between younger and more mature internodes, indicating a similar energy demand in older tissue compared to younger tissue. Therefore, there still exists a demand for sucrose breakdown activity in older tissue for this purpose, while demand for UDP-glucose for fibre synthesis is much lower, judging from ^{14}C incorporation into this component. Uptake of sucrose into sugarcane vacuole preparations is independent of ATP or pyrophosphate (Preisser and Komor 1991), so it seems as if respiration in older internodes simply provides the needed energy and carbon skeletons for normal cellular

metabolism, albeit at lower levels than in younger internodes, e.g. ^{14}C incorporation into protein is about half that in internode 9 than that in internode 3 (Bindon and Botha 2002). The question as to why the increased sucrose breakdown/synthesis ratio of SuSy as the culm matures, coupled with relatively constant levels of neutral invertase in the culm, does not lead to increased cycling between sucrose and hexoses, may be due to compartmentation of most of the sucrose in the vacuole in more mature tissues. Information on compartmentation and localisation of both enzymes and metabolites may provide crucial insights needed for answering many outstanding questions.

The negative correlation of futile cycling with sucrose accumulation need not be because of the energy penalty, it could also be that the futile cycle itself has a regulatory meaning. For example, in *Ricinus communis* seedlings, a doubling in flux through a similar futile cycle was associated with a repartitioning of carbon to starch, instead of the previous mobilisation of starch to sucrose (Geigenberger and Stitt 1991). However, it is unclear how a flux would be sensed, if this is what occurs. The name “futile cycle” is therefore unfortunate, since this cycling probably has a real function/s. There was only a very small (~1.7 %) difference in futile cycling between models containing two different SuSy isoforms (Chapter 4). It would be useful to modify the kinetic model for mature tissue and see what effects different SuSy isoforms, or changes in activity, have on futile cycling.

The use of metabolic modelling to predict the effects of changes in activity, kinetic parameters, or isoform composition of enzymes, has significant application in biotechnology. As long as the system can be modelled, this approach can lead to much faster and more efficient identification of manipulation targets. A work plan consisting of gene expression profiling, determination of enzyme kinetic parameters and subsequent modelling to determine the effects of changes in enzyme levels or composition on a target variable (e.g. sucrose concentration in sugarcane) is much more efficient than a gene-by-gene

“shotgun” manipulation approach. The resources alone required by the latter approach may disqualify it, especially for smaller organisations.

6.3 Localisation studies

No localisation data whatsoever for SuSy in sugarcane were available at the outset of this study. Hence, the presence of SuSy protein in internodes differing in maturity was investigated with an immunohistochemical approach, using a polyclonal anti-SuSy antiserum. Data indicated that SuSy was ubiquitously expressed throughout the internodes, in both vascular and storage parenchyma tissue of young to mature internodes. It is possible that the multiple isoforms in the culm have distinct vascular and storage parenchyma localisation, but this could not be tested with our polyclonal antiserum.

No significant levels of SuSy membrane association were evident in culm tissue, so the effect of membrane-associated SuSy on sucrose metabolism was not considered further.

6.4 Recommendations for further research

A potentially useful area for further research, especially given some of the interesting results obtained in this study, would be to obtain additional kinetic data on SuSy isoforms, especially those in mature internodes, and to investigate the effects of these multiple isoforms, using the kinetic model modified for a mature internode. The kinetic model used in this study requires, in addition to substrate K_m values, K_i values for the substrates. Since they were not available in the literature, the “generic” SuSy in the original kinetic model used estimated K_i values, equal to the substrate K_m values. However, the K_i values obtained for the SuSyC isoform in this study differ substantially from the corresponding K_m values (see Chapter 4). It is therefore not advisable to only determine the substrate K_m values and use these as an approximation of the K_i values. It would be

worthwhile to determine which isoforms, or combinations of isoforms, give the minimum futile cycling. This information would be very useful for possible genetic manipulation of SuSy in sugarcane.

A major restriction encountered in this project was that the SuSy proteins were N-terminal blocked, and so were not correlated or compared on a genetic level to known SuSy genes in sugarcane or other crops. For both fundamental knowledge of SuSy, as well as sugar metabolism (and its possible manipulation) in sugarcane, it is important that the different SuSy genes that are expressed be known. The high ploidy level of sugarcane, as well as the high number of putative SuSy genes in the model plant *Arabidopsis thaliana*, indicates that the picture as far as sugarcane is concerned could be very complex. As an example: although three SuSy isoforms were isolated from leaf roll tissue based on different kinetic and immunological characteristics, four different ESTs from leaf roll are already known. Also, the SuSy isoform composition in the culm changes with maturity. This was indicated by the increase in the sucrose breakdown/synthesis ratio with increasing internode maturity, as well as the concomitant decreasing signal on an immunoblot using an antiserum raised against a purified SuSy protein from leaf roll. Identification and characterisation of these different SuSy isoforms on a genetic level needs to be accomplished before possible manipulation strategies can be considered.

One reason that SuSy isoforms should be identified on a genetic level, is that it could enable very specific manipulation of their activity. Some SuSys apparently fulfil separate sink and vascular functions. The finding that multiple isoforms are present in mature sugarcane internodes means that this separation of functions is also a possibility here. If indeed so, it should theoretically be relatively easy to, say, reduce the levels of a sink-specific SuSy while leaving the vascular function of another isoform intact. The sink-specific SuSy may be an isoform that increases sucrose futile cycling, or lowers sucrose content etc. according to modelling results.

6.5 Concluding remarks

This study succeeded in its stated goals, but apart from that also led to some significant findings, such as the presence of at least three SuSy isoforms in sugarcane sink tissue, and the localisation of SuSy in storage parenchyma in mature tissue. Modelling different SuSy isoforms also demonstrated that different SuSy isoforms may have significant effects on sucrose content.

In addition to the contributions made to our knowledge of SuSy and the regulation of carbohydrate metabolism, aspects that need to be addressed in future studies were also identified. These may significantly impact sucrose yield improvement strategies and, ultimately, the profitability of the sugarcane industry.

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