Expression Analysis and Functional Characterization of *Triticum aestivum* Sucrose Transporter1 (*TaSUT1*) from Winter Wheat

by

Chelsey Jennifer Lynn McDougall

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

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ABBREVIATIONS

µCi	microCuries
µg	microgram
µL	microlitre
ADP	adenosine diphosphate
AGPase	adenosine diphosphate glucose pyrophosphorylase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AtSUC	*Arabidopsis thaliana* sucrose carrier
AtSUT	*Arabidopsis thaliana* sucrose transporter
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
cDNA	complimentary deoxyribonucleic acid
Cl	chloride
CO_2	carbon dioxide
DAH	days after heading
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
E value	expectation value
EDTA	ethylene diamine tetraacetic acid
ETC	electron transport chain
FMN	flavin mononucleotide
g	gravities
gDNA	genomic deoxyribonucleic acid
GmSUT	*Glycine max* sucrose transporter
H^+	proton
HvSUT	*Hordeum vulgare* sucrose transporter

hr	hour
IPTG	isopropyl β-D-1-thiogalactopyranoside
K_M	Michaelis-Menten constant
LB	Luria-Bertani
LeSUT	*Lycopersicon esculentum* sucrose transporter
M	moles
MB	Manitoba
min	minutes
mL	millilitres
mM	millimoles
MN	Minnesota
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodium phosphate
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	nanograms
nm	nanometres
O_2	oxygen
OD_{600}	optical density at 600 nanometres
OsSUT	*Oryza sativa* sucrose transporter
PEG	poly ethylene glycol
pH	potential of Hydrogen
PmSUC	*Plantago major* sucrose carrier
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase – polymerase chain reaction
SD	synthetic complete media with glucose as the sole carbon source
SDS	sodium dodecyl sulphate
SE-CC	sieve element–companion cell

sec	seconds
SS	synthetic complete media with sucrose as the sole carbon source
StSUT	*Solanum tuberosum* sucrose transporter
SUT	sucrose transporter
TAE	tris-acetate ethylene diamine tetraacetic acid
TaSUT	*Triticum aestivum* sucrose transporter
TE	tris ethylene diamine tetraacetic acid
U	units
UBQ	ubiquitin
USA	United States of America
UV	ultraviolet
V	volts
VfSUT	*Vicia faba* sucrose transporter
V_{max}	maximum rate
YPD	yeast peptone dextrose
ZmSUT	*Zea mays* sucrose transporter
ZmBT1	*Zea mays* Brittle1

ABSTRACT

McDougall, Chelsey Jennifer Lynn. M.Sc. The University of Manitoba, August 2008. Expression Analysis and Functional Characterization of *Triticum aestivum* Sucrose Transporter1 (*TaSUT1*) from Winter Wheat. M.Sc. supervisor: Dr. Fawzi Razem.

Photosynthate transport from source to sink organs is a major determinant in overall crop yield. The active transport of sucrose, the most prominent form of transported photosynthate, is achieved by proteins in the sucrose transporter (SUT) family. Interest in developing high yielding wheat (*Triticum aestivum* L.) by manipulating components of the source-sink relationship has created a need for greater understanding of *Triticum aestivum* SUT1 (TaSUT1) expression and function.

With increasing maturity, *TaSUT1* expression slightly decreased in flag leaves, was unaffected in peduncles, and showed a transient increase in developing grains. Both sucrose and glucose treatments slightly reduced *TaSUT1* expression in excised leaves. TaSUT1 operates via a proton-coupled transport mechanism, with sucrose uptake dependent on pH and hindered by electron transport chain inhibitors. Glucose and fructose interfere with sucrose transport by TaSUT1. TaSUT1 is a low affinity transporter with a K_M of approximately 19 mM.

Based on the results of this study, potential approaches for developing high yielding wheat by SUT manipulation are discussed.

1.0 INTRODUCTION

With the current trend of decreasing food supply and increasing use of food items for industrial applications, it is essential to increase crop yield. In cereals, yield is determined by the amount of starch produced per plant, which is determined in part by the efficiency of photosynthesis and the subsequent translocation of photosynthates. The transport of photosynthates, from their production in source tissues to their accumulation in sink tissues, occurs mainly in the form of sucrose. Sucrose transporter (SUT) proteins are known to play many critical roles in this translocation process.

The overall aim of this study was to better understand winter wheat *Triticum aestivum* SUT1 (*TaSUT1)* expression and function. Prior to the current study, no research had been conducted on SUTs from winter wheat, which is an important agronomic crop in Manitoba. In addition, this research was the first to characterize a cereal SUT. The specific objectives of this study were to:

1) Identify *TaSUT1* and analyze both the gene and its expressed protein.
2) Study the expression profile of *TaSUT1* in tissues involved in the source to sink transport of photosynthate during the period of grain maturation.
3) Determine the effect that increased sugar levels have on *TaSUT1* expression.
4) Study the kinetics of TaSUT1 sucrose transport, which includes studying the relationship between protons and sucrose transport, the potential for competing sugars to inhibit or induce sucrose transport, as well as the affinity of TaSUT1 for sucrose.

This research will provide insight into sucrose transport, both in terms of expression of *TaSUT1* genes and characteristics of TaSUT1 proteins, which is required prior to manipulation of this component of the source-sink relationship. Ultimately, this research will provide insight on potential modifications to TaSUT1 or sucrose transport in general which could result in high yield winter wheat.

2.0 LITERATURE REVIEW

2.1 Introduction

Plants are photoautotrophic organisms that produce energy in the form of carbon through reactions involving CO_2 and light energy. Photosynthate transport is an essential process for plant growth and survival, and involves movement of photosynthate from source tissues, where carbon compounds are produced and exported, to sink tissues, which import these compounds. There are multiple sinks within an individual plant and several factors contribute to the partitioning of photosynthate among them. From an agricultural perspective, the ability to manipulate photosynthate partitioning and increase the amount of carbon stored in harvestable sinks, such as the grains of wheat, is important for increasing crop yield. This review will discuss the production of photosynthate, the partitioning of photosynthate among sinks, as well as the transport pathway used to move photosynthate throughout the plant.

Among the many important components of photosynthate transport are SUTs. These proteins function predominantly in the active transport of sucrose into and out of the phloem (Aoki *et al.* 2003). Some SUTs may function in retrieving sucrose leaked from the phloem (Rosche *et al.* 2002; Aoki *et al.* 2004) and others as sucrose sensors (Schulze *et al.* 2000). Sucrose transporters vary in their affinity for sucrose, which is likely related to the diverse roles played by these proteins (Schulze *et al.* 2000). As this research focused on TaSUT1, a more detailed analysis of cereal SUTs is included.

Although sucrose is the form of carbon transported throughout cereal plants, the final storage compound in sink tissues is starch. Prior to starch formation, sucrose is converted into the monosaccharide precursor ADP-glucose (Emes *et al.* 2003). In cereals,

the transport protein Brittle1 is required for moving ADP-glucose from the cytosol into the amyloplast where starch production and accumulation occurs (Shannon *et al.* 1998).

A brief introduction to cultivated hexaploid wheat is presented in this review, including hexaploid wheat's origin in the Fertile Crescent of the Middle East (Feldman 2001), the beginning of its cultivation in Canada, as well as the current status of Canadian hexaploid wheat production.

2.2 Photosynthate Transport in Plants

2.2.1 Photosynthesis and the Source to Sink Relationship

Photosynthesis is essential to nearly all forms of life as it is relied upon for taking up CO_2 from the atmosphere, producing O_2, and creating carbon compounds to be used for energy required during metabolism. Photosynthesis consists of two main reactions: the light reactions, in which ATP and NAPDH are formed and O_2 is evolved, and the carbon-fixation reactions, including the Calvin cycle, in which newly fixed carbon atoms are reduced to a simple sugar. The immediate product of the Calvin cycle is the three carbon compound glyceraldehyde 3-phosphate and the majority of this triose is transported into the cytosol where it is converted into sucrose (Raven *et al.* 2004).

The source to sink relationship describes the main pathway that photosynthate travels through a plant. Source tissues are active photosynthetic organs which produce photosynthate in excess, whereas sink tissues are non-photosynthetic or produce less photosynthate than they require and therefore need to import carbon from the source tissues. Sucrose is a major end product of photosynthetic carbon metabolism and is the

main form of carbohydrate that is transferred throughout higher plants and partitioned among the various sinks (Ishimura *et al.* 2001; Emes *et al.* 2003; and others).

2.2.2 Photosynthate Partitioning into Sinks

Nutrient partitioning among competing sinks is governed by their relative ability to unload nutrients from the importing sieve elements (Patrick and Offler 2001). Carbon partitioning into a sink is largely influenced by the sink's relative size and proximity to the source. Other factors such as hormones, vascular connections and metabolic characteristics of the sink may also play a role in establishing the partitioning pattern (Simmons 1987). In wheat and likely other cereal crops, by two weeks after the grain is set, photosynthate partitioning appears to be mainly regulated by the strength of filling sinks (Gifford *et al.* 1984).

In some plants, the export of sucrose appears to be unaffected by changes in sink demand. Alternatively, other plants have been shown to preferentially partition fixed carbon into sucrose for export during periods of high sink demand or retain carbon in the form of starch during periods of low sink demand (Gifford *et al.* 1984). The activity of fructose-bisphosphatase and sucrose-phosphate synthase, which are key regulators involved in sucrose synthesis, seems to have an important role in the link between sink demand and the rate of carbon partitioning into sucrose and starch. Additionally, Wardlaw and Moncur (1976, as referenced in Simmons 1987) have shown that the speed of assimilate movement through the peduncle was influenced by the spike's requirement for assimilate. The accumulation of photosynthate in sink tissues is of great importance to

agriculture as in the case of cereals, the grains are the portion of the plant which is harvested.

2.2.3 Photosynthate Transport from Source to Sink

The vascular network in leaves is very extensive which enables efficient transport of sucrose from mesophyll to phloem cells (Gifford *et al.* 1984). Phloem is the portion of the vascular system responsible for carbohydrate transport throughout the plant. Two main cell types are found within the phloem: sieve elements and companion cells. Sieve elements are located adjacent to one another and form sieve tubes which run throughout the plant. At maturity, sieve elements lack many elements of the protoplast, including a nucleus and ribosomes. All remaining elements are distributed along the wall, providing an unobstructed pathway for water and dissolved substance movement. Sieve elements are associated with companion cells, which are specialized parenchyma cells containing all components commonly found in living plant cells. Companion cells and sieve elements have many cytoplasmic connections, enabling companion cells to deliver substances such as proteins and ATP to sieve elements (Raven PH *et al.* 2004).

Sucrose moves symplastically from either its site of synthesis or of storage in vegetative tissue through the mesophyll layer to cells that are close to the phloem companion cells (Schnyder 1993). Seventy to ninety percent of the photosynthate incorporated into grain yield is produced after anthesis, with the flag leaf blade and the spike likely contributing the greatest quantity (Simmons 1987). There is evidence to suggest that all photosynthetic organs contain one or more diurnal carbohydrate pools. Long-term carbohydrate reserves in vegetative tissues are often in the form of fructan.

The accumulation of fructan does not compete with grain filling as the fructan pool receives only excess photosynthate. In times when photosynthate production is limited, the pool provides carbohydrate to the developing grains (Schnyder 1993).

In some species, such as cucumber (*Cucumis sativus* L.) and maize (*Zea mays* L.), sucrose may move into the phloem symplastically through plasmodesmata, but this is only true for species that have a high degree of connectivity between mesophyll cells and the sieve element-companion cell (SE-CC) complex (Wardlaw 1990; Riesmeier *et al.* 1994; Truernit 2001). Plasmodesmata are narrow channels, lined by plasma membrane, that join neighbouring cells and provide a pathway for transport of sucrose and other substances (Raven PH *et al.* 2004). Other species, such as potato (*Solanum tuberosum* L.), have limited symplastic connectivity due to the presence of only a limited number of plasmodesmata between their mesophyll and SE-CC complex, and therefore an apoplastic method of transport is assumed (Riesmeier *et al.* 1994). A facilitated efflux mechanism transfers sucrose from the mesophyll cells into an apoplastic solution which has a low concentration of sucrose. Sucrose transport into the phloem is carrier mediated and results in a high concentration of sucrose in the sieve element symplast. In species which exhibit apoplastic phloem loading it is thought that the loading process is tightly regulated, since the rate of photosynthate production varies with environmental conditions and the strength of sink demand varies with developmental stages (Weise *et al.* 2000).

Sieve elements form a continuous path throughout the plant, and due to the absence of one-way valves, sucrose is capable of moving in any direction (Gifford *et al.* 1984; Patrick and Offler 2001). In the sieve elements, sucrose moves by bulk flow under the influence of a hydrostatic pressure gradient, with this gradient being maintained by

regulated loading in source regions and regulated unloading in sink regions (Gifford *et al.* 1984). Sucrose transport through the phloem requires retrieval mechanisms that operate along the pathway to recover any sucrose that has leaked out (Weise *et al.* 2000).

The process of phloem unloading varies among different species and even among different organs within one plant. Young developing leaves likely experience symplastic phloem unloading (Riesmeier *et al.* 1993). In *Vicia faba* L. seeds, solutes move symplastically out of the phloem (Offler and Patrick 1993). Simple plasmodesmata are found in the post-sieve element path in maternal seed tissues and these plasmodesmata could serve as valves which limit the volume flow of phloem sap unloading from the sieve elements (Patrick and Offler 2001). Alternatively, cereal grains have no vascular connections to the parent plant so an apoplastic transport mechanism is used to move sucrose from parental vascular tissue to the endosperm (Riesmeier *et al.* 1993; Weschke *et al.* 2000; Emes *et al.* 2003).

2.2.4 Photosynthate-Based Approach to Increasing Crop Yield

Worldwide crop production is under significant stress due to an ever increasing human population, an increase in using crops for applications other than food, such as fuel ethanol, and a variety of environmental constraints (Rothstein 2007). The quantity and quality of prime agricultural land is decreasing and there is no large land bank available to be used for agricultural crops. To increase crop production, it seems necessary to increase crop yield. Most of plants' dry weight consists of carbon compounds, so an increase in yield is linked with changes in the rate of photosynthesis,

the subsequent partitioning of photosynthate between harvested and non-harvested portions of the crop plant, as well as the rate of respiration (Gifford *et al.* 1984).

Increased sucrose transport could also result in increased crop yield as more sucrose transported to the grain could result in more starch accumulation in the grain. The transport of sucrose from source to sink tissues, as well as the distribution of photosynthate among various parts of the plant, are major determinants for plant productivity and crop yield (Gifford *et al.* 1984; Weise *et al.* 2000). Transgenic pea (*Pisum sativum* L.) plants expressing *StSUT1* from potato in their cotyledons showed increased sucrose influx into the cotyledons and a corresponding increase in growth rate (Rosche *et al.* 2002). Cotyledons play an important role during seed development as their dermal cells retrieve sucrose released from maternal seed coats into the seed apoplasm. In a study which transformed potato with the spinach (*Spinacia oleracea* L.) sucrose transporter *SoSUT1*, plasma-membrane vesicles isolated from these plants exhibited higher rates of sucrose uptake than those isolated from wild-type tissue (Leggewie *et al.* 2003). These plants also had reduced levels of sucrose in their leaves with an unaltered photosynthetic rate, suggesting that more sucrose was transported out of the leaves. Although Rosche et *al.* (2002) observed increased seed growth rates when pea cotyledons expressed StSUT1, the final dry weight of the seed was unaffected, indicating that there are more processes influencing seed biomass than just photosynthate transport.

2.2.5 Sucrose Transporters

2.2.5.1 Sucrose Transporter Structure

All disaccharide transporters identified to date belong to the same gene family, known as the glycoside-pentoside-hexuronide cation symporters family, which is a member of the major facilitator superfamily (Chang *et al.* 2004). The SUT gene family encodes highly hydrophobic proteins with twelve transmembrane-spanning helices, with the greatest sequence conservation among family members being seen in these transmembrane regions (Truernit 2001; Sun *et al.* 2008). Both the N- and C- termini are predicted to be cytoplasmic (Lemoine 2000). There is sequence similarity between the first six transmembrane domains and the last six, suggesting that SUTs may have originated from an ancient gene duplication (Saier 2000). A conserved histidine residue, first identified at position 65 of AtSUC1 from *Arabidopsis thaliana* (L.) Heynh., is present in all functional SUT proteins that have been isolated from plants thus far, and this residue is believed to be involved in sucrose binding during transport (Lu and Bush 1998).

2.2.5.2. Various Sucrose Transporter Functions

Sieve element-companion cells complexes in the phloem of some plant species are believed to be symplasmically restricted, and the presence of SUTs in these locations suggest they have a role in apoplasmic phloem loading (Aoki *et al.* 2004). Sucrose transporters couple the symport movement of sucrose and protons (H^+) across the plasma membrane. The observation that SUTs are energy-dependent and sensitive to protonophores provided evidence that they are sucrose/H^+ symporters (Schulze *et al.*

2000; Takeda *et al.* 2001; and others). Sucrose transport is driven by the transmembrane H^+ gradient generated from proton pumps (Truernit 2001). In regions believed to be the main sites of active sucrose transport to and from the seed apoplasm, a high density of both SUT proteins and proton pumps were observed (Harrington *et al.* 1997). Sucrose transporters are believed to be important for carbon partitioning and have roles in loading sucrose into the phloem in source leaves as well as moving sucrose into storage tissues, such as developing grains, after phloem transport has occurred (Aoki *et al.* 2003). Transformation of potato with antisense *SUT1* provided evidence to show the important role that SUTs play in phloem loading. The transformants had an 100-fold increase in hexoses and a five- to ten-fold increase in sucrose and starch in mature leaves (Riesmeier *et al.* 1994). The root system and tuber yield, which are dependent on sucrose transported from the leaves, were greatly reduced.

Some SUTs appear to play an indirect role in sucrose transport. Sucrose transporters expressed in storage parenchyma cells appear to have a primary role in scavenging sucrose that had leaked out of the symplastic pathway from dermal cells to storage parenchyma cells (Rosche *et al.* 2002). The presence of TaSUT1 in the phloem of internodes, within which sucrose moves symplasmically, suggests that these transporters may retrieve sucrose which has leaked into the apoplasm (Aoki *et al.* 2004).

Not all SUTs have a primary function of sucrose transport. Many of the plants studied thus far contain multiple sucrose transporters, which are orthologous genes but may have evolved to have different functions (Eckardt 2003). In barley (*Hordeum vulgare* L.), *HvSUT2* was found to be expressed in all sink and source tissues studied and is believed to have a housekeeping role (Weschke *et al.* 2000). A unique subgroup of

SUTs, containing the sucrose transporter *AtSUC3/AtSUT2* from *Arabidopsis*, is suggested to have sensory functions, measuring sucrose via conformation changes during transport (Schulze *et al.* 2000). The ability to measure transport rates provides information on sucrose and H^+ concentrations as well as transmembrane gradients. In tomato (*Lycopersicon esculentum* Mill.), *LeSUT2* was shown to co-localize with *LeSUT1* and *LeSUT4*, which are directly involved in sucrose transport, and may regulate the relative activity of sucrose transport into sieve elements (Barker *et al.* 2000). It has been noted that the cytoplasmic domains of SUT2 proteins are extended in comparison with other SUTs and these domains may be involved in effector binding or signal transduction (Barker *et al.* 2000).

2.2.5.3 Mechanism of Transport by Sucrose Transporters

The molar ratio for the transport of sucrose and H^+ by StSUT1 in potato is 1:1 (Boorer *et al.* 1996). Protons are believed to behave as activators and bind to StSUT1 prior to sucrose. When membranes were hyperpolarized, the affinity of StSUT1 for sucrose and H^+ increased. This indicated that StSUT1 is negatively charged and that negative voltage encourages H^+ to bind to their binding site. A model that has been proposed for sucrose/H^+ transport suggests that one H^+ first binds to the external face of the transporter, followed by the binding of one sucrose molecule. The transporter then undergoes a conformational change, releasing the ligands at the cytoplasmic surface. The ligand-binding site returns to the external surface where it is available to bind with another H^+ (Boorer *et al.* 1996). Although this model appears to fit for the activity of

StSUT1, it cannot be applied in all cases. For example, in sugar beets (*Beta vulgaris* L.) the SUT carries a positive charge and sucrose binds prior to the H^+ (Buckhout 1994).

2.2.5.4 Variation in Sucrose Transporters' Affinity for Sucrose

The range of affinities for sucrose exhibited by SUTs is quite broad, which allows plants with multiple SUTs to differentially express certain SUTs depending on extracellular sucrose concentrations and uptake needs (Schulze *et al.* 2000). In potato, StSUT1 has a high affinity for sucrose with a K_M value of approximately 1.0 mM at pH 4.5 and is believed to be involved in phloem loading (Riesmeier *et al.* 1993). In *Plantago major* L., PmSUC3, which is believed to be involved in sucrose retrieval, has a K_M value of 5 mM. The relatively low affinity for sucrose of this SUT may be important in making sucrose retrieval less efficient, allowing some of the leaked sucrose to remain available for lateral sinks (Barth *et al.* 2003). In terms of protein structure, the N-terminus of SUTs appears to highly contribute to differences in substrate affinity (Schulze *et al.* 2000). Since the N-terminus is predicted to be cytosolic, it is unlikely that it binds sucrose directly but instead may influence affinity through intramolecular interactions with other domains in the cytosol.

Some SUTs have an affinity for substances other than sucrose. Both spinach and potato SUT1 were shown to take up maltose but with a different affinity than that reported for sucrose (Riesmeier *et al.* 1993). It appears that the glucose moiety is essential for transport by SUT1 but the fructose moiety found in sucrose can be exchanged with various residues, such as with a glucose moiety as found in maltose. Maltose is believed to be a major product of starch hydrolysis and is therefore one of the

main sugars found in the endosperm during stages with rapid starch degradation. The presence of SUTs in the ground tissue of the scutellum suggests they may have a function in maltose retrieval from the endosperm (Aoki *et al.* 2006).

2.2.5.5 Regulation of Sucrose Transporters by Sucrose

Sucrose transport by SUTs was shown to decrease by 50-65% in sugar beet leaves incubated in 100 mM sucrose compared to water controls (Chiou and Bush 1998). This decrease in activity was not a result of decreased plasma membrane integrity, nor due to an osmotic effect. Although the K_M for sucrose was unaffected by treatment with sucrose, the V_{max} declined in a concentration-dependent fashion, suggesting the presence of fewer symporters in the membrane. These results imply that treatment with sucrose led to changes in transcriptional activity or mRNA turnover rate. The effect of the sucrose treatment was shown to be reversible, indicating that the sucrose signalling pathway is able to respond to dynamic changes in sucrose concentration, and therefore likely has an important role in regulating phloem loading.

Chiou and Bush (1998) have developed an hypothesis to describe how the sucrose-dependent transduction pathway may moderate sucrose allocation. When sink demand is low, the amount of sucrose in the phloem is high. Less sucrose will be loaded into the phloem, resulting in higher sucrose levels in the mesophyll. The high sucrose levels in the phloem down-regulate symporter activity, while the high sucrose levels in the mesophyll down-regulate photosynthetic activity. Alternatively, when sink demand is high, sucrose levels in the phloem will be low, which up-regulates transporter activity and increases the capacity for phloem loading.

2.2.5.6 Cereal Sucrose Transporters Form a Phylogenetic Group

Sucrose transporter genes have been isolated from many dicotyledonous and monocotyledonous species and phylogenetic analysis of deduced amino acid sequences have suggested SUTs are separated into at least five groups: cereal-SUT1, cereal-SUT2, dicot-SUT1, dicot-SUT2, and dicot-SUT4 (Aoki *et al.* 2003). Analysis of cereal SUT1 genes have shown greater than 80% identity in the primary nucleotide sequences of rice (*Oryza sativa* L.), maize, barley and wheat SUT1s (Aoki *et al.* 2003). Correspondingly, the protein sequences are also highly similar, being 86-97% similar among SUT homologues from maize, rice and barley (Aoki *et al.* 2002).

Triticum aestivum, with the genomic designation AABBDD, has three SUT1s, *TaSUT1A, 1B* and *1D*, each located on chromosome four of their respective genome. These genes are more than 96% identical at the cDNA level and more than 98% identical at the amino acid level (Aoki *et al.* 2002). Expression levels of all three genes vary among different tissues, but in any tissues, each of the three genes are expressed equally relative to one another (Aoki *et al.* 2002; Aoki *et al.* 2004). Recently, a putative SUT2 has been identified in wheat (submitted to GenBank by Liu *et al.*, May 2008).

2.2.5.7 Yeast Complementation Test: a Tool Used to Study Sucrose Transporters

Wild-type *Saccharomyces cerevisiae* (Meyen) is able to grow on sucrose due to the activity of a secreted invertase, which breaks sucrose into monosaccharides that are taken up by hexose transport systems (Riesmeier *et al.* 1992). By knocking out the invertase gene and inserting a sucrose synthase gene, the yeast strain SUSY7 was developed, which is able to metabolize internal but not external sucrose. SUSY7 was later

modified by deletion of the *URA3* gene, rendering it unable to produce uracil (Barker *et al.* 2000). Vectors which express *ura3* can be transformed into this yeast strain, designated SUSY7/*ura3*, and uracil production is then used as a selection marker for positive transformation. Insertion of a functional SUT into SUSY7/*ura3* provides a mechanism for taking up sucrose into the yeast, enabling the yeast to grow on media with sucrose as the sole carbon source (Riesmeier *et al.* 1992).

Another mutant yeast strain, SEY6210, has proven to be useful for SUT uptake experiments (Schulze *et al.* 2000). This mutant is also lacking invertase activity and is therefore unable to hydrolyze internal sucrose. After transformation with a functional SUT, sucrose is taken up and accumulates in SEY6210 cells. Using radiolabelled sucrose enables quantitative measurements of sucrose uptake by this yeast strain.

2.2.6 Brittle1 is an Additional Sugar Transporter Required for Starch Biosynthesis

Sucrose transport towards sink tissues is critical for starch production to occur within these organs, but in addition to the requirement of transporter proteins for sucrose translocation, transporter proteins for ADP-glucose are also needed. ADP-glucose, which is made from sucrose, is the basic monosaccharide unit of starch and the production of this metabolite is a key factor influencing the rate of starch synthesis (Emes *et al.* 2003). In cereals, ADP-glucose is a product of the phosphorylation reaction mediated by cytosolic ADP-glucose pyrophosphorylase (AGPase) (Bowsher *et al.* 2007). In maize, ZmBT1 is a carrier that mediates the transfer of ADP-glucose from the cytosol into the amyloplast (Shannon *et al.* 1998). *In vitro*, ADP-glucose transport could be achieved with the co-transport of either ADP or AMP, with the transporter having a similar affinity for

both substrates. Under physiological conditions, it is likely that ADP-glucose is exchanged with ADP since high concentrations of ADP are to be expected as it is a by-product of the starch synthase reaction involved in starch biosynthesis in the plastid (Bowsher *et al.* 2007; Kirchberger *et al.* 2007). Amino acid analysis of ZmBT1 revealed the presence of a putative ADP-glucose-binding motif likely important for ADP-glucose transport (Shannon *et al.* 1998). The affinity of ZmBT1 for ADP-glucose is relatively low but is sufficient for effective transport due to high concentrations of ADP-glucose in the cytosol of the maize endosperm as a result of the highly active cytosolic AGPase (Kirchberger *et al.* 2007).

Homologues of *BT1* have been found in other plants, such as *Arabidopsis* and potato. In non-cereal plants, ADP-glucose is not imported into storage plastids because it is already produced there via plastidic AGPase, so BT1 must have a different role than it has in maize (Kirchberger *et al.* 2007). Further analysis of various BT1 proteins suggests there are two distinct groups: one group acts as ADP-glucose carriers, and the other as AMP/ADP/ATP carriers. Maize was shown to have two *BT1* homologues. The homologue involved in ADP-glucose transport, *ZmBT1*, is expressed exclusively in endosperm tissues during starch synthesis, whereas the homologue involved in AMP/ADP/ATP transporter, *ZmBT1-2*, is expressed ubiquitously in both heterotrophic and autotrophic tissues.

2.3 Cultivation of Hexaploid Wheat in Western Canada

2.3.1 Origins of Cultivated Hexaploid Wheat and of Wheat Production in Western Canada

The origin of the wild progenitors of cultivated wheat is believed to be the Fertile Crescent in the Middle East (Feldman 2001). As stated previously, hexaploid wheat has the genomic designation AABBDD. It is widely accepted that the A genome originated from *Triticum monococcum* L. and the D genome from *T. tauschii* (Coss.) Schmal. (also known as *Aegilops squarrosa* L.). The origin of the B genome is not as well known and potential candidates include *T. urartu* Thumanian ex Gandilyn, *T. speltoides* (Tausch) Gren. Ex K Richt., *T. longissimum* (Schweinf. & Muschl.) Bowden and *T. searsii* (Feldman & Kislev ex K. Hammer) Feldman (Kimber and Sears 1987). The creation of hexaploid wheat occurred in multiple steps. First, tetraploidy resulted from chromosome doubling of crosses made between diploids. These tetraploids then crossed with another diploid, and after chromosome doubling, these hybrids evolved into a hexaploid species. All three genomes in hexaploid wheat are genetically related as shown by each chromosome having a homolog in each of the other two genomes.

The origin of wheat production in western Canada is credited to the settlers who came from Scotland in 1812 and established the Red River Settlement in Manitoba. The arrival of Mennonites in southern Manitoba in 1875 increased wheat production in Manitoba as well as increased wheat diversity as they brought with them many cultivars from their homeland, Russia. In 1877, the first shipment of wheat was sent directly from Canada to Britain. From this point onwards, wheat production on the prairies increased at

a rapid rate, rising from 235,000 hectares planted in 1885 to 1,675,000 hectares planted in 1905 (DePauw and Hunt 2001).

2.3.2 Recent Progress in Wheat Production in Canada

In 2005, Canada was the sixth greatest producer of wheat worldwide (Canadian Wheat Board 2007). The majority of wheat produced in Canada is in the western provinces, with approximately 20,393,000 acres of land in western Canada devoted to wheat production in 2007, comprising 40% of the land in western Canada that was devoted to principal grains (Canadian Wheat Board 2007). The total production of wheat in the western provinces in 2007 reached 18,395,000 tonnes, which was approximately 92% of the wheat produced nationwide. From 1991-2001, the distribution of wheat grown nationwide was approximately 80% spring wheat, 15-20% durum, and 3-4% winter wheat, with most winter wheat production occurring in the eastern provinces (Canadian Food Inspection Agency 1999; DePauw and Hunt 2001).

Research and development of winter wheat varieties is ongoing. Some desirable features that are associated with new varieties of winter wheat include improved cold hardiness, higher yields, and reduced input costs (Canadian Wheat Board 2006). Due at least in part to these desirable characteristics, the acreage of winter wheat grown across the prairies is increasing. The winter wheat cultivar McClintock was selected for this study due to its high yield potential, excellent stem and leaf rust resistance, and its adaptation to the winter wheat production area of western Canada (Anita Brûlé-Babel, personal communication).

2.4 Conclusion

Wheat is an agronomically important crop known to produce great quantities of carbohydrate for both food and industrial applications. Sucrose transporters play an important role in carbohydrate accumulation in harvestable sinks and it is therefore desirable to understand them in more detail. This study focused on *TaSUT1* in winter wheat, specifically its gene expression and protein function. The main objectives of this research were to study the expression of *TaSUT1* during grain maturation, to see the impact that high sugar concentrations have on *TaSUT1* expression, and to study the ability of TaSUT1 protein to transport sucrose.

3.0 MATERIALS AND METHODS

3.1 Plant Growth Conditions and Tissue Collection

Wheat plants (*Triticum aestivum* cv. McClintock) were grown under two different conditions: in the experimental field plots of The Point or in the Crop Technology Centre's greenhouse, both located at the University of Manitoba. Seeds were provided by Dr. Anita Brûlé-Babel's wheat breeding program. Tissue collected from the field was used for expression profiling and cloning of *TaSUT1,* whereas greenhouse grown plants were used for studying the impact of sugar treatments on *TaSUT1* expression. Tissue used for *TaSUT1* expression profiling was collected from the head, peduncle and flag leaf of one tiller during the grain maturation stage, with collection dates ranging from June 22 to July 28, 2007, corresponding to thirty-five to zero days, respectively, prior to fully hardened kernels. After being removed from the plant, tissue was immediately frozen in liquid nitrogen and then stored at -80 °C until further analysis.

To grow plants in the greenhouse, wheat seeds stored at 4 °C were imbibed in darkness for four days on moist filter paper. The seeds were then planted in professional growing mix (Sunshine, LG3 mix) and watered every second day. Greenhouse conditions were 20 °C/16 °C, 17/7 hr day/night.

3.1.1 Treatment of Wheat Leaves with Various Sugars

Leaves were harvested from approximately six week old plants grown in the greenhouse, cut into 1 cm^2 sections and placed into Petri dishes containing one of the following treatments: 0, 25, 50, 75, 100 or 250 mM D-mannitol (Fisher, M-120-3), D-(+)-glucose (Sigma, G7528) or sucrose (Fisher, ICN19401891), with 5 μM of ethylene

diamine tetraacetic acid (EDTA) (Fisher, BP118-500) to prevent callose formation. Cut leaves were submerged in the solution, and then the Petri dishes were sealed and left in the greenhouse for 24 hrs. Each solution received multiple sections from multiple leaves to compensate for variation among leaves. Following this incubation period, the leaves were removed from the solutions, quickly patted dry with paper towel, placed into individual collection tubes, and immediately frozen in liquid nitrogen. Tissue was then stored at -80 °C until further analysis.

3.2 RNA Extraction from Wheat Tissue

RNA was extracted from wheat as described by Krapp et al. (1993), with some modifications. In brief, frozen tissue was ground to a fine powder in a pre-chilled mortar and homogenized with 7.5 mL of guanidine RNA extraction buffer per gram of tissue. The extraction buffer consisted of 8 M guanidine-hydrochloride (Fisher, BP178), 20 mM 2-(4-morpholino)-ethane sulfonic acid (Fisher, BP300-100), 50 mM EDTA, pH 7.0 and 50 mM 2-mercaptoethanol (Fisher, PI35602) added at the time of use. After vortexing for 10 min, one volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH 4.3 (Fisher, BP1754I), was added to the mixture, which was then briefly vortexed and then centrifuged at 3800 g for 10 min. The aqueous phase was transferred to a new tube and 0.1 volume of 3 M sodium acetate (Sigma, S7545) and 2.5 volume of 95% ethanol (Commercial Alcohols) were added. The mixture was centrifuged at 8000 g for 15 min at 4 °C, the supernatant removed and the pellet resuspended in 1 mL resuspension buffer (2 M lithium chloride (Sigma, 310468), 10 mM sodium acetate, pH 5.2). The solution was transferred to a new tube and incubated for 2 hrs at 4 °C. After incubation, the solution

was centrifuged at 13800 g for 15 min and the pellet briefly washed with 70% ethanol before being dried in a speed vacuum (Thermo Electron Corporation, Savant DNA 120 Speed Vac Concentrator). The pellet was resuspended in 50 μL of RNase free water and either used immediately or stored at -80 °C.

RNeasy® Plant Mini Kit (Qiagen, 74904) was used to isolate small quantities of high quality RNA used for semi-quantitative reverse transcriptase – polymerase chain reaction (RT-PCR). As described in the manufacturer's protocol, 450 μL guanidinium thiocyanate (Buffer RLT) containing 2-mercaptoethanol was added to 100 mg of frozen, powdered tissue. This mixture was transferred to a QIAshredder spin column and centrifuged at full speed for 2 min. The supernatant was transferred to a clean tube, and 0.5 volume of 100% ethanol was added, followed by immediate mixing. The mixture was then transferred to an RNeasy spin column, the column centrifuged at 8000 g for 15 sec and the flow-through discarded. To the column, 700 μL guanidinium thiocyanate and ethanol (Buffer RW1) was added followed by 500 μL 100% ethanol (Buffer RPE), with centrifugation at 8000 g for 15 sec and disposal of flow-through following each buffer addition. A final wash with 500 μL Buffer RPE was performed, followed by centrifugation at 8000 g for 2 min which ensured that no ethanol would be eluted with the RNA. The column was transferred to a new tube and RNA was eluted from the column by the addition of 30 μL RNase-free water and centrifugation at 8000 g for 1 min. RNA was stored at -80 °C until future use.

3.3 Cloning *TaSUT1* cDNA

3.3.1 Cloning *TaSUT1* into pGEM®-T Easy

Messenger RNA (mRNA) was isolated from total RNA using the Absolutely mRNA Purification Kit (Stratagene, 400806). RNA was quantified using an Ultrospec 3100 pro spectrophotometer (Biochrom). In brief, total RNA (approximately 1 μg/μL in RNase-free water) was heated to 65 °C for 3 min, placed on ice temporarily, and then transferred to a tube containing oligo (dT) magnetic particles, which had been washed twice in hybridization buffer. The mixture was incubated at room temperature for 7 min with gentle agitation to allow mRNA to hybridize with the magnetic particles. The magnetic particle/mRNA complexes were collected with a magnetic stand and the supernatant removed. The mRNA-bound magnetic particles were washed four times with wash buffer, each time the particles being resuspended and then collected by the magnetic stand. Following the final wash and removal of wash buffer, Absolutely mRNA elution buffer was added and the particles incubated at room temperature for 5 min with gentle agitation. The magnetic particles were collected by the magnetic stand and the mRNA drawn off and stored at -80 °C until required for future analysis.

Complimentary DNA (cDNA) was synthesized with AccuScript® High Fidelity Reverse Transcriptase (Stratagene, 600089) following the manufacturer's protocol. In brief, 1X AccuScript buffer, 500 ng oligo(dT) primers, 10 mM deoxyribonucleotide triphosphate (dNTP) and 0.1-100 ng mRNA were mixed in a microcentrifuge tube and allowed to incubate at 65 °C for 5 min. The mixture was then cooled to room temperature, allowing the oligo (dT) primers to anneal to the RNA, and 100 mM

dithiothreitol (DTT) and 25 U AccuScript high fidelity reverse transcriptase were added. All components were mixed and incubated at 42 °C for 1 hr. The transcriptase reaction was inactivated at 70 °C for 15 min and the cDNA was placed either on ice for immediate use or at -20 °C for longer term storage.

TaSUT1 cDNA was amplified by PCR using a thermostable DNA polymerase (obtained from Dr. Genyi Li, Department of Plant Science, University of Manitoba, Winnipeg, MB, Canada). The reaction mixture consisted of 1X reaction buffer (10X stock containing 500 mM potassium chloride (Sigma, P5405), 100 mM Tris (Fisher, BP152), 1% Triton (Fisher, BP151), 15 mM magnesium chloride (Aldrich, 208337), pH 9.3, 0.2 mM dNTP mix (from a stock of 10 mM dNTP mix (BioRad, 170-8874)), 0.5 μM of each of TaSUT-D-F-Full-173 and TaSUT-D-R-Full-1835 primers (Table 3.1.), approximately 25 ng cDNA and 0.5 U DNA polymerase per 20 μL reaction. PCR amplification was carried out with a thermal cycler (MyCycler™, Bio-Rad) under the following conditions: initial denaturation at 94 °C for 90 sec, 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 105 sec, followed by a final extension at 72 °C for 10 min.

Amplified *TaSUT1* cDNA was mixed with 1X DNA loading buffer (0.126% (w/v) xylene cyanol FF (Fisher, BP565-10), 0.126% (w/v) bromophenol blue (Fisher, B-392-5), 6.25% (v/v) 10% sodium dodecyl sulphate (SDS; Fisher, BP166), 62.5% glycerol (Fisher, BP229-1)) (Sambrook and Russell 2001, with modification), and then loaded into a 1.4% (w/v) agarose (Fisher, BP1356-100) gel containing 1X TAE buffer (1 L of 50X stock contained 242 g Tris (Fisher BP152), 57.1 mL glacial acetic acid (Fisher, A-38), 100 mL of 0.5 M EDTA, pH 8.0) (Sambrook and Russell 2001) stained with ethidium

bromide (Fisher, BP1302-10). Gel electrophoresis was carried out in 1X TAE buffer at 140 V. Ultraviolet light emitted from a transilluminator (Fisher Scientific, FisherBiotech Transilluminators, FB-TIV-816A) was used to visualize the band corresponding to *TaSUT1* expected size, and the DNA was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, 28704). The DNA fragment was cut out of the gel and placed into a microcentrifuge tube. Three volumes of guanidinium thiocyanate (Buffer QG) were added to 1 volume of gel, followed by incubation at 50 °C until the gel had dissolved. One gel volume of isopropanol was added and mixed in. The mixture was transferred to a QIAquick spin column which was centrifuged at full speed for 1 min to bind the DNA to the column. To remove any residual gel, 0.5 mL Buffer QG was added to the column followed by a wash with 0.75 mL 100% ethanol (Buffer PE) with centrifugation at full speed for 1 min between additions. The column was centrifuged for an additional min to remove any residual buffer and then transferred to a clean tube. Nuclease-free water was added to the centre of the QIAquick membrane within the column and the column was centrifuged at full speed for 1 min to elute the DNA, which was then stored at -20 °C until subsequent use.

TaSUT1 cDNA was cloned into the pGEM®-T Easy vector (Promega, A1360) via a ligation reaction containing 1X ligation buffer, 50 ng pGEM®-T Easy vector, 75 ng *TaSUT1* insert (corresponding to a 3:1 insert:vector molar ratio) and 3 Weiss units of T4 DNA ligase, to a final reaction volume of 10 µL. The ligation reaction was incubated at 4 °C overnight and then either used directly to transform *E. coli* competent cells, or stored at -20 °C for future transformations.

3.3.2 Cloning *TaSUT1* into pDR196

TaSUT1 amplification was achieved by PCR using iProof DNA polymerase (BioRad, 172-5300). The source of DNA for the PCR mixture was *TaSUT1*-pGEM®-T Easy. The primers TaSUT-D-F-*SmaI*-link and TaSUT-D-R-*XhoI*-link (Table 3.1.) were used to amplify the full length *TaSUT1* cDNA and link sequences containing restriction enzyme recognition sites, specifically *SmaI* prior to the start of *TaSUT1* or *XhoI* after the end of *TaSUT1*. The reaction conditions for *TaSUT1* linked with *SmaI* and *XhoI* were as follows: 98 °C for 1 min, 35 cycles of 98 °C for 15 sec, 65 °C for 30 sec, 72 °C for 1 min, followed by 72 °C for 10 min. Following the addition of 1X DNA loading buffer the PCR product was electrophoresed and the DNA band corresponding to *TaSUT1* was excised, all as described previously.

In order to ligate *TaSUT1* into the plasmid pDR196 (provided by Dr. John Ward, Department of Plant Biology, University of Minnesota, St. Paul, MN, USA), both the *TaSUT1* cDNA and the plasmid were digested with FastDigest™ *SmaI* (Fermentas, #FD0663) and *XhoI* (Fermentas, #FD0694) restriction enzymes. The digestion reaction for the plasmid consisted of 1X Fast Digest buffer, up to 1 µg DNA, and 1 FastDigest® Unit each of *SmaI* and *XhoI*. The digestion reaction of the linked *TaSUT1* cDNA consisted of 1X Fast Digest buffer, approximately 200 ng cDNA, and 1 FastDigest® Unit of each of *SmaI* and *XhoI*. The reaction components were mixed gently and then incubated at 37 °C for 10 min. After incubation, the reaction was run on an agarose gel which effectively terminated the digestion reaction. Both *SmaI* and *XhoI* have only one cut site within the multiple cloning site of pDR196 and neither cut the open reading frame

of *TaSUT1*. Digestion of both the cDNA and the plasmid provides sites of sequence complementarity at which ligation can occur.

DNA loading buffer was added following digestion, and electrophoresis of both the digested insert and the digested plasmid was carried out, followed by DNA extraction, both as described previously. Ligation of the digested *TaSUT1* cDNA into the digested pDR196 plasmid consisted of 1X ligation buffer, 45 ng pDR196, 30 ng *TaSUT1* insert and 3 Weiss units of T4 DNA ligase, to a final reaction volume of 10 µL. The ligation reaction was incubated at 4 °C overnight and then either used directly to transform *E. coli* competent cells or stored at -20 °C for future transformations.

3.4 Transformation of DH5α with *TaSUT1*

TaSUT1-pGEM®-T Easy and *TaSUT1*-pDR196 were cloned into Subcloning Efficiency™ DH5α™ Competent *E. coli* Cells (Invitrogen, 18265-017). Fifty microlitres of DH5α cells, previously stored at -80 °C, were placed on ice for 20 min to thaw. At the same time, the *TaSUT1*-pGEM®-T Easy ligation reaction was removed from its incubation at 4 °C and placed at room temperature for 20 min. One to 10 ng of ligation reaction was added and gently mixed into the competent cells, which were then allowed to incubate for 30 min on ice. Cells were heat shocked at 37 °C for 20 sec and then immediately placed on ice, causing the cells to take in the transformed plasmids. Nine hundred and fifty microlitres of pre-warmed Luria-Bertani (LB) medium (10 g tryptone (Fisher, BP1421), 5 g yeast extract (Fisher, BP1422), 10 g NaCl (Fisher, BP358) per litre of media, pH 7.0) (Sambrook and Russell 2001) was added to the transformed cells. The tube was placed horizontally in an incubator shaker (New Brunswick Scientific, Excella

E24 Incubator Shaker Series) and shaken at 200 rpm at 37 °C for 1-2 hrs. Two hundred microlitres of cells were plated on LB plates containing 0.1 mg ampicillin (Sigma, A5354), 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Fermentas, R0391) and 0.08 mg X-gal (Promega V394) per 1 mL of LB. The presence of IPTG and X-gal in the media allowed for blue-white screening, with white colonies generally containing the insert. The plates were inverted and then incubated at 37 °C overnight.

3.4.1 Verification of *TaSUT1* Positive Clones

To confirm that white colonies grown on ampicillin/X-gal/IPTG-containing agar plates were positive for the full *TaSUT1* insert, three verification methods were followed. First, PCR verification was performed using the same reaction mixture, reaction conditions and gene-specific *TaSUTD* primers initially used for cDNA amplification, with the exception that the initial denaturation time was slightly increased to enable breakdown of the *E. coli* cells. An additional PCR reaction was carried out with all components the same as previously mentioned except different primers, designed to amplify only a partial length of *TaSUT1*, were used.

Second, plasmid digestion of the cloned TaSUT1-pGEM®-T Easy was carried out. Plasmids were isolated from transformed DH5α by the QIAprep Spin Miniprep Kit (Qiagen, 27104). In brief, transformed DH5α were centrifuged at 3800 g for 5 min and the supernatant removed. The pelleted cells were resuspended in 250 μL RNase A (Buffer P1), and then 250 μL SDS and sodium hydroxide (NaOH) (Buffer P2) were added, followed by 350 μL guanidinium chloride and acetic acid (Buffer N3), with thorough mixing after each step. The mixture was centrifuged at 16200 g for 10 min and

the supernatant containing the isolated plasmids was transferred to a QIAprep spin column. Following centrifugation at 16200 g for 60 sec, the column was first washed with 0.5 mL guanidinium chloride and isopropanol (Buffer PB) and then with 0.75 mL 100% ethanol (Buffer PE), with centrifugation at 16200 g for 60 sec and disposal of flow-through after each addition. After an additional 1 min centrifugation to remove any residual buffer, the column was transferred to a clean tube. To elute the DNA, 10 mM Tris-chloride (Buffer EB) was added to the centre of the column. Following incubation for 1 min at room temperature, the column was centrifuged at 16200 g for 1 min.

Plasmid digestion was carried out using FastDigest™ *EcoRI* (Fermentas, #FD0274). *EcoRI* has cut sites on either side of the multiple cloning region of the pGEM®-T Easy vector, thus allowing the removal of the cloned insert. The digestion reaction contained 1X FastDigest™ buffer, up to 1 μg DNA, and 1 FastDigest® Unit of *EcoRI*. The reaction was carried out as described for digestion by FastDigest™ *SmaI* and *XhoI*. Following digestion, the reaction mixture was run on agarose gel and the size of the digested fragment determined.

The third verification technique involved sequencing isolated plasmids, which was performed commercially by Macrogen Corporation (Maryland, USA) using plasmid specific M13 forward and reverse primers (Table 3.1.). Sequence results were compared with other cloned genes using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to determine genes with highest homology to the cloned insert.

3.5 *TaSUT1* Expression Studies

3.5.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Semi-quantitative RT-PCR was carried out with SuperScript™ One-Step RT-PCR Kit, containing SuperScript™ reverse transcriptase and Platinum® Taq DNA polymerase (Invitrogen, 10928-042), as described in the manufacturer's protocol. Fifteen nanograms of RNA were used per 25 μL reaction and the primer combinations used were TaSUT-F-Partial and TaSUT-R-Partial to amplify *TaSUT1* cDNA, and TaUBQ-F-Partial and TaUBQ-R-Partial to amplify *ubiquitin* cDNA (Table 3.1.). The supplied 2X reaction mix provided the buffer, magnesium, dNTPs and stabilizers required for the reaction. The RT-PCR reaction program was as follows: 50 °C for 30 min and then 94 °C for 2 min for initial cDNA synthesis and pre-denaturation, followed by 30 cycles of 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 105 sec for PCR amplification, and a final 72 °C for 10 min for final extension.

After the RT-PCR was completed, 1X DNA loading buffer was added to the RT-PCR reaction. Ten microlitres of this mixture was loaded into a 1.2% (w/v) agarose gel stained with ethidium bromide and the gel was run in 1X TAE buffer. Visualization of the bands in the gel was achieved using the BioRad Universal Hood II gel documentation system. The intensity of the bands was determined with BioRad's Quantity One® Software. For each data point, the intensity of the *SUT* band was standardized against the *ubiquitin* band to correct for any unequal loading. Expression analysis of leaves and peduncles included two biological replicates, one of which with three technical replicates. Expression analysis of head tissue included one biological replicate with three technical

replicates. For studying the impact of sugars on *TaSUT1* expression in leaves, experiments included three biological replicates, two of which included three technical replicates.

3.6 Preparation of Yeast Competent Cells

Two yeast (*Saccharomyces cerevisiae*) invertase-knockout mutant strains named SUSY7/*ura3* and SEY6210, provided by Dr. John Ward, were grown in 100 mL of yeast peptone dextrose (YPD) media (20 g peptone, 10 g yeast extract, 20 g glucose in 1 L distilled water; YPD Broth, Fisher, BP2469) shaking at 250 rpm at 30 °C until an optical density at 600 nm (OD_{600}) of 0.7 was reached. Cells were made competent according to Dohmen *et al.* (1991), with modification. In brief, cells were pelleted at 1370 g for 7 min and then the pellet was washed with 0.5 volume of Solution 1 (1.0 M sorbitol (Fisher, BP439), 3% poly ethylene glycol (PEG_{1000}, Fluka, 72393), 10 mM bicine-NaOH (Sigma, B3876 and S8263), pH 8.35), and then resuspended in 0.02 volume of the same solution. Competent cells were stored in aliquots of 200 µL at -80 °C.

3.7 Transformation of Yeast with *TaSUT1*-pDR196

Technical assistance for the transformation of SUSY7/*ura3* and SEY6210 was provided by Dr. Anke Reinders (Department of Plant Biology, University of Minnesota, St. Paul, MN, USA), based on a protocol by Dohmen *et al.* (1991), with minor modifications made. Approximately 0.1 to 5.0 µg of *TaSUT1*-pDR196 DNA was added to 200 µL of competent yeast cells. To this cell suspension, 1.2 mL of Solution 2 (33.35% PEG_{1000}, 0.166 M bicine-NaOH, pH 8.35) was added and the tubes were vortexed until

the contents were well mixed. The mixture was then left to incubate at 30 °C for 1 hr without shaking. After incubation, the cells were pelleted at 3000 g for 15 sec, and then resuspended in 1.0 mL of Solution 3 (0.15 M sodium chloride (Fisher, BP358), 10 mM bicine-NaOH, pH 8.35). Transformed SUSY7/*ura3* cells were grown at 30 °C on synthetic complete media with sucrose as the sole carbon source (SS), containing 1.7 g/L yeast nitrogen base without ammonium sulphate and amino acids (Sigma, Y1251), 5 g/L ammonium sulphate (Fisher, BP212R), 20 g/L sucrose, and 10 mL/L of each of 100X L-histidine (Fisher, BP382-100), L-leucine (Sigma, L1512), L-lysine (Fisher, BP386-100) and L-tryptophan (Fisher, BP395-100). Transformed SEY6210 cells were grown at 30 °C on synthetic complete medium with glucose as the sole carbon source (SD), which contained 1.7 g/L yeast nitrogen base without ammonium sulphate and amino acids, 5 g/L ammonium sulphate, 20 g/L glucose, and 10 mL/L of each of 100X histidine, leucine, lysine and tryptophan.

3.7.1 Verification of SUSY7/*ura3* and SEY6210 Positive Clones

Successful transformations of SUSY7/*ura3* and SEY6210 with *TaSUT1*-pDR196 were verified in multiple ways. First, yeast cells were grown on media lacking uracil, which is a pyrimidine derivative they require for survival. Both mutant yeast strains are unable to produce uracil, whereas pDR196 possesses URA3 which encodes for orotidine 5-phosphate decarboxylase, an enzyme involved in uracil synthesis. SUSY7/*ura3* and SEY6210 which have been successfully transformed with pDR196 will be able to survive on media lacking uracil. An additional verification approach used for SUSY7/*ura3* was to grow the cells on SS media. Untransformed SUSY7/*ura3* cells have no ability to

internalize sucrose and therefore are unable to grow on SS media. Transformation with a functional SUT provides a mechanism to transport sucrose into the yeast where, in the case of SUSY7/*ura3*, the sucrose is broken down by sucrose synthase into usable glucose. Verification by growth on SS media cannot be used for SEY6210 since this strain does not contain sucrose synthase.

The second verification technique was based on PCR amplification using transformed SUSY7/*ura3* and SEY6210 as the DNA templates. Genomic DNA was isolated from yeast according to Philippsen et al. (1991), with minor modification. In brief, 1.3 mL of overnight yeast culture was pelleted and then resuspended in 1 mL of 1.2 M sorbitol (Fisher, BP439) and 50 mM EDTA. Thirty-five microlitres of lyticase (12.25 U; Sigma, L4025) was added to the tube, which was then incubated at 30 °C for 30 min with gentle agitation. Cells were again pelleted and the supernatant discarded. The pellet was resuspended in 0.5 mL 50 mM EDTA, pH 8.5 and 0.2% SDS and then heated at 65-70 °C for 15 min. Fifty microlitres of 5 M potassium acetate (Fisher, BP364) was added to the cells and then the tube was placed on ice until the contents got thick and cloudy. Unwanted sediment was precipitated by centrifugation at full speed for 10 min, and then the supernatant was transferred to a clean tube. Approximately 1 mL of cold 95% ethanol was added to the supernatant, followed by centrifuging at full speed for 15 min at 4 °C. The pellet was washed with 1 mL of 70% ethanol, dried, and then resuspended in 10 µL TE buffer (100 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0).

Genomic DNA from both SUSY7/*ura3* and SEY6210 previously transformed with *TaSUT1*-pDR196 was then used to transform DH5α via the transformation reaction previously described. DH5α cells containing *TaSUT1*-pDR196 from the yeast were then

used in a PCR reaction with both full and partial *TaSUT1* primer combinations, as described in 3.4.1.

3.8 Sucrose Uptake Experiments with *TaSUT1*-Transformed Yeast

Sucrose uptake experiments were carried out as described by Weise et al. (2000) with some modifications as described below. Three technical replicates were performed for each assay.

3.8.1 Preparation of Yeast Cells for Assays

SEY6210 yeast cells previously transformed with pDR196-*TaSUT1* were grown in YPD media until an OD_{600} of 1.0, corresponding to approximately 10^7 cells, was reached. The cells were pelleted by centrifugation at 950 g for 5 min and then resuspended in 0.5 volume of 25 mM sodium phosphate buffer (NaH_2PO_4; Fisher BP329), pH 4.0. Cells were again centrifuged at 950 g for 5 min, resuspended in 0.05 volume of NaH_2PO_4 buffer, and then kept on ice until the assays were carried out. Yeast cells that had previously been transformed with pDR196-*StSUT1* or with empty pDR196 were also prepared and used as positive and negative controls, respectively.

3.8.2 Sucrose Uptake Assays

Fifty microlitres of SEY6210 transformed cells in NaH_2PO_4 buffer (pH 4.0) were transferred to a 1.5 mL tube and placed into a 30 °C heatblock. After 2 min of heating with gentle agitation, 5 µL of 120 mM glucose (final concentration 10 mM glucose) was added to energize the cells. The contents of the tube were mixed by pipetting and then

incubated at 30 °C for 2 min. Twenty five microlitres of ^{14}C-sucrose (PerkinElmer, NEC100X050UC) hot mix (final concentration 1 mM) was added to the cells, which were then mixed well and allowed to incubate at 30 °C for 5 min. The total ^{14}C-sucrose activity added to the cells was 0.05 μCi. The entire contents of the tube were placed on pre-moistened Whatman GF/A filter paper (25mm Ø Pall filters, Fisher, 09-874-12A) which was placed onto a low vacuum manifold. The cells were washed with 4 mL of ice-cold 10 mM sucrose. The filter paper and collected cells were placed into a scintillation vial and the activity of the ^{14}C-sucrose taken up by the cells was counted for 5 min.

To determine the relationship between sucrose uptake and cell number, the above protocol was slightly modified: the number of cells which were added to the filter paper ranged from 2 x 10^6 to 3 x 10^7. To determine the relationship between sucrose uptake and uptake assay time, a different modification was made to the above protocol: the final incubation at 30 °C ranged from 0.5 to 6.5 min.

To test the affinity of TaSUT1 sucrose transport, uptake assays were carried out as described above but with the minor modification that the concentration of ^{14}C-sucrose added ranged from 0.1 to 100 mM.

3.8.3 Determination of Sucrose/Proton Coupled Transport

To determine if sucrose transport was coupled with proton transport, two assays were carried out. First, the effect of pH on sucrose uptake was determined. The preparation of yeast cells for this assay required that the cells were washed and resuspended in NaH_2PO_4 buffer of the appropriate pH, which ranged from 3.0 to 7.0. The second study carried out was to see the effect that electron transport chain (ETC)

inhibitors antimycin A (Sigma, A8674), rotenone (Sigma, R8875) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma, C2759) had on sucrose uptake. The protocol described in 3.8.2 was followed with the minor modification of the addition of 25 μg Antimycin A per 80 μL reaction, 50 μM rotenone or 10 μM CCCP added simultaneously with ^{14}C-sucrose hot mix.

3.8.4 Determination of TaSUT1 Uptake Specificity for Sucrose

The determination of TaSUT1 specificity followed the protocol described in 3.8.2 with one modification. Simultaneous with the addition of 1 mM ^{14}C-sucrose hot mix was the addition of 10 mM of one of the following sugars: glucose, β-D-(-)fructose (Sigma F3510), sorbitol (Fisher, BP439-500), mannitol, D-(+)-maltose (Fisher, ICN19470180), D-(+)-galactose (Fisher, ICN1017471), D- + -raffinose (Fisher, ICN10279780), stachyose tetrahydrate (Fisher, AC22608-5000), or sucrose.

3.9. Statistical analysis

Two-sample *t* procedures were used to compare sample means. Statistical differences were based on a 95% confidence interval ($P \leq 0.05$).

Table 3.1. DNA primer sequences, intended uses, and anticipated amplicon sizes.

Name	Sequence	Intended Uses	Fragment size
TaSUT-F-Full-173	5'- CCGTAGAATTGATAGGCGAAC -3'	TaSUT1 full length amplification	1682 bp
TaSUT-R-Full-1835	5'- GCGAAAATTTGGTAAATGGC -3'		
TaSUT-F-*SmaI*-link	5'- CTCCCGGGATGGCGCGCGGC -3'	TaSUT1 insertion into pDR196	1572 bp
TaSUT-R-*XhoI*-link	5'- CTCGAGTCAGTGACCGCCGC -3'		
TaSUT-F-Partial	5'- TCGGCTTCTCGGCTGACAT -3'	RT-PCR	288 bp
TaSUT-R-Partial	5'- GGAACGGAAACCACTTGTGC -3'		
TaUBQ F-Partial	5'- AAGACCCTCACCGGCAAGA -3'	RT-PCR	280 bp
TaUBQ R-Partial	5'- GGATACCGGAGACACCGAGA -3'		
M13 forward	5'- GTAAAACGACGGCCAGT -3'	Sequencing insert in pGEM®-T Easy	N/A
M13 reverse	5'- GCGGATAACAATTTCACACAGG -3'		

4.0 RESULTS

4.1 A Full Length *TaSUT1* was Cloned into pGEM®-T Easy and pDR196

Full length *TaSUT1* cDNA was amplified by PCR and then cloned into pGEM®-T Easy. Multiple verification techniques were applied to confirm that cloning was successful. First, *TaSUT1*-pGEM®-T Easy was digested with the restriction enzyme *EcoRI*, which has recognition sites on both sides of the multiple cloning site, effectively removing the *TaSUT1* cDNA which had been inserted into the plasmid. When the digestion product was run on an agarose gel, a band of approximately 1700 base pairs (bp) was observed (Figure 4.1.1.). This band corresponds to the full length *TaSUT1* coding sequence, which is reported in GenBank to be between 1569-1572 bp, in addition to 110 nucleotides from the untranslated regions upstream and downstream of the *TaSUT1* coding sequence, due to the locations of the gene-specific primers, as well as approximately 18 nucleotides from pGEM®-T Easy, due to the location of the *EcoRI* recognition sites.

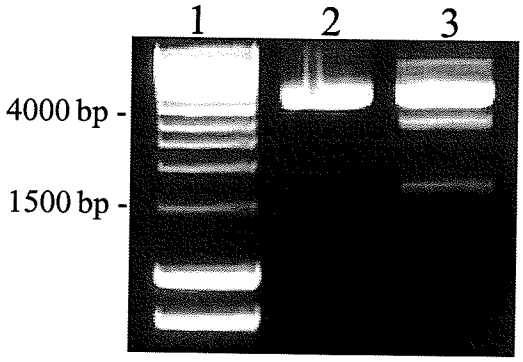

Figure 4.1.1. Digestion analysis of *TaSUT1*-pGEM®-T Easy with the restriction enzyme *EcoRI*, run on 1.2% agarose gel. Lane 1 contains the DNA marker HyperLadder I. Lane 2 shows undigested *TaSUT1*-pGEM®-T Easy. Lane 3 contains *EcoRI* digested *TaSUT1*-pGEM®-T Easy. The band of approximately 1700 bp corresponds to the full-length *TaSUT1* cDNA, in addition to 110 nucleotides from the untranslated region of *TaSUT1* and approximately 18 nucleotides from pGEM®-T Easy.

Verification was also carried out by performing PCRs using *TaSUT1*-pGEM®-T Easy as the template DNA and primer combinations designed to amplify either the full or partial length of *TaSUT1*. As shown in Figure 4.1.2., when the primer pair designed for partial length amplification (TaSUT-F-Partial and TaSUT-R-Partial) was used, the corresponding amplified band matched the anticipated length of approximately 300 bp. In addition, when the primer set designed for full length amplification (TaSUT-F-Full-173 and TaSUT-R-Full-1835) was used, a band of approximately 1600 bp, again corresponding to the anticipated length, was seen.

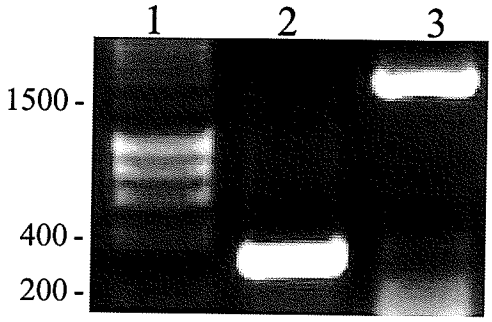

Figure 4.1.2. PCR amplification of *TaSUT1* from cloned *TaSUT1*-pGEM®-T Easy template. Two primer combinations were used for PCR amplification: TaSUT-F-Partial and TaSUT-R-Partial, corresponding to amplification of approximately 300 bp (lane 2) and TaSUT-F-Full-173 and TaSUT-R-Full-1835, corresponding to amplification of approximately 1600 bp (lane 3). HyperLadder I is shown in Lane 1.

To confirm that the cloned cDNA insert was *TaSUT1* and to be able to compare it with other available SUTs in GenBank, *TaSUT1*-pGEM®-T Easy was sequenced. The nucleotide sequence obtained was compared to other available SUT sequences using the BLAST feature on the NCBI website and the degree of homology between the sequenced insert and other SUT genes was determined. As shown in Table 4.1. and Appendix 1., the cloned insert had the highest sequence similarity with *TaSUT1B*, followed closely by

TaSUT1D and *TaSUT1A*, providing further verification that *TaSUT1* was successfully cloned.

Table 4.1. Sequence comparison results obtained from performing a nucleotide BLAST on the *TaSUT1* cDNA insert cloned into pGEM®-T Easy. Maximum score indicates the amount of homology, with a higher score corresponding to higher homology, between the cloned *TaSUT1* sequence and other sequences available on the NCBI website, each designated by their accession number. The expectation value (E value) indicates the significance of the alignment score, with a lower E value corresponding to a more significant score.

Accession	Description	Maximum Score	E Value
AF408843.1	*Triticum aestivum SUT1B*	2652	0.0
AF408844.1	*Triticum aestivum SUT1D*	2536	0.0
AF408842.1	*Triticum aestivum SUT1A*	2525	0.0
AM055812.1	*Hordeum vulgare SUT1*	2296	0.0
EU255258.1	*Lolium perenne SUT*	1777	0.0
NM_001111370.1	*Zea mays SUT1*	1301	0.0
AY780256.1	*Saccharum hybrid SUT1*	1290	0.0

Two of the previously mentioned verification techniques were also used to confirm successful cloning of *TaSUT1* cDNA into pDR196. PCRs carried out using primers designed to amplify either the full length (TaSUT1-F-*SmaI*-link and TaSUT1-R-*XhoI*-link) or partial length (TaSUT1- F-Partial and TaSUT-R-Partial) *TaSUT1* resulted in the anticipated band lengths of approximately 1600 bp and 300 bp, respectively (Figure 4.1.3.). The *TaSUT1* insert from *TaSUT1*-pDR196 was also sequenced and the results compared to other gene sequences via a nucleotide BLAST on NCBI. Once again, the highest sequence similarities to the *TaSUT1* insert were seen with publically available *TaSUT1* gene sequences (data not shown).

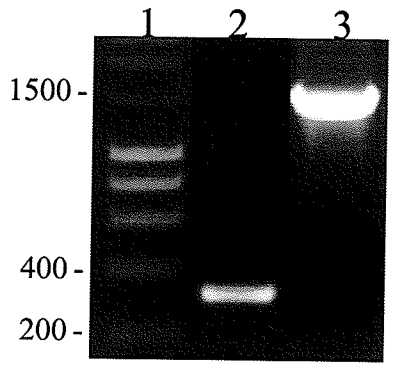

Figure 4.1.3. PCR amplification of *TaSUT1* from cloned *TaSUT1*-pDR196 template. Two primer combinations were used for PCR amplification: TaSUT-F-Partial and TaSUT-R-Partial, corresponding to a fragment of *TaSUT1* of approximately 300 bp (lane 2), and TaSUT-F-*Sma1*-link and TaSUT-R-*Xho1*-link, corresponding to full length *TaSUT1* which is approximately 1600 bp. HyperLadder I is shown in Lane 1.

4.2 *TaSUT1* is Expressed in Flag Leaves, Peduncles and Developing Grains Throughout the Grain Maturation Phase

Semi-quantitative RT-PCR was used to determine the expression level of *TaSUT1* in wheat flag leaves, peduncles and developing grains at different times during grain maturation. Following electrophoresis of the RT-PCR product, the intensities of the bands corresponding to *TaSUT1* were measured using Quantity One® software. The intensities of the *TaSUT1* bands were then standardized against the bands corresponding to *ubiquitin* to normalize for any variation in loading among the samples. A slight, but not significant, decrease in the expression of *TaSUT1* was observed in flag leaves from 28 to 11 days prior to the hard kernel stage (Figure 4.2.). Peduncles showed fluctuating levels of *TaSUT1* expression. In developing grains, *TaSUT1* expression increased slightly from 35 to 23 days prior to hard kernel stage and then slightly decreased at 17 days prior to hard kernel stage.

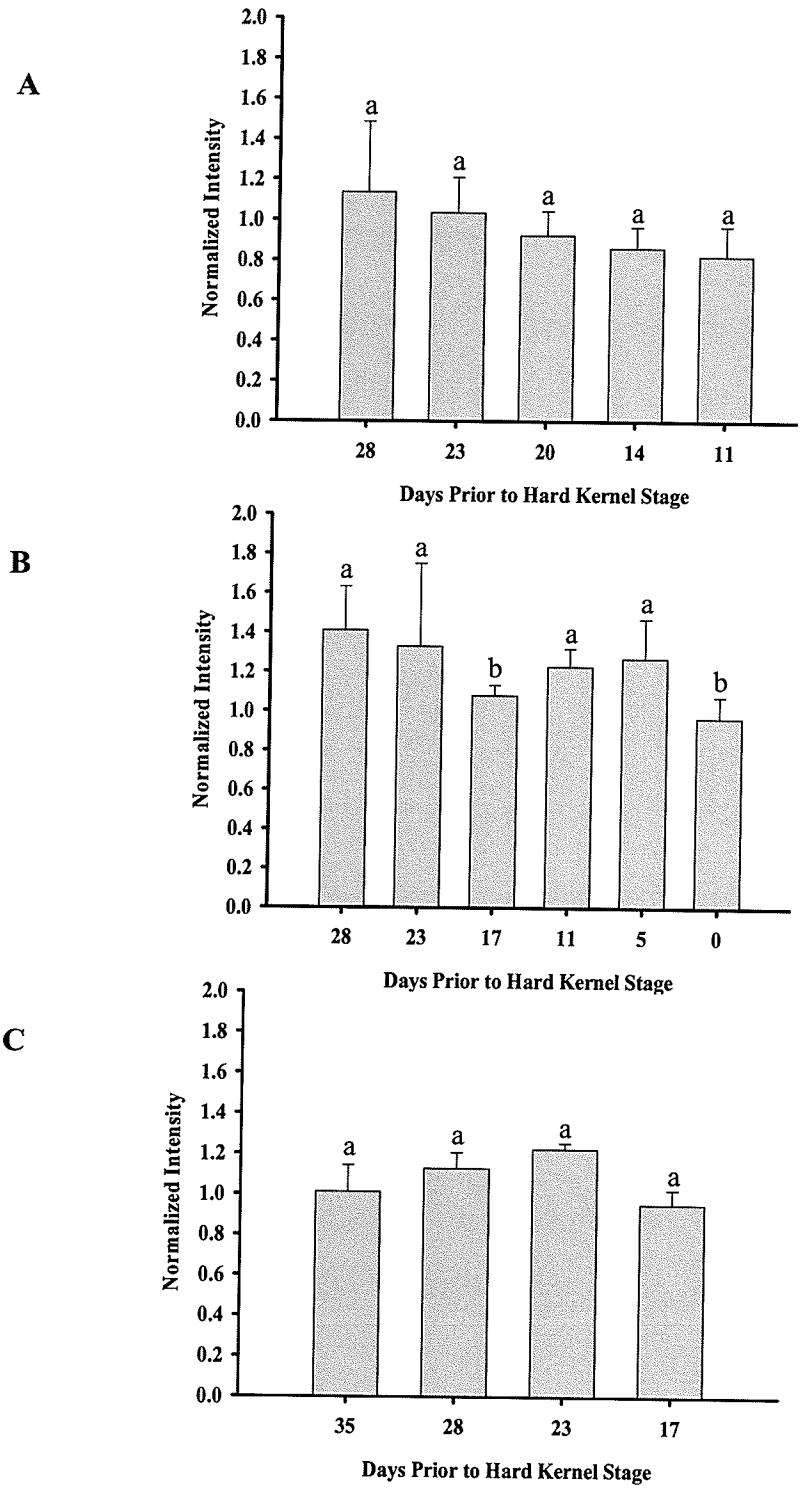

Figure 4.2. Expression of *TaSUT1* in (A) flag leaves, (B) peduncles, and (C) grains at increasing time points during the grain maturation phase. Normalized intensity corresponds to the intensity of *TaSUT1* amplification relative to that of *ubiquitin*. Bars represent the mean value with error bars representing standard deviation ($n \geq 3$). Bars labelled with different superscripts (a and b) are statistically different than the first time point measured for that tissue ($P \leq 0.05$).

4.3 High Concentrations of Glucose and Sucrose Slightly Decreased *TaSUT1* Expression in Leaves

Excised leaves were treated with mannitol, glucose or sucrose in concentrations ranging from 0 to 250 mM. Following a 24 hr incubation period, RNA was extracted and used in semi-quantitative RT-PCR analysis. The RT-PCR products were run on an agarose gel, and the intensities of the bands analyzed using Quantity One® software. The intensities of the *TaSUT1* bands were normalized with that of *ubiquitin* to correct for unequal loading. For all concentrations of mannitol, there were no statistical differences in *TaSUT1* expression and no obvious trend in expression levels (Figure 4.3.). Although the differences were not significant, there was a slight reduction in *TaSUT1* expression below that of the 0 mM control in all glucose treatments. Similarly, all concentrations of sucrose slightly reduced *TaSUT1* expression below that of the 0 mM control.

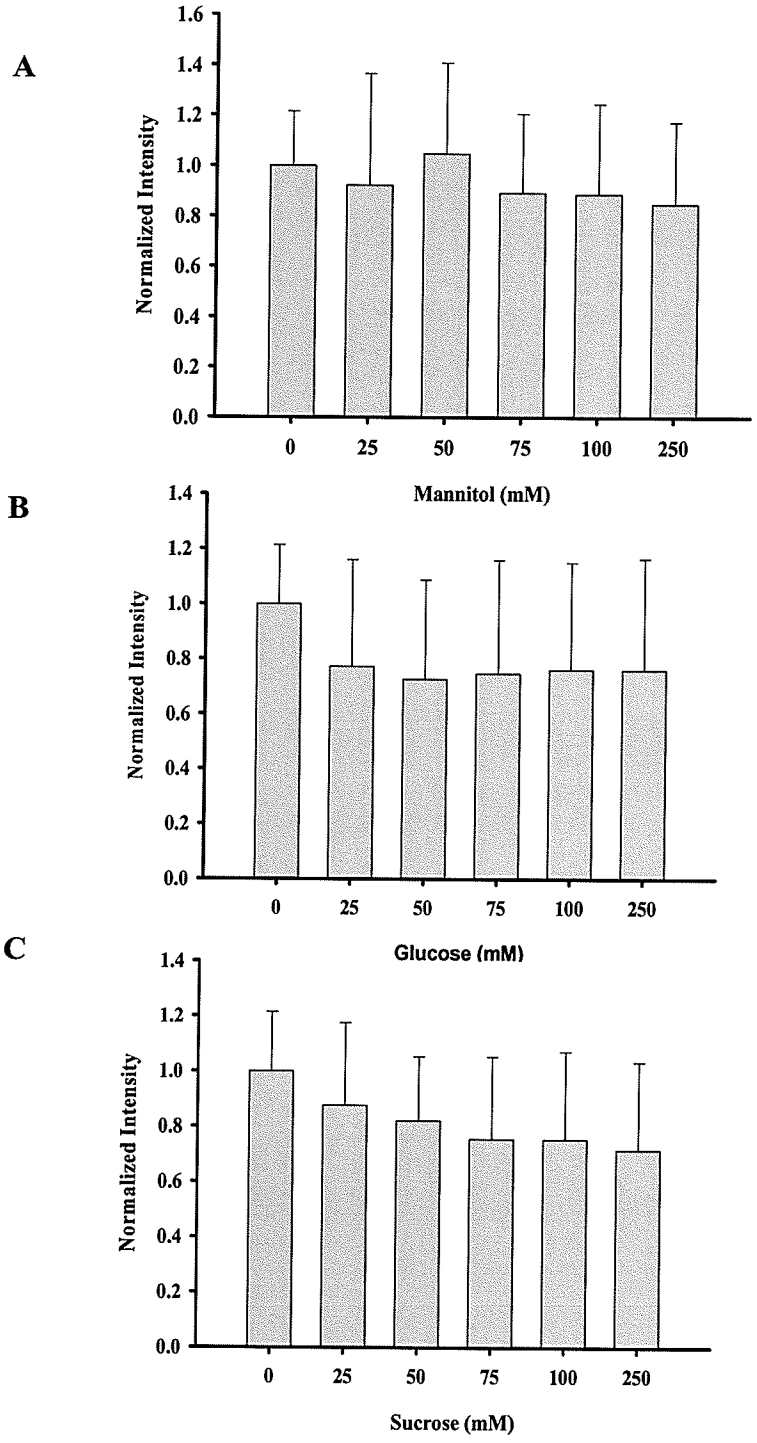

Figure 4.3. Expression of *TaSUT1* in leaves after treatment with (A) mannitol, (B) glucose, or (C) sucrose. Leaves were incubated in 0-250 mM sugar solutions for 24 hrs. Normalized intensity corresponds to the intensity of *TaSUT1* amplification relative to that of *ubiquitin*. All values represent intensity relative to that of the 0 mM control. Bars represent the mean value with error bars representing standard deviation (n = 7). No statistical differences were observed ($P \leq 0.05$).

4.4 *TaSUT1* was Successfully Cloned into SUSY7/*ura3* and SEY6210

PCR verification was used to confirm that SUSY7/*ura3* and SEY6210 were successfully transformed with *TaSUT1*-pDR196. Genomic DNA was isolated from the transformed yeast and cloned into *E. coli*. A PCR was performed with the transformed *E. coli* as the DNA template and the primer combination TaSUT1-F-*SmaI*-link and TaSUT1-R-*XhoI*-link. For *E. coli* containing DNA from either SUSY7/*ura3* or SEY6210, an amplification band of approximately 1600 bp was observed, corresponding to the full length *TaSUT1* which had previously been cloned into the yeast (Figure 4.4.).

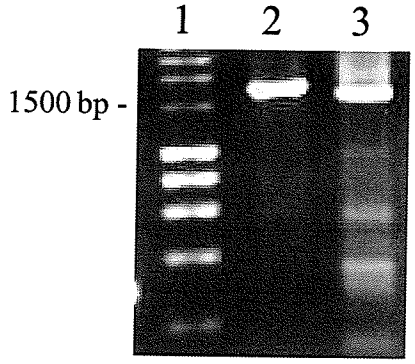

Figure 4.4. PCR verification of successful transformation of SUSY7/*ura3* and SEY6210 with *TaSUT1*-pDR196. Genomic DNA was isolated from yeast, transformed into *E. coli* and then the *E. coli* used in a PCR with the primer combination TaSUT-F-*Sma1*-link and TaSUT-R-*Xho1*-link. HyperLadder I is shown in lane 1. The band of approximately 1600 bp shown in lane 2 is full length *TaSUT1* amplified from SUSY7/*ura3*. The 1600 bp band in lane 3 is full length *TaSUT1* amplified from SEY6210.

4.5 TaSUT1 is Functional in Sucrose Transportation

The mutant yeast strain SUSY7/*ura3*, which has no ability to internalize sucrose, was used to determine if the cloned *TaSUT1* is capable of transporting sucrose. Following transformation with a functional SUT, sucrose will be transported into the yeast cell and be broken down by sucrose synthase into glucose which the yeast can metabolize. Transformation with a functional SUT will therefore enable SUSY7/*ura3* to survive on

SS media. Following infection of SS media with approximately 3 x 10^5 cells, the growth rates of SUSY7/*ura3* cells transformed with either TaSUT1-pDR196 or empty pDR196, as a control, were measured spectrophotometrically at 600 nm over a period of five days. SUSY7/*ura3* cells transformed with *TaSUT1*-pDR196 grow more rapidly than those cells transformed with empty pDR196, indicating that *TaSUT1* was functioning to transport sucrose into the yeast, thus providing a carbon source for growth (Figure 4.5.).

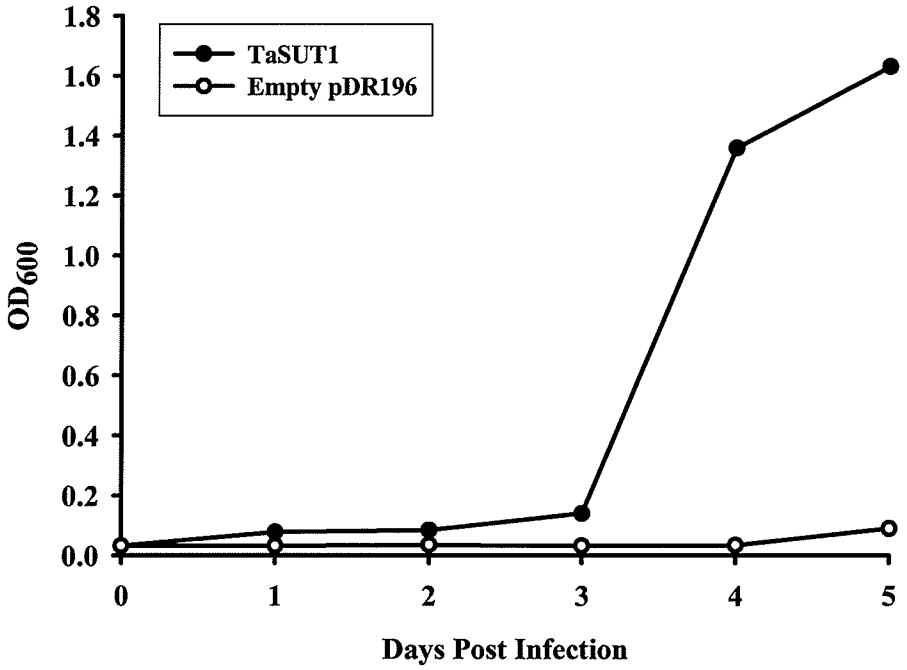

Figure 4.5. Growth of SUSY7/*ura3* transformed with either *TaSUT1*-pDR196 or empty pDR196 over five days following infection of SS media. Cell number was measured by taking the absorbance at 600 nm (OD_{600}). An OD_{600} of one is equivalent to approximately 10^7 cells/mL.

4.6 Sucrose Uptake is Positively Correlated with Cell Number and Uptake Time

The mutant yeast strain SEY6210 was transformed with a functional *TaSUT1* and the amount of radiolabelled sucrose taken into the yeast was measured by liquid scintillation counting. A positive linear correlation was observed between sucrose uptake

and cell number, as well as between sucrose uptake and uptake time (Figure 4.6.1. and 4.6.2., respectively).

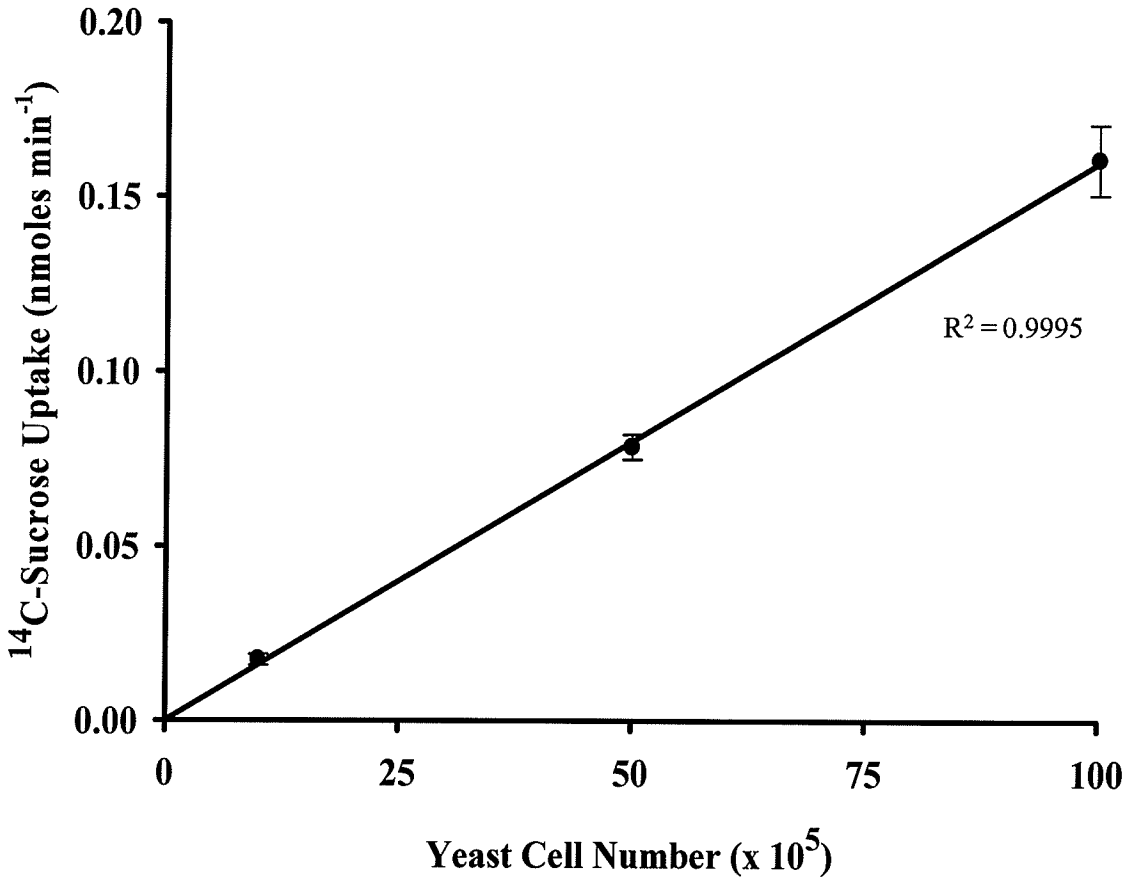

Figure 4.6.1. Uptake rate of ^{14}C-sucrose by SEY6210 yeast cells expressing *TaSUT1*. Assays were carried out using 1 mM ^{14}C-sucrose at pH 4.0. Error bars represent means +/- standard deviation (n = 3).

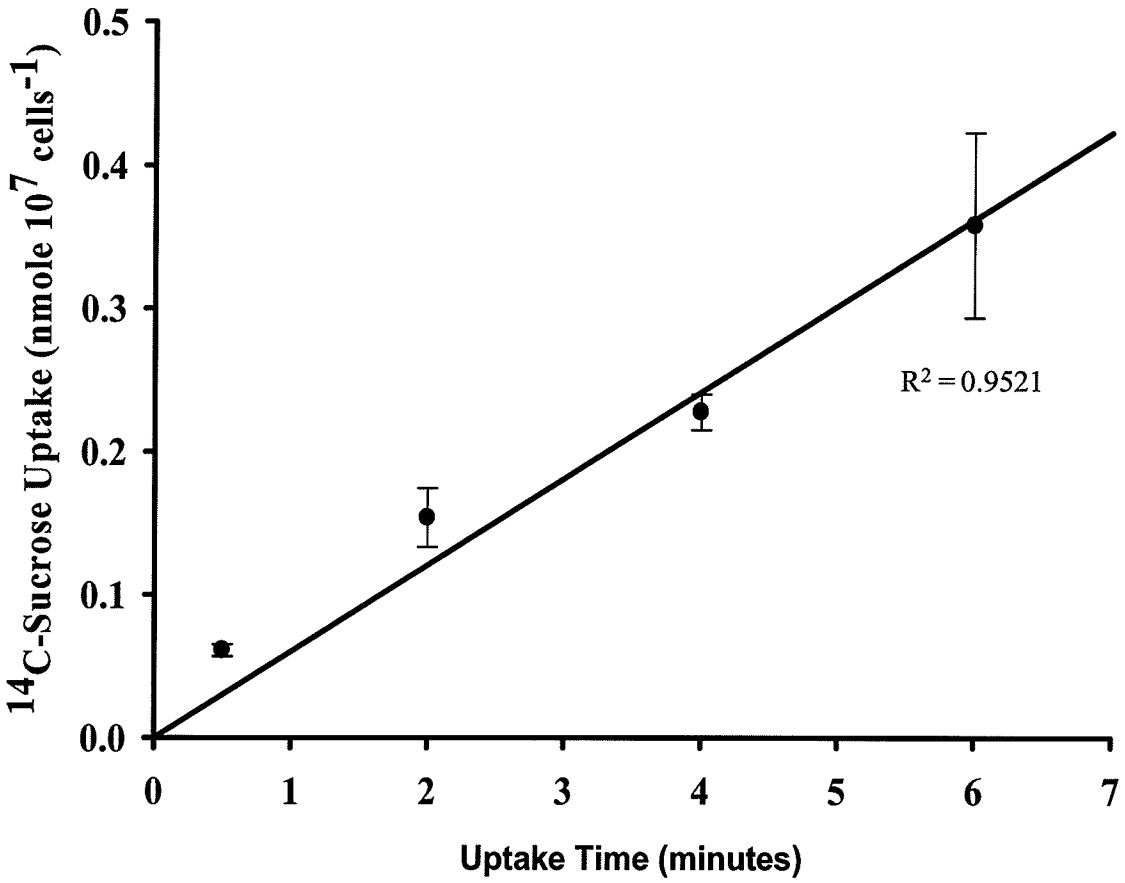

Figure 4.6.2. Uptake of ^{14}C-sucrose by SEY6210 yeast cells expressing *TaSUT1* relative to uptake time. Assays were carried out using 1 mM ^{14}C-sucrose at pH 4.0. Error bars represent means +/- standard deviation (n = 3).

4.7 Transport of Sucrose by TaSUT1 is Coupled with Proton Transport

Sucrose transport by TaSUT1 was shown to be pH dependent, with optimal sucrose uptake rates by *TaSUT1*-transformed SEY6210 in NaH_2PO_4 buffer of pH 4.0 (Figure 4.7.1.).

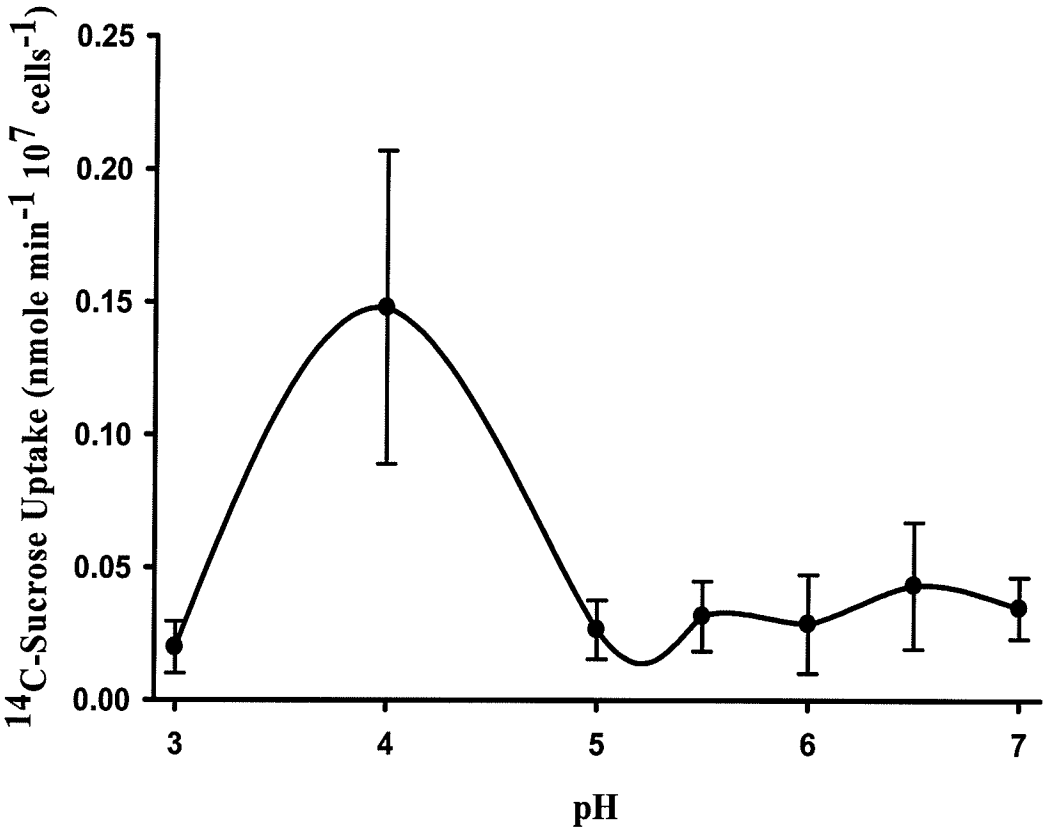

Figure 4.7.1. Uptake of ^{14}C-sucrose by *TaSUT1*-transformed SEY6210 yeast cells in NaH_2PO_4 buffer with pH ranging from 3.0 to 7.0. Assays were carried out using 1 mM ^{14}C-sucrose. Error bars represent means +/- standard deviation (n = 3).

The requirement of protons for sucrose transport by TaSUT1 was indirectly shown by the addition of ETC inhibitors antimycin A, CCCP, and rotenone, all of which disrupt the proton gradient. Sucrose uptake measurements were taken five minutes after the simultaneous addition of ^{14}C-sucrose and one of each of the ETC inhibitors indicated above. All ETC inhibitors tested reduced the amount of sucrose taken uptake by *TaSUT1*-transformed SEY6210 yeast cells (Figure 4.7.2.).

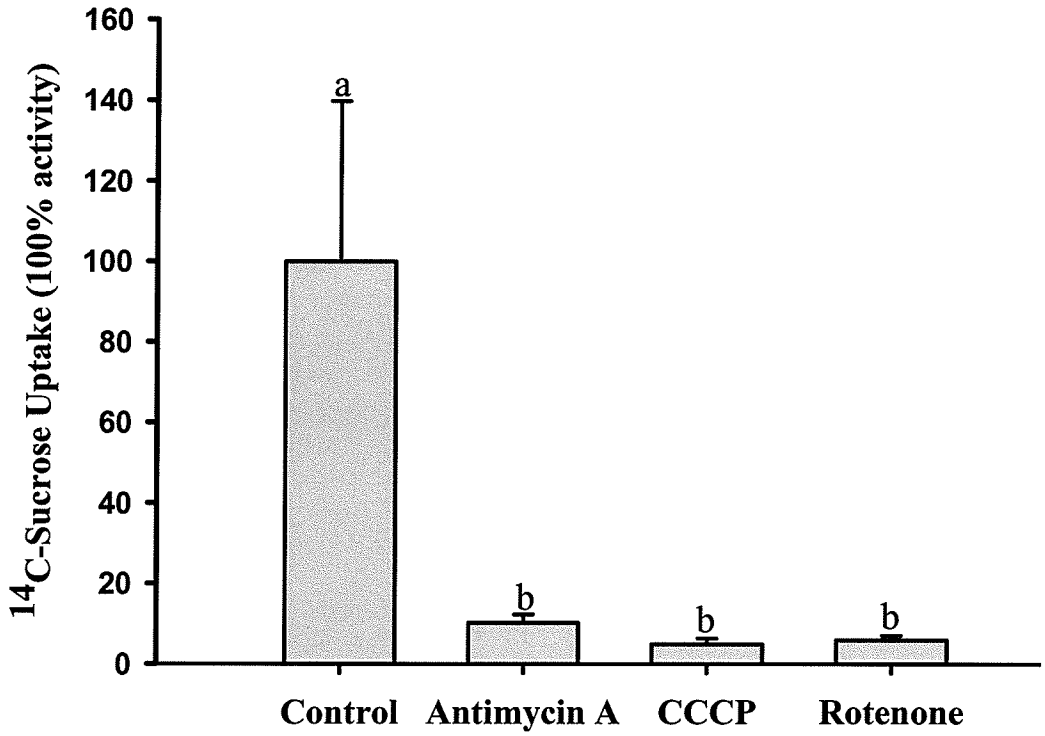

Figure 4.7.2. Uptake of ^{14}C-sucrose by *TaSUT1*-expressing SEY6210 yeast after five minutes following the addition of the ETC inhibitors antimycin A, CCCP or rotenone simultaneously with ^{14}C-sucrose. The ^{14}C-sucrose uptake rate of the control reaction, which received only 1 mM ^{14}C-sucrose, was considered as 100% and all other treatments were compared relative to it. Reactions were carried out in 1mM sucrose at pH 4.0. Inhibitors were added at concentrations of 25 μg Antimycin A per 80 μL reaction, 50 μM rotenone or 10 μM CCCP. Bars represent the means plus standard deviation (n = 3). Bars labelled with different superscripts (a and b) are statistically different ($P \leq 0.05$).

4.8 TaSUT1 is Highly Specific for Sucrose

To determine the specificity of TaSUT1 for sucrose, various sugars at 10 mM concentration were added to *TaSUT1*-transformed SEY6210 cells simultaneous with the addition of 1 mM ^{14}C-sucrose, and the uptake rate of ^{14}C-sucrose was subsequently measured. Both glucose and fructose significantly reduced ^{14}C-sucrose uptake relative to

the control (Figure 4.8.). All other sugars tested had no significant effect on ^{14}C-sucrose uptake.

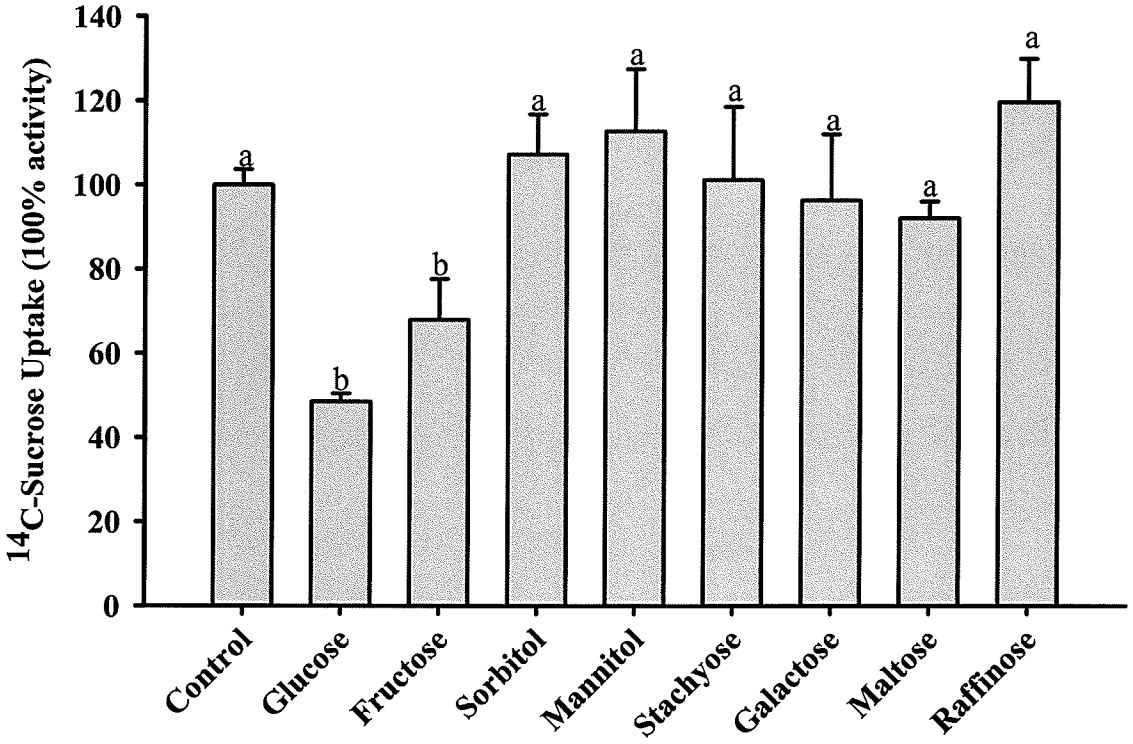

Figure 4.8. Competitive effect of sugars and sugar alcohols on ^{14}C-sucrose uptake by SEY6210 yeast cells expressing *TaSUT1*. The control treatment received 1mM ^{14}C-sucrose, whereas the cells in all other treatments simultaneously received 10 mM of the competitor and 1 mM of ^{14}C-sucrose. The ^{14}C-sucrose uptake rate of the control reaction was considered as 100% and all other treatments were compared relative to it. Reactions were carried out at pH 4.0. Bars represent the means plus standard deviation (n=3). Bars with different superscripts (a and b) are significantly different ($P \leq 0.05$)

4.9 TaSUT1 is a Low Affinity Sucrose Transporter

The rate of ^{14}C-sucrose uptake by SEY6210 cells expressing TaSUT1 was measured at sucrose concentrations ranging from 0.1-100 mM. The resulting data were used to create a Michaelis-Menten curve which revealed a K_M value of approximately 19 mM, indicating that TaSUT1 is a low affinity sucrose transporter (Figure 4.9.).

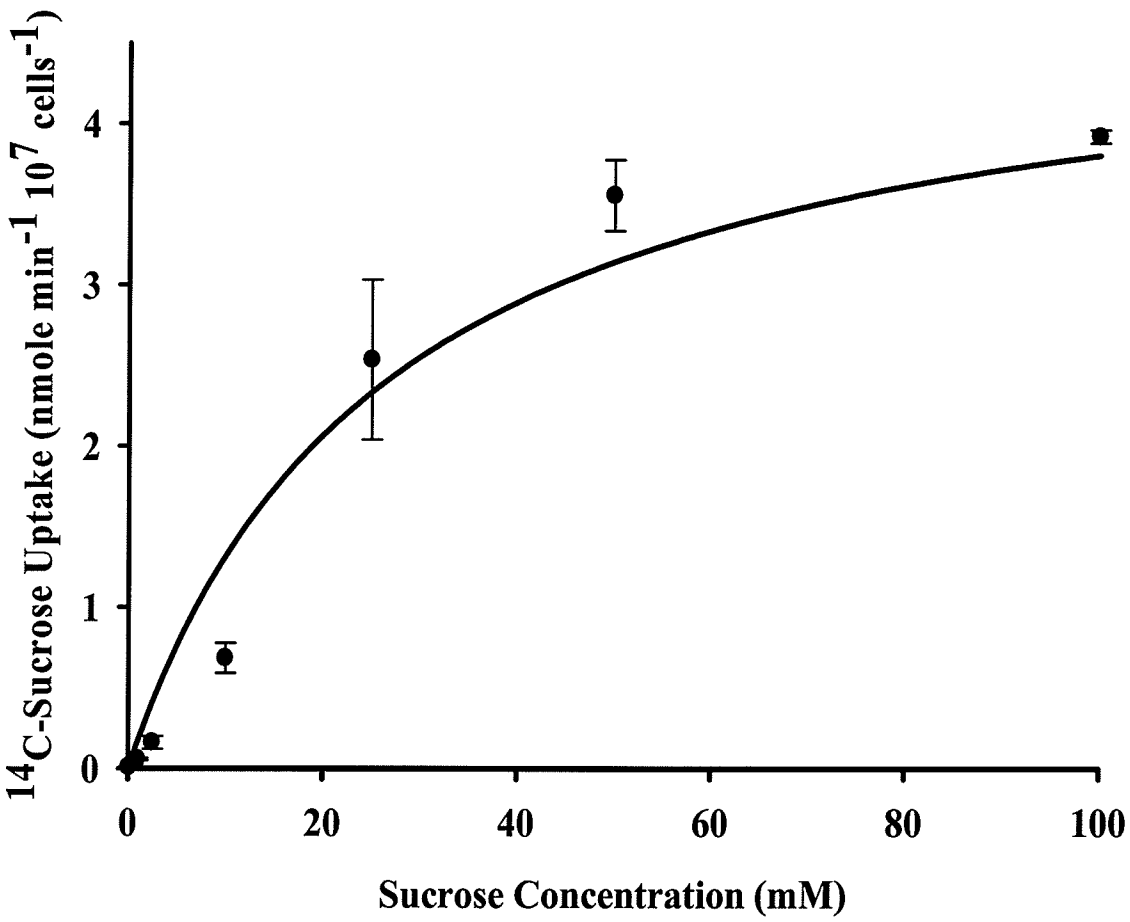

Figure 4.9. Michaelis-Menten analysis of TaSUT1, measured by ^{14}C-sucrose uptake rates of *TaSUT1*-expressing SEY6210 at sucrose concentrations ranging from 0.1 to 100 mM. Reactions were carried out at pH 4.0. Data points represent the means +/- standard deviation (n=3).

5.0 DISCUSSION

5.1. *TaSUT1* was Successfully Cloned into *E. coli.*

Successful cloning of *TaSUT1* into *E. coli* was shown in a variety of ways. Following cloning into pGEM®-T Easy, digestion with *EcoRI* removed a fragment of approximately 1700 bp, corresponding to *TaSUT1* cDNA, some of the untranslated regions of *TaSUT1* and a portion of pGEM®-T Easy (Figure 4.1.1.). Verification was also shown by PCR reactions in which gene specific primers used with *TaSUT1*-pGEM®-T Easy as the DNA template amplified fragments of the anticipated lengths for both partial and full length *TaSUT1* (Figures 4.1.2. and 4.1.3.). These primers lack homology with pGEM®-T Easy and therefore the amplification visible is due to their homology with the inserted *TaSUT1*.

A nucleotide BLAST was performed using the sequence of winter wheat *TaSUT1* which had been cloned into pGEM®-T Easy, and it was found that the gene with highest homology to *TaSUT1* was *TaSUT1B* previously cloned from *Triticum aestivum* spring wheat (Table 4.1.). Sequence alignment of the two genes revealed a section of approximately sixty nucleotides missing from the sequence of the cloned winter wheat *TaSUT1* near the 5' end of the coding sequence (Appendix 1.). Apart from this section, the cloned *TaSUT1* and *TaSUT1B* sequences are highly homologous. *TaSUT1B* is 1569 bases in length with twelve transmembrane domains, and encodes a 522 amino acid protein that weighs 55.1 kilodaltons (http://www.ncbi.nlm.nih.gov/). Located at position 70 of TaSUT1B is a histidine residue, which is likely the histidine that is conserved among all functional SUTs identified in plants. Analysis of conserved domains reveals that this protein contains a sugar transporter superfamily domain near the N-terminus.

The N-terminus is predicated to be cytoplasmic, suggesting that the sugar transporter domain is located on the outer face of the plasma membrane to facilitate sucrose binding.

5.2 *TaSUT1* is Expressed in Flag Leaves, Peduncles, and Developing Grains

Expression analysis of *TaSUT1* was carried out for three different tissues, all of which showed some level of expression (Figure 4.2.). Tissues studied were flag leaves, which are source tissues, developing grains, which are sink tissues, and peduncles, which form the pathway between flag leaves and developing grains. Since *TaSUT1* was expressed in each of these tissues, it is likely that the protein has multiple functions. In flag leaves, the major role of TaSUT1 is likely phloem loading as this is the tissue where sucrose is produced and exported. In the peduncle, TaSUT1 likely plays a role in phloem loading as well. Wheat plants temporarily store carbon, mainly in the form of fructans, in the peduncle and other parts of the stem during the early stages of grain filling (Aoki *et al.* 2004). Remobilized fructans, in the form of sucrose, likely depend upon SUTs for loading into the phloem. Additionally, any sucrose that leaks out of the phloem requires SUTs for retrieval and reloading. Alternatively, in the grains TaSUT1 likely has a role in phloem unloading, sink loading and, later, during germination, in transporting sucrose across the scutellar epidermis from the endosperm to the embryo (Aoki *et al.* 2002; Aoki *et al.* 2006). The expression of *TaSUT1* in virtually all parts of the wheat plant, such as leaves, peduncles and grains, as shown here, and roots, as shown by Aoki *et al.* (2006), suggests that SUTs play a critical role in plant development.

5.2.1 During the Grain Maturation Phase, *TaSUT1* Expression Patterns Vary Among Flag Leaves, Peduncles, and Developing Grains

TaSUT1 expression in flag leaves slightly decreased as the grains became more mature (Figure 4.2.). This reduction is likely correlated with leaf age. As time progressed through the grain maturation phase, the flag leaves began to suffer from chlorosis and became dry and brittle. This reduced the leaves' ability to perform photosynthesis, resulting in decreased photosynthate production and export, therefore reducing the need for transporter proteins. The reduction observed in *TaSUT1* expression is likely just one example of many genes whose expression decreased as the leaves were senescing. Aoki *et al.* (2002) reported that *TaSUT1* transcript levels accumulated to high levels in flag-leaf blades four days before heading but declined at twelve days after heading (DAH), corresponding to approximately thirty days prior to hard kernel stage, which is slightly less mature than the earliest time point analyzed in the current study. Their finding of decreased *TaSUT1* expression during the grain filling period was unexpected as this is the period of substantial sucrose export from the leaves to the filling grains.

No obvious overall trend was observed for *TaSUT1* expression in peduncles during the grain maturation phase (Figure 4.2.). The fluctuations observed in *TaSUT1* expression may be correlated with either variations in the flow rate of sucrose, which corresponds to the amount of sucrose leaked out of the phloem, or potentially with the rate of fructan remobilization.

Although not statistically significant, the expression of *TaSUT1* in the developing grains slightly increased from thirty-five to twenty-three days prior to the hard kernel stage and then slightly decreased (Figure 4.2.). The peak in expression at twenty-three

days prior to the hard kernel stage suggests that this may be a period of rapid grain filling, beyond which there is less demand for starch and therefore less demand for sucrose to be loaded into the grain. The reduction in *TaSUT1* expression may be related to this decrease in sink demand, as was suggested by Chiou and Bush (1998) and summarized below.

Similar results were reported by Aoki *et al.* (2002) for *TaSUT1* expression in developing grains ranging in maturity from eight to thirty-two DAH, corresponding to approximately thirty-four to ten days prior to the hard kernel stage. *TaSUT1* transcripts were detected at eight DAH, reached a maximum level at sixteen to twenty DAH, and then declined and remained low through thirty-two DAH. As stated previously, there is no symplastic connection between the maternal and filial tissues, so TaSUT1 likely plays an important role in transporting sucrose into filial cells, which may be maximal at sixteen to twenty DAH.

Although the current study did not continue past the hard kernel stage, it is expected that *TaSUT1* expression in the grains will likely increase as the seeds begin to germinate. Prior to the initiation of starch breakdown, sucrose stored in the aleurone layer as well as that produced from oil catabolism is actively transported into the endosperm to provide an early energy source to the embryo (Aoki *et al.* 2006). *TaSUT1* expression will likely increase in the scutellar epidermis during this time as this tissue is a symplastic barrier to sucrose movement located between the endosperm and the embryo. Later in the germination process, glucose and maltose levels in the endosperm will greatly increase as starch degradation is occurring (Aoki *et al.* 2006). At this stage, *TaSUT1* expression will likely increase in the scutellar epidermis and ground tissue to function in transporting maltose from the endosperm to the embryo.

5.3 High Concentrations of Glucose and Sucrose Slightly Decreased *TaSUT1* Expression in Leaves

To determine the impact sugars have on *TaSUT1* expression, excised leaves were treated with various concentrations of mannitol, sucrose or glucose. For all concentrations of mannitol tested, *TaSUT1* expression was not significantly affected and there was no trend visible in the expression levels (Figure 4.3.). Combined with the knowledge that the mannitol solutions maintained the same osmotic potentials as the other sugar solutions of equal concentrations, mannitol was considered to be another control in addition to the 0 mM treatment.

For all concentrations of glucose tested, *TaSUT1* expression in the leaves was slightly reduced relative to the 0 mM control and to the equivalent concentrations of mannitol, although these results were not statistically significant (Figure 4.3.). Similar results were observed for *VfSUT1* in fava bean cotyledons, in which high levels of glucose resulted in decreased *VfSUT1* transcript levels (Weber *et al.* 1997). Conversely, Barker et al. (2000) reported that potato *StSUT1* and *StSUT2* expression was unaltered in excised source leaves following the application of 100 mM glucose. Treatment of detached sugar beet leaves with 100 mM glucose also had no effect on sucrose transport, suggesting that the expression level of SUT was unaltered (Chiou and Bush 1998). Even more dissimilar to the current findings on *TaSUT1* expression was shown by Matsukura et al. (2000) where glucose induced *OsSUT1* transcription in rice embryos.

Data from this study show that all concentrations of sucrose slightly reduced *TaSUT1* expression below that of the 0 mM control as well as the equivalent concentration of mannitol (Figure 4.3.). Weber *et al.* (1997) reported that fava bean

cotyledons treated with 150 mM sucrose showed decreased *VfSUT1* transcript levels. Decreased sucrose transport in sugar beet leaves fed 100 and 250 mM sucrose also supports the current findings since decreased transport was likely related to decreased SUT expression (Chiou and Bush 1998). *StSUT1* expression remained unaltered in the presence of 100 mM sucrose, but interestingly, *StSUT2* expression was induced (Barker *et al.* 2000). In tomato, *LeSUT2* expression was induced in sink leaves but not in source leaves, and in either type of leaves *LeSUT1* expression was unaltered. As seen previously with the glucose treatments, sucrose treatments also induced *OsSUT1* transcription in rice embryos (Matsukura *et al.* 2000).

The results of this component of the study may best be explained by the sucrose-specific response pathway described by Chiou and Bush (1998). *In vivo*, when sink demand is low, high amounts of sucrose will accumulate in the vascular tissue. Low sink demand indicates that sucrose transport is minimal, and the plant is somehow able to down-regulate SUT expression. In the current study, treating leaves with sucrose solutions would likely have increased sucrose in the vascular tissue, mimicking decreased sink demand, leading to decreased SUT expression. Leaves treated with glucose may have experienced a similar response, since glucose is one component of sucrose and the high levels of available glucose may have been converted to high levels of sucrose. Mannitol is not readily converted into sucrose and therefore mannitol application did not decrease SUT expression.

The findings of this study have implications for future work aimed at manipulating overall plant yield. Two approaches for increasing yield include increasing photosynthate production and/or increasing *TaSUT1* expression. Increasing photosynthate

production would result in more carbon available to be transported and ultimately stored in the sinks, but this may require an increase in SUT activity. If in wildtype plants the SUTs were not saturated with sucrose, they may be able to accommodate the increase in sucrose resulting from increased photosynthetic activity. But, if the SUTs were saturated, they will not be able to accommodate increased levels of sucrose. A way of rectifying this problem would be to increase SUT expression, but as the current study has shown, exposing leaves to high sucrose concentrations led to a decrease in *TaSUT1* expression. The expression of *TaSUT1* will not increase in response to the increased sucrose, which is the desired response because then the levels of *TaSUT1* would correlate with the amount of substrate present. A modification to the approach of increasing yield by increasing photosynthate production would be to do so in combination with transformation of wheat plants with either *StSUT2* or *OsSUT1*. Both of the genes showed increased expression following treatment with high concentrations of sucrose (Barker *et al.* 2000; Matsukura *et al.* 2000).

The second approached to increase yield as mentioned above would be to increase *TaSUT1* expression. This would be an appropriate approach if the amounts of SUTs are a limiting factor in sucrose transport, i.e. there is excess sucrose produced in source tissues which is not able to be transported to sink tissues. Increasing the expression of *TaSUT1*, possibly by modifying the promoter to increase its activity or by transforming the plant with a more active promoter, would result in more proteins available to transport sucrose.

5.4 *TaSUT1* was Successfully Cloned into Yeast

PCR verification was used to confirm successful cloning of *TaSUT1* into yeast, but since it is difficult to perform PCR on yeast directly, verification was a multi-step process. First, genomic DNA (gDNA), which consists of all yeast-specific DNA in addition to the *TaSUT1*-containing plasmid DNA, was isolated from *TaSUT1*-transformed yeast. This DNA was cloned into *E. coli* cells which were subsequently used as the DNA template for PCR amplification using *TaSUT1*-specific primers. A strong band was visible at approximately 1600 bp (Figure 4.4.), corresponding to full length *TaSUT1,* confirming that *TaSUT1* was successfully cloned into yeast.

5.5 TaSUT1 is Functional in Transporting Sucrose

The plasmid pDR196 was used for all yeast characterization work. This plasmid contains the plasma membrane ATPase promoter upstream of the cloning site which results in expression of the cloned gene on the plasma membrane. Localization on the plasma membrane is important for *TaSUT1* since it is a transmembrane protein. This plasmid also contains the alcohol dehydrogenase terminator which is very powerful and prevents any transcription beyond that of the cloned gene, thereby preventing any unwanted transcription of plasmid DNA.

To determine if TaSUT1 was functional in transporting sucrose, SUSY7/*ura3* cells expressing TaSUT1 were grown on SS media. SUSY7/*ura3* can metabolize internal sucrose but has no ability to internalize sucrose itself. Since the only carbon source available in SS media is sucrose, SUSY7/*ura3* will only be able to survive if there is a means of transporting in sucrose. As shown in Figure 4.5., *TaSUT1*-expressing

SUSY7/*ura3* cells were able to grow, increasing from approximately 320,000 cells to 16,300,000 cells (corresponding to an OD_{600} of 0.032 and 1.63, respectively) over five days following infection. This increase in cell number indicates that the transformed SUSY7/*ura3* cells were able to metabolize sucrose from the media, indicating that TaSUT1 was functional in transporting sucrose into the yeast cells. By way of a control, SUSY7/*ura3* transformed with empty pDR196 was also grown on SS media and its growth was shown to be much less than that of TaSUT1-SUSY7/*ura3*. This result is to be expected since SUSY7/*ura3* transformed with empty pDR196 has no ability to internalize sucrose and therefore no ability to metabolize sucrose from the media.

5.6 Sucrose Uptake is Positively Correlated with Cell Number and Uptake Time

As the number of *TaSUT1*-expressing SEY6210 cells increased so did the quantity of ^{14}C-sucrose taken up by the yeast, as shown in Figure 4.6.1. A positive linear relationship was also observed between ^{14}C-sucrose uptake and ^{14}C-sucrose uptake time (Figure 4.6.2.). Collectively, these data suggest that sucrose uptake is governed by a biological process.

5.7 Transport of Sucrose by TaSUT1 is Coupled with Proton Transport

Two approaches were taken to determine if sucrose transport by TaSUT1 is coupled with H^+ transport. First, the sucrose uptake rate by TaSUT1 was shown to vary with pH, with optimal sucrose uptake at pH 4.0 (Figure 4.7.1.). This optimal pH for sucrose uptake by TaSUT1 is the same as was reported for other characterized SUTs,

notably GmSUT1 from soybean (*Glycine max* L. Merr.) (Mike *et al.* 2003) and AtSUT2 and AtSUT4 from *Arabidopsis* (Schulze *et al.* 2000; Weise *et al.* 2000).

The second approach taken to show that sucrose and H^+ transport were coupled was to add one of three ETC inhibitors, specifically antimycin A, CCCP or rotenone, concurrently with radiolabelled sucrose and observe the corresponding sucrose uptake rate. Antimycin A inhibits electron transfer activity of the bc_1 complex, disrupting normal electron flow though the ETC (Boumans *et al.* 1998). CCCP is a protonophore which uncouples oxidative phosphorylation, disrupts ATP production, and increases the proton conductance of membranes, all of which interrupt the previously established proton gradient (Beauvoit *et al.* 1991). Rotenone is an inhibitor of NADH-FMN oxidoreductase, an enzyme which catalyzes the reduction of FMN simultaneously with the oxidation of NADH (Aliverdieva 2001). NADH is required for subsequent steps in the ETC and rotenone prevents its production. As shown in Figure 4.7.2., the sucrose uptake rate of *TaSUT1*-expressing SEY6210 treated with any of the inhibitors was significantly lower than that of the control, providing strong evidence that sucrose transport is coupled with H^+ transport.

5.8 Glucose and Fructose Reduce Transport of Sucrose by TaSUT1

A variety of monosaccharides, disaccharides, polysaccharides and sugar alcohols were studied to see if they were capable of interfering with sucrose transport by TaSUT1. Only glucose and fructose were able to significantly reduce the amount of ^{14}C-sucrose taken up by SEY6210 cells (Figure 4.8.). The addition of glucose or fructose may have improved the energy status of the yeast cells, therefore reducing their need for sucrose.

This may have consequently reduced their sucrose uptake rate, although it is unknown if cells have control over the transport process. The inability of the other sugars to compete with sucrose transport, even when present at concentrations 10X greater than that of sucrose, suggests that TaSUT1 is highly specific for sucrose.

5.9 TaSUT1 is a Low Affinity Sucrose Transporter

The sucrose uptake rate of TaSUT1 varies with the concentration of sucrose available to the transporter. As shown in Figure 4.9., the ^{14}C-sucrose uptake rate increased with increasing sucrose concentration until a certain level, at which point the uptake rate levelled off, approaching the maximum uptake rate. The sucrose concentration which corresponds to half of the maximum uptake rate is designated as the K_M value and provides information on the affinity of SUTs to sucrose. Results from this study in combination with Michaelis-Menten analysis indicate that the K_M of TaSUT1 is approximately 19 mM at pH 4.0. In similar assay conditions, the K_M of potato StSUT1 was shown to be 1.7 mM whereas the K_M of AtSUT2 from *Arabidopsis* was found to be 11.7 mM (Schulze *et al.* 2000). When these results were originally published, AtSUT2 had the lowest affinity of all characterized SUTs, but the findings of the current study suggest that TaSUT1 actually has a lower affinity for sucrose than AtSUT2. An inverse relationship exists between affinity and capacity of SUTs as was shown when mutations in histidine-65 of AtSUT1 caused an increase in its K_M for sucrose and a higher V_{max} (Lu and Bush 1998). Wheat plants typically have high sucrose levels so the high capacity of TaSUT1 is likely important for effective transport of the large amount of sucrose in the plants.

There is interest in developing high yielding wheat plants through manipulation of components of the source-sink relationship. One potential approach would be to increase the expression of *SUT* genes with the thought that this may increase the amount of sucrose transport. To achieve maximum sucrose uptake across a wide range of sucrose concentrations, it may be necessary to express a SUT of higher affinity. Transformation of wheat plants with a higher affinity SUT, such as StSUT1, may improve sucrose transport as this transporter functions at its maximum transport rate at a lower sucrose concentration. For plants transformed with higher-affinity transporters, it would likely be important to maintain the activity of TaSUT1 in order to accommodate the large amount of sucrose found in wheat. Although high affinity transporters are desirable for their ability to achieve maximum transport rates at lower sucrose concentrations, their low capacity for sucrose makes them inappropriate as the sole transporters in wheat. A more effective approach for increasing yield would be to have both low and high affinity transporters as this combination should provide optimized uptake rates across a range of sucrose concentrations while still accommodating the vast amount of sucrose.

Another approach to express high affinity SUTs in wheat would be to modify TaSUT to increase its affinity. Previous work has shown that the K_M of AtSUT2 was increased by replacing its N-terminal domain with that of StSUT1 (Schulze *et al.* 2000). The same approach could also be used to increase the affinity of TaSUT1. Alternatively, there may be a higher affinity transporter in wheat, yet unknown, which could be over-expressed. Winter wheat has a very large genome yet only two SUTs have been identified, whereas other species with much smaller genomes have had many sucrose transporters identified (Kuhn *et al.* 1999; Aoki *et al.* 2003). Multiple SUTs within one

plant vary in their affinity for sucrose, allowing plants to differentially express certain SUTs depending on sucrose concentrations (Schulze *et al.* 2000). Future research focused on identifying additional wheat SUTs, possibly using SUT-specific primers designed from homologous regions of other crop species, as well as characterizing the putative TaSUT2, may uncover higher affinity SUTs which can subsequently be manipulated.

The expression of a higher-affinity transporter is desirable if the sucrose concentration drops low enough that the lower-affinity *TaSUT1* is unable to function at its maximum transport rate. But, as long as the sucrose concentration remains sufficiently high, *TaSUT1* will remain at its maximum transport rate and therefore its overexpression should increase sucrose transport. Detailed knowledge on the plant's sucrose concentrations of sucrose as well as fluctuation in sucrose levels should provide insight into whether increased expression of high- or low-affinity transporters is more desirable.

6.0 CONCLUSIONS

The goal of this study was to develop a greater understanding of *TaSUT1* expression and function, ultimately providing insight into potential modifications to sucrose transport which could increase winter wheat yield.

The *TaSUT1* gene was identified in winter wheat and its cDNA successfully cloned into *E. coli*. The sequence of this gene is very similar to that of *TaSUT1B,* previously cloned from *Triticum aestivum* spring wheat, which is 1569 nucleotides in length, has twelve transmembrane domains, and encodes a protein which consists of 522 amino acids and has a molecular mass of 55.1 kilodaltons.

Expression profiling revealed that flag leaves, peduncles, and developing grains all express *TaSUT1* during the grain maturation phase but expression levels vary. In flag leaves, *TaSUT1* expression slightly declined from twenty-eight to eleven days prior to hard kernel stage, likely due to leaf senescence and reduction in photosynthate production. In peduncles, the level of *TaSUT1* expressed fluctuated from twenty-eight to zero days prior to hard kernel stage. The fluctuation was speculated to relate to variations in the amount of sucrose in the phloem or the rate of fructan remobilization. For developing grains, *TaSUT1* expression was observed to increase up until twenty-three days prior to hard kernel stage, beyond which levels started to decline. The peak in *TaSUT1* expression may correspond to a period of maximal phloem unloading or sink loading, beyond which sink demand is lessening and therefore the need for sucrose transport decreasing.

Increasing *TaSUT1* expression during the grain filling stage may result in increased sucrose transport and ultimately increased starch yield. Increased expression

may be achieved by modifying *TaSUT1*'s promoter or transforming wheat with a more active promoter. Logically, it would be beneficial to target tissues and/or developmental stages where TaSUT1 is most limited. Studying *TaSUT1* expression across a broader time scale than that of the current study could provide insight into what stage and/or what tissue would be best to target. In the future, *TaSUT1* could be expressed in the ideal tissue at the ideal stage through the use of tissue specific or developmental stage specific promoters. Alternatively, transformation of wheat with a higher affinity SUT may increase grain filling as this transporter would be able to maintain a high transport rate even at low sucrose concentrations. In this scenario, likely the best option is to express both a high affinity and a high capacity transporter, since a high affinity transport alone may not have the capacity to handle the large quantities of sucrose present in wheat.

High concentrations of either glucose or sucrose resulted in slight decreases in *TaSUT1* expression in excised wheat leaves. Elevated sucrose levels in the vascular tissue of leaves, due either to the direct application of sucrose or as a result of high glucose concentrations, potentially mimicked low sink demand and resulted in decreased *TaSUT1* expression. When sink demand is low there is limited need for sucrose transport, which may down-regulate *TaSUT1* expression. Mannitol is not readily converted into sucrose and correspondingly did not have the same effect on *TaSUT1* expression.

Another approach that may be taken in attempt to develop high yield winter wheat is to increase the amount of sucrose produced by increasing the amount of photosynthesis occurring. Plants would then have to transport larger amounts of sucrose, and if the SUT levels remained the same as in the wildtype, they may not be able to accommodate these increased sugar levels. Ideally, if *TaSUT1* expression increased in response to increased

sucrose levels, the plant should be able to handle the greater amounts of photosynthate. The current study has shown that for *TaSUT1* this is not the case; instead, increased sucrose concentrations resulted in decreased *TaSUT1* expression. To develop wheat plants that are capable of increasing SUT expression when sucrose levels are higher, two approaches may be taken. First, wheat plants could be transformed with a SUT that has been shown to experience increased expression when sucrose levels increase, such as StSUT2 (Barker *et al.* 2000). Alternatively, determination of the reason why TaSUT1 and StSUT2 expression varies in response to sucrose levels may provide insight into modifications that could be made to TaSUT1 to behave like StSUT2, increasing its expression when sucrose levels increase.

TaSUT1 cDNA was successfully cloned into two different yeast strains, SUSY7/*ura3* and SEY6210. As mentioned previously, TaSUT1 is a transmembrane protein and as such required the plasma membrane ATPase promoter to express it into the proper subcellular location. SUSY7/*ura3* was used to determine if TaSUT1 was functional in transporting sucrose. As a yeast strain which is unable to take up sucrose but able to metabolize internal sucrose, transformation with *TaSUT1* and subsequent growth on media containing sucrose as the sole carbon source provided evidence showing that TaSUT1 was capable of transporting sucrose.

Kinetic analyses were carried out to study characteristics of TaSUT1 function. The second transformed yeast strain, SEY6210, was used for all TaSUT1 kinetic assays due to its inability to metabolize sucrose. Sucrose transport by TaSUT1 was shown to be a biological process since the uptake rate increased linearly with both cell number and uptake time. TaSUT1 functions as a sucrose/proton symporter and as such shows pH

dependent sucrose uptake. The uptake rate was optimal at pH 4.0 and sucrose uptake was greatly reduced by electron chain inhibitors which ultimately interfere with the proton gradient.

TaSUT1 is highly specific for sucrose as shown by minimal variation in sucrose uptake rates even when in the presence of 10X higher concentrations of other sugars. The only exceptions observed were glucose and fructose. These two monosaccharides may serve as energy metabolites for the yeast cells, reducing the yeast's need for sucrose, and subsequently resulting in less sucrose taken up by the yeast cells.

Kinetic analyses have revealed that TaSUT1 is a low affinity transporter with a K_M of approximately 19 mM at pH 4.0. As affinity and capacity are known to be inversely related, TaSUT1's high capacity for sucrose may be important for enabling transport of the large quantities of sucrose present in wheat.

Various approaches based on SUT affinity could be carried out in attempt to develop high yielding winter wheat. As a high affinity transporter is able to achieve its maximal sucrose uptake rate at lower sucrose concentrations, it may be desirable, if sucrose concentrations are sufficiently low, to increase the activity of high affinity transporters. This could be achieved by transforming wheat plants with a higher affinity SUT from another species, or by replacing the N-terminus of TaSUT1 with that of a higher affinity transporter. Also, there may be additional SUTs in wheat, yet undiscovered, that have a higher affinity for sucrose and could be subsequently manipulated. Alternatively, if sucrose concentrations remain sufficiently high that TaSUT1 is able to function at its maximal transport rate, overexpression of *TaSUT1* may be desirable.

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Appendix 1. Nucleotide alignment of *TaSUT1A, TaSUT1B, TaSUT1D* and cloned *TaSUT1*. Nucleotides in bold indicate variation among the sequences. Underlined nucleotides indicate the location of the sugar transport superfamily domain.

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TaSUT1-A        ATGGCGCGCG GCGGCGGCAA CGGCGAGGTG GAGCTCTCGG TGGGGGT---
TaSUT1-B        ATGGCGCGCG GCGGCGGCAA CGGCGAGGTG GAGCTCTCGG TGGGGGT---
TaSUT1-D        ATGGCGCGCG GCGGAGGCAA CGGCGAGGTG GAGCTCTCGG TCGGGGTCGG
Cloned TaSUT1   ATGGCGCGCG GCGGCGGCAA CGGCGAG--- ---------- ----------

TaSUT1-A        CGGCGGAGGC GGCGCCGGCG CCGGCGGGGC GGACGCCCCC GCCGTGGACA
TaSUT1-B        CGGCGGAGGC GGCGCCGGCG CCGGCGGGGC GGACGCCCCC GCCGTGGACA
TaSUT1-D        CGGCGGAGGC GGCGGCGCCG CCGGCGGGGG GGAGCAACCC GCCGTGGACA
Cloned TaSUT1   ---------- ---------- ---------- ---------- ---GTGGACA

TaSUT1-A        TCAGCCTCGG CAGGCTCATC CTCGCCGGCA TGGTCGCCGG CGGCGTGCAG
TaSUT1-B        TCAGCCTCGG CAGGCTCATC CTCGCCGGCA TGGTCGCCGG CGGCGTGCAG
TaSUT1-D        TCAGCCTCGG CAGACTCATC CTCGCCGGCA TGGTCGCCGG CGGCGTGCAG
Cloned TaSUT1   TCAGCCTCGG CAGGCTCATC CTCGCCGGCA TGGTCGCCGG CGGCGTGCAG

TaSUT1-A        TACGGATGGG CGCTCCAGCT CTCCCTGCTC ACCCCCTACG TCCAGACTCT
TaSUT1-B        TACGGATGGG CGCTCCAGCT CTCCCTGCTC ACCCCCTACG TCCAGACTCT
TaSUT1-D        TACGGATGGG CGCTCCAGCT CTCCCTGCTC ACCCCCTACG TCCAGACTCT
Cloned TaSUT1   TACGGATGGG CGCTCCAGCT CTCCCTGCTC ACCCCCTACG TCCAGACTCT

TaSUT1-A        GGGACTTTCG CATGCTCTGA CTTCATTCAT GTGGCTCTGC GGCCCTATTG
TaSUT1-B        GGGACTTTCG CATGCTCTGA CTTCATTCAT GTGGCTCTGC GGCCCTATTG
TaSUT1-D        GGGACTTTCG CATGCTCTGA CTTCATTCAT GTGGCTCTGC GGCCCTATTG
Cloned TaSUT1   GGGACTTACG CATGCTCTGA CTTCATTCAT GTGGCTCTGC GGCCCTATTG

TaSUT1-A        CTGGATTAGT GGTTCAACCA TGCGTTGGGC TCTACAGTGA CAAGTGCACT
TaSUT1-B        CTGGATTAGT GGTTCAACCA TGCGTTGGGC TCTACAGTGA CAAGTGCACT
TaSUT1-D        CTGGATTAGT GGTTCAACCA TGCGTTGGGC TCTACAGTGA CAAGTGCACA
Cloned TaSUT1   CTGGATTAGT GGTTCAACCA TGCGTTGGGC TCTACAGTGA CAAGTGCACT

TaSUT1-A        TCAAGATGGG GAAGACGCAG ACCGTTCATT CTGACAGGAT GTATCCTCAT
TaSUT1-B        TCTAGATGGG GAAGACGCAG ACCGTTTATT CTGACAGGAT ATATCCTCAT
TaSUT1-D        TCTAGATGGG GAAGACGCAG ACCGTTTATT CTGACAGGAT GCATCCTCAT
Cloned TaSUT1   TCTAGATGGG GAAGACGCAG ACCGTTTATT CTGACAGGAT GTATCCTCAT

TaSUT1-A        CTGCATTGCT GTCGTGGTCG TCGGCTTCTC GGCTGACATT GGAGCTGCTC
TaSUT1-B        CTGCATTGCT GTCGTAGTCG TCGGCTTCTC GGCTGACATT GGAGCTGCTC
TaSUT1-D        CTGCATTGCT GTTGTGGTCG TCGGCTTCTC GGCTGACATT GGAGCTGCTC
Cloned TaSUT1   CTGCATTGCT GTCGTAATCG TCGGCTTCTC GGCTGACATT GGAGCTGCTC

TaSUT1-A        TGGGTGACAG CAAGGAAGAG TGCAGTCTCT ATCATGGGCC TCGTTGGCAC
TaSUT1-B        TGGGTGACAG CAAGGAAGAG TGCAGTCTCT ATCATGGGCC TCGTTGGCAC
TaSUT1-D        TGGGTGACAG CAAGGAAGAG TGCAGTCTCT ATCATGGGCC TCGTTGGCAC
Cloned TaSUT1   TGGGTGACAG CAAGGAAGAG TGCAGTCTCT ATCATGGGCC TCGTTGGCAC

TaSUT1-A        GCTGCAATTG TGTATGTTCT TGGATTCTGG CTCCTTGACT TCTCCAACAA
TaSUT1-B        GCTGCAATTG TGTATGTTCT TGGATTCTGG CTCCTTGACT TCTCCAACAA
TaSUT1-D        GCTGCAATTG TGTATGTTCT TGGATTCTGG CTCCTTGACT TCTCCAACAA
Cloned TaSUT1   GCTGCAATTG TGTATGTTCT TGGATTCTGG CTCCTTGACT TCTCCAACAA
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TaSUT1-A        CACAGTGCAA GGACCAGCGC GTGCTCTGAT GGCTGATTTA TCAGCCCAGC
TaSUT1-B        CACTGTGCAA GGTCCAGCGC GTGCTCTGAT GGCTGATTTA TCAGCTCAAC
TaSUT1-D        CACTGTGCAA GGTCCAGCGC GTGCTCTGAT GGCTGATTTA TCAGCTCAGC
Cloned TaSUT1   CACTGTGCAA GGTCCAGCGC GTGCTCTGAT GGCTGATTTA TCAGCTCAAC

TaSUT1-A        ATGGACCCAG TGCAGCAAAT TCAATCTTCT GTTCTTGGAT GGCACTGGGA
TaSUT1-B        ATGGACCCAG TGCAGCAAAT TCAATCTTCT GTTCTTGGAT GGCACTAGGA
TaSUT1-D        ATGGACCCAG TGCAGCAAAT TCAATCTTCT GTTCTTGGAT GGCGCTAGGA
Cloned TaSUT1   ATGGACCCAG TGCAGCAAAT TCAATCTTCT GTTCTTGGAT GGCACTAGGA

TaSUT1-A        AATATCCTAG GATACTCATC TGGTTCCACA AATAACTGGC ACAAGTGGTT
TaSUT1-B        AATATCCTTG GATACTCCTC TGGTTCCACA AATAACTGGC ACAAGTGGTT
TaSUT1-D        AATATCCTTG GATACTCCTC TGGTTCCACA AACAACTGGC ACAAGTGGTT
Cloned TaSUT1   AATATCCTTG GATACTCCTC TGGTTCCACA AATAACTGGC ACAAGTGGTT

TaSUT1-A        TCCGTTCCTC CGGACAAGGG CTTGCTGTGA AGCCTGCGCA AATCTGAAAG
TaSUT1-B        TCCGTTCCTC CGGACAAGGG CTTGCTGTGA AGCCTGCGCA AATCTGAAAG
TaSUT1-D        TCCGTTCCTC CGGACAAGGG CTTGCTGTGA AGCCTGCGCA AATCTGAAAG
Cloned TaSUT1   TCCGTTCCTC CGGACAAGGG CTTGCTGTGA AGCCTGCGCA AATCTGAAAG

TaSUT1-A        GCGCATTTCT GGTGGCAGTG CTGTTCCTGG CCTTCTGTTT GGTGATAACC
TaSUT1-B        GCGCATTTCT GGTGGCAGTG CTGTTCCTGG CCTTCTGTTT GGTGATAACT
TaSUT1-D        GCGCATTTCT GGTGGCAGTG CTGTTCCTGG CCTTCTGTTT GGTGATAACT
Cloned TaSUT1   GCGCATTTCT GGTGGCAGCG CTGTTCCTGG CCTTCTGTTT GGTGATAACT

TaSUT1-A        GTGATCTTCG CCAAGGAGAT ACCGTACAAG GCGATTGCGC CCCTCCCAAC
TaSUT1-B        GTGATCTTCG CCAAGGAGAT ACCGTACAAG GCGATCGCGC CCCTCCCAAC
TaSUT1-D        GTGATCTTCG CCAAGGAGAT ACCGTACAAG GCGATTGCGC CCCTCCCAAC
Cloned TaSUT1   GTGATCTTCG CCAAGGAGAT ACCGTACAAG GCGATCGCGC CCCTCCCNAC

TaSUT1-A        AAAGGCCAAT GGCCAGGTTG AAGTCGAGCC CACCGGGCCG CTCGCCGTCT
TaSUT1-B        AAAGGCCAAT GGCCAGGTTG AAGTCGAGCC CACTGGGCCG CTCGCCGTGT
TaSUT1-D        AAAGGCCAAT GGCCAGGTTG AAGTCGAGCC CACCGGGCCG CTCGCCGTGT
Cloned TaSUT1   A-AGGCCCAT GGCCAGGTTT GAGTCGAG-C CACTGGGCCG CTCGCCGTGT

TaSUT1-A        TCAAAGGCTT CAAGAACTTG CCTCCTGGAA TGCCGTCAGT GCTCCTCGTC
TaSUT1-B        TCAAAGGCTT CAAGAACTTG CCTCCTGGAA TGCCATCAGT GCTCCTCGTC
TaSUT1-D        TCAAAGGCTT CAAGAACTTG CCTCCTGGAA TGCCGTCGGT GCTCCTCGTC
Cloned TaSUT1   TC-AAGGCTT CAAGAGCTTG CCTCCTGGAA TGCCATCAGT GCTCCTCGTC

TaSUT1-A        ACCGGCCTCA CCTGGCTGTC CTGGTTCCCC TTCATCCTGT ACGACACCGA
TaSUT1-B        ACTGGCCTCA CCTGGCTGTC CTGGTTCCCC TTCATCCTCT ACGACACCGA
TaSUT1-D        ACTGGCCTCA CCTGGCTGTC CTGGTTCCCC TTCATCCTGT ACGACACCGA
Cloned TaSUT1   ACTGGCCTCA CCTGGCTGTC CTGGTTCCCC TTCATCCTCT ACGACACCGA

TaSUT1-A        CTGGATGGGT CGTGAGATCT ACCACGGTGA CCCCAAGGGA ACCCCCGACG
TaSUT1-B        CTGGATGGGT CGTGAGATCT ACCACGGTGA CCCCAAGGGA ACCCCCGACG
TaSUT1-D        CTGGATGGGT CGTGAGATCT ACCACGGTGA CCCCAAGGGA ACCCCCGACG
Cloned TaSUT1   CTGGATGGGT CGTGAGATCT ACCACGGTGG CCCCAAGGGA ACCCCCGACG

TaSUT1-A        AGGCCAACGC GTTCCAGGCA GGTGTCAGGG CCGGGGCGTT CGGCCTGCTA
TaSUT1-B        AGGCCAACGC GTTCCAGGCA GGTGTCAGGG CCGGGGCGTT CGGCCTGCTA
TaSUT1-D        AGGCCAACGC GTTCCAGGCA GGTGTCAGGG CCGGGGCGTT CGGCCTGCTA
Cloned TaSUT1   AGGCCAACGC GTTCCAGGCA GGTGTCAGGG CCGGGGCGTT CGGCCTGCTA
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TaSUT1-A        CTCAACTCGG TCGTCCTGGG GTTCAGCTCG TTCCTGATCG AGCCGCTGTG
TaSUT1-B        CTCAACTCGG TCGTCCTGGG GTTCAGCTCC TTCCTGATCG AGCCGCTGTG
TaSUT1-D        CTCAACTCGG TCGTCCTGGG GTTCAGCTCG TTCCTGATCG AGCCGCTGTG
Cloned TaSUT1   CTCAACTCGG TCGTCCTGGG GTTCAGCTCC TTCCTGATCG AGCCGCTGTG

TaSUT1-A        CAAGAGGCTA GGCCCGCGGG TGGTGTGGGT GTCAAGCAAC TTCCTCGTCT
TaSUT1-B        CAAGAGGCTA GGCCCGCGGG TGGTGTGGGT GTCGAGCAAC TTCCTCGTCT
TaSUT1-D        CAAGAGGCTA GGCCCGCGGG TGGTGTGGGT GTCGAGCAAC TTCCTCGTCT
Cloned TaSUT1   CAAGAGGCTA GGCCCGCGGG TGGTGTGGGT GTCGAGCAAC TTCCTCGTCT

TaSUT1-A        GCCTCTCCAT GGCCGCCATT TGCATCATAA GCTGGTGGGC CACTCAGGAC
TaSUT1-B        GCCTCTCCAT GGCGGCCATT TGCATCATAA GCTGGTGGGC TACTCAGGAC
TaSUT1-D        GCCTCTCCAT GGCCGCGATT TGCATCATAA GCTGGTGGGC TACTCAGGAC
Cloned TaSUT1   GCCTCTCCAT GGCGGCCATT TGCATCATAA GCTGGTGGGC TACTCAGGAC

TaSUT1-A        CTGCATGGGT ACATCCAGCA CGCCATCACC GCCAGCAAGG AGATCAAGAT
TaSUT1-B        ATGCATGGGT ACATCCAGCA CGCCATCACC GCCAGCAAGG AGATCAAGAT
TaSUT1-D        TTGCATGGGT ATATCCAGCA CGCCATCACC GCCAGCAAGG AGATCAAGAT
Cloned TaSUT1   ATGCATGGGT ACATCCAGCA CGCCATCACC GCCAGCAAGG AGATCAAGAT

TaSUT1-A        CGTCTCCCTC GCCCTCTTCG CCTTCCTCGG AATCCCTCTC GCCATTCTGT
TaSUT1-B        CGTCTCCCTC GCCCTCTTCG CCTTCCTCGG AGTCCCTCTC GCCATTCTGT
TaSUT1-D        CGTCTCCCTC GCCCTCTTCG CCTTCCTCGG AATCCCTCTC GCCATTCTGT
Cloned TaSUT1   CGTCTCCCTC GCCCTCTTCG CCTTCCTCGG AGTCCCTCTC GCCATTCTGT

TaSUT1-A        ACAGTGTCCC TTTCGCGGTG ACGGCGCAGC TGGCGGCGAA CAGAGGCGGT
TaSUT1-B        ACAGTGTCCC TTTCGCGGTG ACGGCGCAGC TGGCGGCGAA CAGAGGCGGT
TaSUT1-D        ACAGTGTCCC TTTCGCGGTG ACGGCGCAGC TGGCGGCGAA GAGAGGCGGT
Cloned TaSUT1   ACAGTGTCCC TTTCGCGGTG ACGGCGCAGC TGGCGGCGAA CAGAGGCGGT

TaSUT1-A        GGCCAAGGGC TGTGCACGGG CGTGCTGAAC ATCGCCATCG TGATACCCCA
TaSUT1-B        GGCCAAGGGC TGTGCACGGG CGTGCTGAAC ATCGCCATCG TGATACCCCA
TaSUT1-D        GGCCAAGGGC TGTGCACGGG CGTGCTCAAC ATCGCCATCG TGATACCCCA
Cloned TaSUT1   GGCCAAGGGC TGTGCACGGG CGTGCTGAAC ATCGCCATCG TGATACCCCA

TaSUT1-A        GGTGATCATC GCGGTGGGGG CGGGGCCGTG GGACGAGCTG TTCGGCAAGG
TaSUT1-B        GGTGATCATC GCGGTGGGGG CGGGGCCGTG GGACGAGCTG TTCGGCAAGG
TaSUT1-D        GGTGATCATC GCGGTGGGGG CGGGGCCGTG GGACGAGCTG TTCGGCAAGG
Cloned TaSUT1   GGTGATCATC GCGGTGGGGG CGGGGCCGTG GGACGAGCTG TTCGGCAAGG

TaSUT1-A        GCAACATCCC GGCGTTCGGC GTGGCGTCCG CCTTCGCGCT CATCGGCGGC
TaSUT1-B        GCAACATCCC GGCGTTCGGC ATGGCGTCCG CCTTCGCGCT CATCGGCGGC
TaSUT1-D        GCAACATCCC GGCGTTCGGC ATGGCCTCCG CCTTCGCGCT CATCGGCGGC
Cloned TaSUT1   GCAACATCCC GGCGTTCGGC ATGGCGTCCG CCTTCCCGCT CATCGGCGGC

TaSUT1-A        ATCGTCGGCA TATTCCTGCT GCCCAAGATC TCCAGGCGCC AGTTCCGGGC
TaSUT1-B        ATCGTCGGCA TATTCCTGCT GCCCAAGATC TCCAGGCGCC AGTTCCGGGC
TaSUT1-D        ATCGTCGGCA TATTCCTGCT GCCCAAGATC TCCAGGCGCC AGTTCCGGGC
Cloned TaSUT1   ATCGTCGGCA TATTCCTGCT GCCCAAGATC TCCAGGCGCC AGTTCCGGGC

TaSUT1-A        CGTCAGCGGC GGCGGTCACT GA
TaSUT1-B        CGTCAGCGGC GGCGGTCACT GA
TaSUT1-D        CGTCAGCGGC GGCGGTCACT GA
Cloned TaSUT1   CGTCAGCGGC GGCGGTCACT GA
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