การแสดงออกของยีนตัวขนส่งซูโครสจากมันสำปะหลัง *Manihot esculenta* Crantz. ในยีสต์ *Saccharomyces cerevisiae* และลักษณะสมบัติของโปรตีน

นายยุทธนา วรวุธ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EXPRESSION OF CASSAVA *Manihot esculenta* Crantz. SUCROSE TRANSPORTER GENES IN YEAST *Saccharomyces cerevisiae* AND CHARACTERIZATION OF THE PROTEINS

Mr. Yuttana Worawut

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By	Mr. Yuttana Worawut
Field of Study	Biochemistry
Thesis Advisor	Associate Professor Tipaporn Limpaseni, Ph.D.
Thesis Co-Advisor	Malinee Suksangpanomrung, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

…….. Chairman
(Professor Anchalee Tassanakajon, Ph.D.)

……..……. Thesis Advisor
(Associate Professor Tipaporn Limpaseni, Ph.D.)

…….. Thesis Co-Advisor
(Malinee Suksangpanomrung, Ph.D.)

…….. Examiner
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

…….. Examiner
(Saowarath Jantaro, Ph.D.)

…….. External Examiner
(Assistant Professor Thidarat Eksittikul, Ph.D.)

ยุทธนา วรวุธ : การแสดงออกของยีนตัวขนส่งซูโครสจากมันสำปะหลัง *Manihot esculenta* Crantz. ในยีสต์ *Saccharomyces cerevisiae* และลักษณะสมบัติของโปรตีน. (EXPRESSION OF CASSAVA *Manihot esculenta* Crantz. SUCROSE TRANSPORTER GENES IN YEAST *Saccharomyces cerevisiae* AND CHARACTERIZATION OF THE PROTEINS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ทิพาพร ลิมปเสนีย์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.มาลินี สุขแสงพนมรุ้ง, 117 หน้า.

ตัวขนส่งซูโครส (Sucrose transporters: SUTs) เป็นโปรตีนที่มีบทบาทสำคัญในกระบวนการขนส่งซูโครสแบบใช้พลังงานในพืชชั้นสูงซึ่งส่งผลต่อการเจริญและการให้ผลผลิตของพืช งานวิจัยก่อนหน้านี้ได้ทำการแยกยีนตัวขนส่งซูโครสสี่ชนิดได้แก่ *MeSUT1, MeSUT2, MeSUT4* และ *MeSUT5* จากห้องสมุดดีเอ็นเอของหัวและใบมันสำปะหลัง (*Manihot esculenta* Crantz.) งานวิจัยนี้ทำการศึกษาสมบัติทางชีวเคมีของโปรตีนขนส่งซูโครสแต่ละชนิดโดยได้ทำการแสดงออกยีนในระบบยีสต์ SUSY7/ura3 (*Saccharomyces cerevisiae*) ซึ่งถูกดัดแปลงพันธุกรรมให้ไม่สามารถสร้างเอนไซม์ invertase ได้ ยีนตัวขนส่งซูโครสจากมันสำปะหลังทั้งสี่ยีนได้ถูกทรานส์ฟอร์มเข้าเซลล์ยีสต์และพบว่าโปรตีนที่ถูกสร้างขึ้นสามารถทำงานได้ในระบบนี้ การขนส่ง ^{14}C-sucrose เข้าเซลล์ยีสต์ที่มีการแสดงออกของยีน *MeSUTs* ทุกชนิดขึ้นกับ pH แต่มีแอคติวิตี้ที่เหมาะสมที่ pH ต่างกันโดย MeSUT1 ทำงานได้ดีที่สุดที่ pH 6 ในขณะที่การนำเข้าซูโครสโดย MeSUT2, MeSUT4 และ MeSUT5 มีอัตราสูงสุดที่ pH ประมาณ 7 MeSUT1 จัดเป็นตัวขนส่งซูโครสชนิด high affinity/low capacity โดยมีค่า K_m และ V_{max} เท่ากับ 1.15±0.11mM และ 0.06 nmol/mg FW/min ในขณะที่ MeSUT2 จัดอยู่ในกลุ่ม low affinity/high capacity ซึ่งมีค่า K_m และ V_{max} เท่ากับ 12.55±3.58 mM และ 0.36±0.10 nmol/mg FW/min ตามลำดับ ค่า K_m และ V_{max} ของ MeSUT4 เท่ากับ 42.25±1.54 mM และ 1.58±0.13 nmol/mg FW/min และของ MeSUT5 เท่ากับ 51.37±5.30 mM และ 1.10±0.12 nmol/mg FW/min ตามลำดับ ซึ่งแสดงว่าโปรตีนทั้งสองชนิดเป็นตัวขนส่งซูโครสแบบ low affinity/high capacity ตัวขนส่งซูโครสทั้งสี่ชนิดมีการตอบสนองต่อตัวยับยั้งและน้ำตาลชนิดต่างๆที่แตกต่างกัน อย่างไรก็ตาม ตัวขนส่งซูโครสทั้งสี่ชนิดถูกยับยั้งอย่างรุนแรงด้วย CCCP และ antimycin A ซึ่งสอดคล้องกับลักษณะของตัวขนส่งซูโครสแบบใช้พลังงาน จากผลการตอบสนองต่อ pH และตัวยับยั้งชนิดต่างๆ สามารถสันนิษฐานได้ว่า MeSUT1 น่าจะเป็นตัวขนส่งซูโครสแบบ sucrose/H^+-symporter ในขณะที่ MeSUT2, MeSUT4 และ MeSU5 น่าจะเป็นตัวขนส่งซูโครสชนิด sucrose/H^+-antiporter เยื่อหุ้มเซลล์ที่สกัดจากหัวมันสำปะหลังมีอัตราการจับซูโครสสูงสุดที่ pH 8 และมีค่า K_m และ V_{max} เท่ากับ 19.16±6.44 mM และ 1.47±0.54 μmol/mg protein/h ตามลำดับ ซึ่งสอดคล้องกับข้อมูลจากการขนส่งซูโครสในแผ่นหัวมันสำปะหลัง ในการสังเคราะห์ *MeSUTs* cRNA ทั้งสี่ชนิดและแสดงออกในไข่กบ (*Xenopus laevis* oocytes) พบว่า *MeSUT2* ไม่สามารถแสดงออกได้ในระบบนี้ ในขณะที่ไข่กบที่มีการแสดงออกของยีน *MeSUT1* และ *MeSUT5* มีปริมาณกระแสไฟฟ้าเหนี่ยวนำน้อยมากและไม่สามารถใช้ในการวิเคราะห์ต่อไปได้ ไข่กบที่มีการแสดงออกของยีน *MeSUT4* มีปริมาณกระแสไฟฟ้าเหนี่ยวนำที่ชัดเจนและมีค่า $K_{0.5}$ ต่อซูโครสเท่ากับ 12.35 mM ซึ่งสนับสนุนว่า MeSUT4 เป็นตัวขนส่งซูโครสชนิด low affinity

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา..... 2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4773830323 : MAJOR BIOCHEMISTRY
KEYWORDS: SUCROSE TRANSPORTER / CASSAVA / *Manihot esculenta* Crantz / YEAST / FUNCTIONAL CHARATERIZATION

YUTTANA WORAWUT: EXPRESSION OF CASSAVA *Manihot esculenta* Crantz. SUCROSE TRANSPORTER GENES IN YEAST *Saccharomyces cerevisiae* AND CHARACTERIZATION OF THE PROTEINS. THESIS ADVISOR: ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D. THESIS CO-ADVISOR: MALINEE SUKSANGPANOMRUNG, Ph.D., 117 pp.

Sucrose transporter proteins (SUTs) play a pivotal role in active sucrose transport processes in higher plants which affect the plant maturation and crop production. Four sucrose transporter genes in cassava (*Manihot esculenta* Crantz.) designated as *MeSUT1*, *MeSUT2*, *MeSUT4* and *MeSUT5*, have been isolated from leaf and storage root cDNA libraries. In order to obtain the biochemical properties of each MeSUTs, plasmids containing each of MeSUT cDNAs were constructed and used for heterologous expression in an invertase deficient SUSY7/ura3 yeast (*Saccharomyces cerevisiae*) mutant. All four cassava sucrose transporter genes were successfully transformed and they were able to complement the yeast SUSY7/ura3 phenotypes, suggesting the proteins were functional in yeast. ^{14}C-sucrose uptake by yeast expressing *MeSUT*s was pH dependent but their optimum activities were observed at different pH. MeSUT1 was highly active at pH 6, while the highest sucrose uptake activities of MeSUT2, MeSUT4 and MeSUT5 were observed at pH~7. MeSUT1 was a high affinity/low capacity sucrose transporter with the K_m and V_{max} of 1.50±0.11 mM and 0.06 nmol/mg FW/min, while those of MeSUT2 were 12.55±3.58 mM and 0.36±0.10 nmol/mg FW/min, indicating that MeSUT2 belonged to low affinity/high capacity type. MeSUT4 and MeSUT5, the isoforms of cassava SUT4, showed the K_m and V_{max} of 42.25±1.54 mM and 1.58±0.13 nmol/mg FW/min and 51.37±5.30 mM and 1.10±0.12 nmol/mg FW/min, respectively, suggesting that they were low affinity/high capacity sucrose transporters. The responses to various inhibitors and sugars of these four MeSUTs were different, however, all were strongly inhibited by CCCP and antimycin A, supporting the characteristics of active transport carriers. From the sensitivity towards pH and several metabolic inhibitors, it was proposed that MeSUT1 might be sucrose/H^+-symporter, while MeSUT2, MeSUT4 and MeSUT5 were possibly sucrose/H^+ -antiporters. The sucrose binding activity of plasma membrane of cassava tubers was pH dependent with the highest activity observed at pH 8 and its K_m and V_{max} were 19.16±6.44 mM and 1.47 ± 0.54 μmol/mg protein/h, respectively. cRNA of all four *MeSUTs* were synthesized and expressed in *Xenopus laevis* oocytes. Expression of MeSUT2 was not successful whereas *MeSUT1* and *MeSUT5* expressing oocytes showed small induced currents but too small to be used for further analysis. MeSUT4 expressing oocytes showed clear inward currents and the $K_{0.5}$ for sucrose transport was 12.35 mM, supporting its low affinity characteristics.

Department : Biochemistry Student's Signature .....
Field of Study : Biochemistry Advisor's Signature .....
Academic Year : 2010 Co-Advisor's Signature .....

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CONTENTS

Pages

Pages

Pages

LIST OF TABLES

LIST OF FIGURES

Figure **Pages**

Figure **Pages**

LIST OF ABBREVIATIONS

Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
Bq	Baquerel
CCCP	carbonyl cyanide-*m*-chlorophenylhydrazone
°C	degree Celsius
cDNA	complementary DNA
cRNA	complementary RNA
Ci	curies
cm	centimeter
DMSO	dimethysulfoxide
dNTP	deoxyribonucleotide-5´-triphosphate
DNA	deoxy ribonucleic acid
2, 4-DNP,	2, 4-dinitrophenol
dpm	disintegration per minute
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	disodium ethylenediaminetetraacetic acid
FW	fresh weight
g	gram
g	gravity force
h	hour
kb	kilobase pair
kDa	kilodalton
K_m	Michaelis constant
l	litre
LB	Luria-Bertani medium
M	molar
m	meter
μM	micromolar
mg	milligram

LIST OF ABBREVIATIONS (Continued)

min	minute
ml	milliliter
mM	millimolar
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger RNA
NEM	*N*-metylmaleimide
ng	nanogram
nmol	nanomole
mm	millimeter
OD	optical density
PCMBS	*p*-chloromecuribenzene sulphonate
PCR	polymerase chain reaction
PEG	polyethylene glycol 3350
%	percentage
PMSF	phenylmethysulfonyf fluoride
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
rpm	round per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris-hydrochloric acid
U	unit
V	volt
v/v	volume per volume
w/v	weight per volume

CHAPTER I
INTRODUCTIONS

Sucrose plays pivotal roles in the plant kingdom. Apart from being the primary product of photosynthesis and the main form of carbon transport in plants, sucrose represents the most abundant form of soluble storage carbohydrate and also serves as a signaling molecule that triggers essential metabolic events. Furthermore, it plays a major role in plant reproduction and propagation. There are other soluble sugars present in plants, however, they are usually accompanied by high levels of sucrose in all conditions. Moreover, sucrose is the basic carbohydrate that is important for building all other organic compounds in plants and most other carbohydrates in nature, supporting the position of plants as the foundation of the energy food chain.

The reason that sucrose is present in every part in plants is unobvious. In comparison with trehalose and raffinose-based saccharides which are commonly found in various plant species, several hypotheses have been postulated (Pontis, 1977). Based on the process of natural selection to perform equivalent functions, the assumption is that these molecules must share some properties crucial for physiological relevance. These saccharides possess a common characteristic that they are non reducing sugars. Non-reducing molecules are less reactive and less susceptible to breakdown by the cellular enzymatic environments. The high energy released from hydrolysis of glycosidic linkage makes these molecules more valuable as energy currency and are readily available as carbon sources. Maltose and lactose are the other two disaccharides found in plants and they also have glycosidic linkages, however, these sugars yielded less than half the energy of hydrolysis of sucrose.

1.1 Two pathways of sucrose transport: symplastic movement and apoplastic transport

In chlorophyll containing cells, glucose 1-phosphate and fructose 6-phosphate are synthesized in the cytosol from triose-phosphates produced in the Calvin cycle and exported from the chloroplast. Sucrose is synthesized from UDP-glucose and fructose-6-phosphate in a sequence of two reactions catalyzed by sucrose phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP, Figure 1.1). Both enzymes

are localized in the cytosol and appear to form a metabolic unit during synthesis (Echeverria *et al.*, 1997).

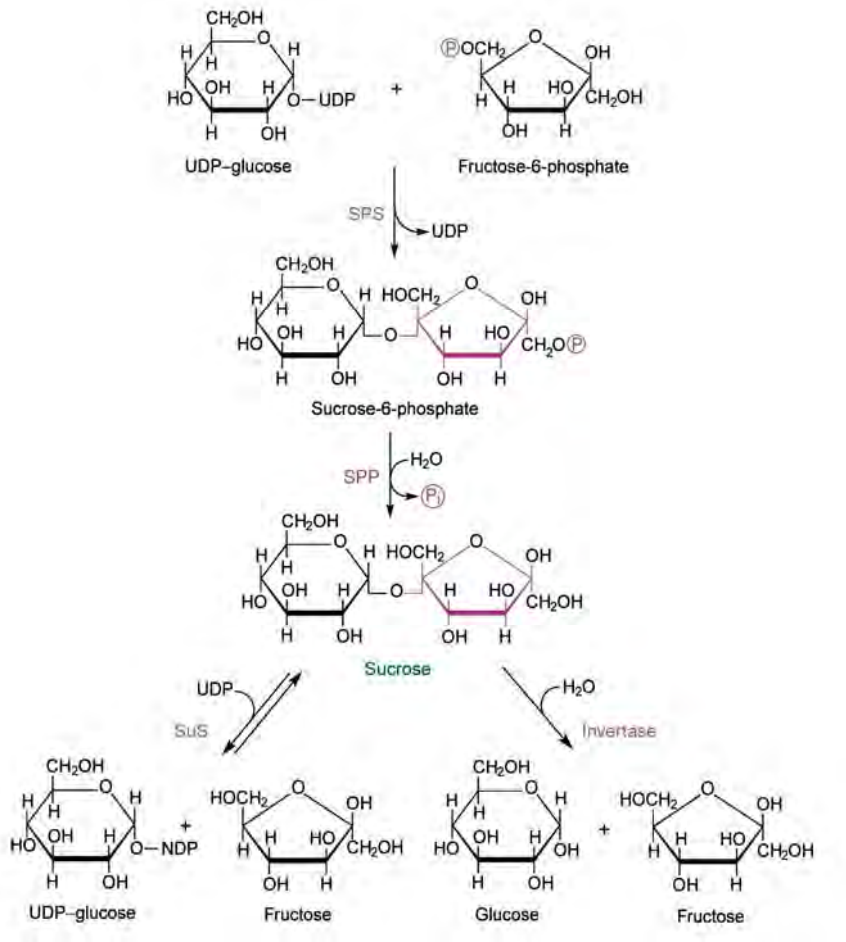


Figure 1.1 Sucrose synthesis pathways in plants. In photosynthetically active tissues, sucrose is synthesized from a sequence of two continuing reactions catalyzed by sucrose phosphate synthase (SPS) and sucrose phosphate phosphathase (SPP). Sucrose is then hydrolyzed by several pathways such as cleavage by sucrose synthase (SuS) or invertase to yield the hydrolysis products for further plant utilization (Salerno and Curatti, 2003).

In photosynthetic cells, newly synthesized sucrose has two potential fates depending on cellular, physiological, and environmental factors. Sucrose is either stored in the vacuole and/ or exported to supply carbon to heterotrophic cells. In the time of high photosynthetic activity and limited phloem loading capacity, excess sucrose is temporary stored in the vacuole. The movement of sucrose from mesophyll cells to the phloem elements can take various routes depending on plant species, and it involves different cell types. Figure 1.2 illustrates two pathways of sucrose transport in higher plants. The first pathway is symplastic transport which involves the movement of sucrose from the exporting cells to another via connection between cells called plasmodesmata. The second is apoplastic transport, which sucrose is released into the extracellular between cells namely apoplast by diffusion across cell wall (Saure, 2007). After reaching the sieve element/companion cell complex (SE/CC), sucrose is actively taken up by the plasma membrane bound sucrose transporters and the accumulation of sucrose in the SE/CC increases the hydrostatic pressure which drives mass flow of phloem sap for long distance transport to other plant organs via the phloem (Lalonde *et al.*, 1999).

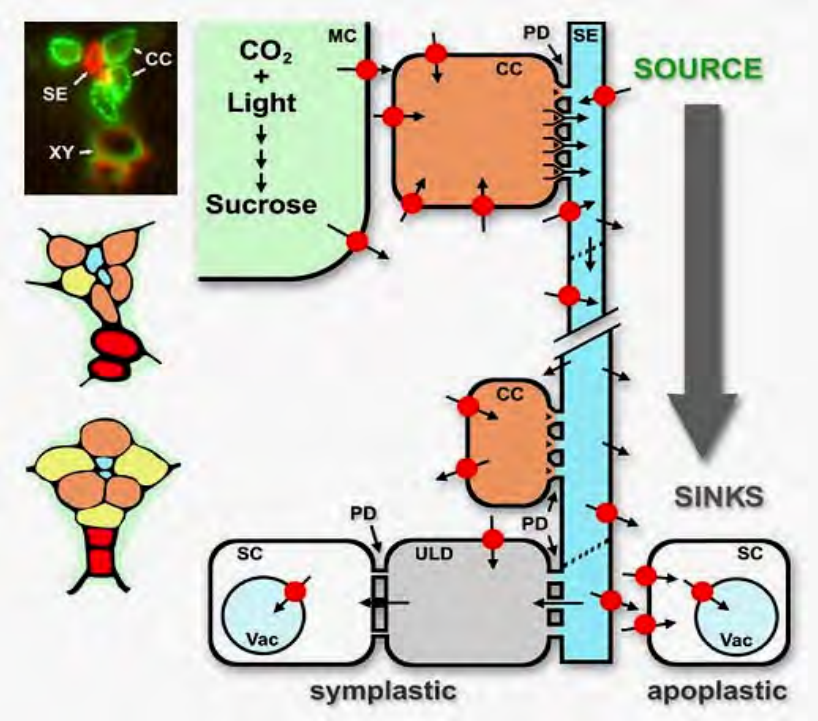


Figure 1.2 Pathway for sucrose transport in higher plants. The sucrose synthesized from photosynthetically active tissues (referred as source organs) is transported to other organ unable to perform photosynthesis (sink organs) via two pathways. Symplastic transport is the pathway that sucrose moves from one cell to another via plasmodesmata connectivity using water potential as a driving force. Another fate is apoplastic movement which sucrose is released from source organs to apoplast and the process is active transport carriers (closed circles). Depending on the plant species, the arriving sucrose is then taken up by two sink types either symplastic loading sink or apoplastic sink. Abbreviations: XY, xylem; SE, sieve element; CC, companion cell; MC, mesophyll cell; PD, plasmodesmata (branched or non-branched); ULD, unloading domain; SC, sink cell; Vac, vacuole (Sauer, 2007).

1.2 The importance of plasmalemma localized sucrose transporters in phloem loading, maintenance of phloem flux and releasing of sucrose into apoplastic loaders

Sucrose partitioning between the mesophyll layers in source leaf (photosynthesizing site) and several green and non-green sink tissues of an apoplastically loading plant begins at the highly specialized SE/CC complex of the phloem. Membrane-localized, energy-dependent, H^+-symporting sucrose transporter proteins (typically named SUC for sucrose carrier or SUT for sucrose transporter) are responsible for the loading of sucrose into these cells (Figure 1.3). Furthermore, they are important for maintaining the sucrose concentration within the phloem and probably also for the release of sucrose into the apoplast to feed certain apoplastic sinks such as guard cells, developing pollen grains, pollen tubes or developing embryos (Stadler *et al.*, 2005a). The other sinks such as root tips or the outer integuments of seed coats are called symplastic sinks or unloading domains (ULDs), which are connected to the phloem via plasmodesmata (Stadler *et al.*, 2005a; 2005b). In these cases, the plasmodesmata between the terminal SEs of the sink phloem and the cells of the ULDs have large size exclusion limits (>60 kDa) that allow effective symplastic movement of sucrose into these ULDs (Stadler *et al.*, 2005a; 2005b). Therefore, phloem-localized sucrose transporters do not seem to be involved and the transport from the ULDs into the sinks is subsequently mediated by smaller size exclusion (<30 kDa) plasmodesmata (Patrick and Offler, 1995; Stadler *et al.*, 2005a; 2005b). In apoplastic loaders, newly synthesized sucrose is released from the mesophyll into the apoplastic space where extracellular sucrose concentrations of 2–7 mM were observed (Livingston and Henson, 1998; Lohaus *et al.*, 2000). This initial step of sucrose partitioning is not fully understood whether it is mediated by specific sucrose transporter in any plant species. However, sucrose uptake into mesophyll specific leaf plasma membrane vesicles extracted from potato wild type plants showed proton-motive force dependent (Lemoine *et al.*, 1996). The loading of sucrose into the SE/CC complex has been characterized in detail and responsible transporters were identified in several plant species (Riesmeier *et al.*, 1994; Stadler *et al.*, 1995; Lemoine *et al.*, 1996; Stadler and Sauer, 1996; Kühn *et al.*, 1997; Bürkle *et al.*, 1998).

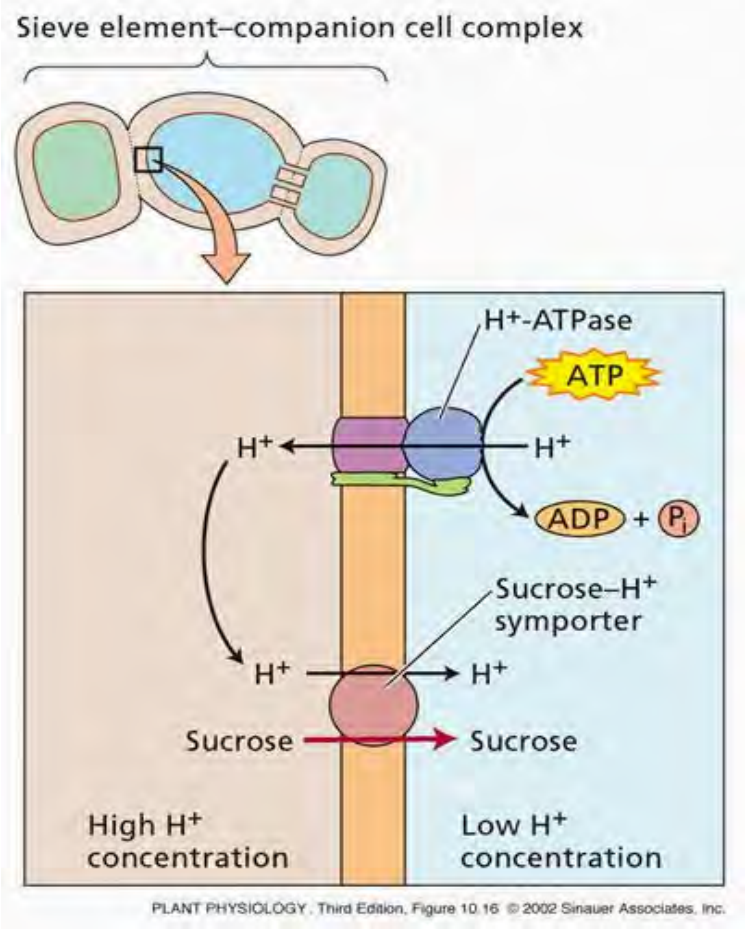


Figure 1.3 Proposed schematic model of active sucrose transport mediated by sucrose/H^+-symporter. One ATP is hydrolyzed by ATPase to release energy for pumping proton against its concentration gradient from inside to outside of the cells. The proton is moved down its concentration gradient together with one sucrose molecule to inside of the cell via sucrose/H^+-symporter (Taiz, 2002).

Definite proof for essential role of these transporters in phloem loading was obtained with transgenic potato plants expressing antisense *StSUT1* cDNA (Riesmeier *et al.*, 1994), tobacco plants expressing *NtSUT1* in antisense (Bürkle *et al.*, 1998) and in *Arabidopsis* plants carrying a T-DNA insertion in the *AtSUC2* gene (Gottwald *et al.*, 2000). These plants exhibited partial or complete blockage of sucrose export from

source leaves which subsequently increase the levels of soluble carbohydrates and starch in their source leaves. The reduction of carbon export led to chlorotic lesions in the leaves as well as inhibition of sink development and consequently reduced plant growth. Unexpectedly, promoter/reporter gene analyses as well as immunolocalization studies also showed the expression of these transporter genes in the phloem of stems, roots and several non-green sink tissues. These indicated that phloem-loading sucrose transporters may also be responsible for the retrieval of sucrose from the extracellular space and they may even catalyze the export of sucrose from the phloem (Truernit and Sauer, 1995; Kühn *et al.*, 2003). The hypotheses supported by electrophysiological analyses of *Xenopus laevis* oocytes expressing the cRNA of maize ZmSUT1demonstrated that sucrose transporters can mediate both sucrose uptake and release (Carpaneto *et al.*, 2005).

1.3 The electrogenic transport and several substrates recognitions by functional plant sucrose transporters

The sucrose transporter cDNAs was firstly cloned and expressed in bakers' yeast (*Saccharomyces cerevisiae*) which allowed determining of the kinetic properties of individual sucrose transporters. Typically, phloem-loading sucrose transporters, have a low pH optimum for sucrose transport and they are sensitive to proton gradients uncoupling agents as well as thiol group inhibitors [e.g. p-chloro-mercuribenzenesulphonic acid (PCMBS)]. Also, their K_m values are in the range upto 11.5 mM (Riesmeier *et al.*, 1992; Gahrtz *et al.*, 1994; Sauer and Stolz, 1994; Knop *et al.*, 2004). Although several of these characteristics described support the idea of proton and membrane potential-driven sucrose uptake, the definite evidence is confirmed by analyses of sucrose-induced proton currents in *Xenopus* oocytes. According to analyses on *Xenopus* oocytes, sucrose and proton are co-transported in a 1:1 stoichiometry and provided detailed information on the affinity of sucrose transporters to protons. Moreover, it helps explaining the complex interrelationship among membrane potential, pH gradient and substrate concentration, and the effects of these parameters on the direction of sucrose transport (Boorer *et al.*, 1996; Zhou *et al.*, 1997; Carpaneto *et al.*, 2005). The analyses of substrate specificities for sucrose

transporters were generally based on inhibitory effects of potential substrates on the uptake of ^{14}C-labelled sucrose (Riesmeier *et al.*, 1992; Gahrtz *et al.*, 1994; Sauer and Stolz, 1994). The results from these experiments suggested that maltose might also be a substrate for plant sucrose transporters and the other α- and β-glucosides may be accepted as substrates. No evidence was obtained for transport of the trisaccharide raffinose. Additional analyses in *Xenopus* oocytes confirmed the broad selectivity towards several substrates and revealed the proton induced currents also for other naturally occurring glucosides such as arbutin, an aryl-β-glucoside found in coffee plants (*Coffea arabica* L.), and salicin, a salicylic acid derivative frequently found in willow (Chandran *et al.*, 2003; Sivitz *et al.*, 2005) or the synthetic sweetener sucralose, a chlorinated sucrose derivative (Sivitz *et al.*, 2007). Surprisingly, a cDNA for the *Arabidopsis* sucrose transporter *AtSUC5* was identified during a complementation screening for plant biotin transporters in a biotin uptake-defecient yeast mutant (Ludwig *et al.*, 2000). Beside sucrose, AtSUC5 protein was also able to catalyze uptake of biotin across yeast plasma membranes and biotin transport was not inhibited by sucrose. Thus, it is possible that biotin uptake may be a general property of all plant sucrose transporters (Ludwig *et al.*, 2000). The fact that other complementing cDNAs could not be found in this screening suggested that sucrose transporter-mediated transport of biotin may be physiologically relevant.

1.4 Classification of SUTs

Based on amino acid sequences homology and phylogenetic analysis, SUTs can be classified into three types designated as SUT1/SUC2 type, SUT2/SUC3 type and SUT4 type. In dicot plants, each type corresponds with its structural or functional differences: SUT1/SUC2 has a high affinity for sucrose with the Km value ~1 mM, SUT4 has a tenfold lower affinity and SUT2 which contains a highly conserved domain within the extended central loop in most members of the family, is a low affinity system (Figure 1.4). Usually, *SUT2* and *SUT4* exist as single copies in dicotyledon genomes while *SUT1* is often present in multiple copies. For example, nine genes were discovered in *Arabidopsis* genome which composed of seven SUT1-types and one each of SUT2/SUC3-type and SUT4 type. Among these, seven genes

were demonstrated to encode functional proteins (Stadler *et al.*, 1999; Barker *et al.*, 2000; Gottwald *et al.*, 2000; Meyer *et al.*, 2000; Weise *et al.*, 2000; Sauer *et al.*, 2004; Baud *et al.*, 2005).

1.4.1 Sucrose transporter SUT1/SUC2 type

SUT1/SUC2 members typically possess a high affinity for sucrose with K_m between 0.3-1.8 mM (Riesmeier *et al.*, 1992; Sauer and Stolz, 1994; Zhou *et al.*, 1997; Shakya and Sturm, 1998). In solanaceous species, SUT1 localizes on the phloem plasma membrane of leaves, petioles, and stems in source and sink tissues and all along the translocation path. There are evidences from various plant species suggesting that *SUT1*mRNA is synthesized in CCs and transports through plasmodesmata into SE (Kühn *et al.*, 1997; Ruiz-Medrano *et al.*, 1999; Knop *et al.*, 2001). The expression of *SUT1/SUC2* along the translocation path suggests its role in keeping high osmotic pressure in SE, by retrieval of sucrose that lost through passive leakage. SUT1/SUC2 must be important for regulating plant development because transgenic lines or mutants showed a reduction of sucrose export from leaves and subsequently reduced the supplying of carbohydrate to sink organs (Riesmeier *et al.*, 1994; Bürkle *et al.*, 1998; Gottwald *et al.*, 2000). NtSUC3 and AtSUC1 which are the orthologs of SUT1/SUC2 seem to play a role in distribution of nutrients to pollen and they are derived from gene duplication after separation of the plant families (Lemoine *et al.*, 1999; Stadler *et al.*, 1999). In comparison with other plant species, the *Arabidopsis* genome possesses additional members of SUT1 type which distribute across seven paralogs. Nevertheless, AtSUC2 paralog plays the most pivotal SUT1 function since knockout of this gene leads the plant to be lethal (Gottwald *et al.*, 2000).

Most of functionally characterized SUT1/SUC2 are identified as sucrose/H^+ symporters since they are highly active at acidic environments, with almost no sucrose transports observed at more alkaline pH. Moreover, SUT1 activity is strongly inhibited by proton uncouplers and highly sensitive to sulfhydryl modifying reagents (Bürkle *et al.*, 1998; Stadler *et al.*, 1999; Knop *et al.*, 2004). However, some evidences suggested the confliction of typical SUT1 characteristics. Even in the same plant, sucrose uptake by yeast expressing *AtSUC1* showed the highest rate at pH 5-6

and was assigned to be neutral transporter whereas AtSUC2 was highly active at acidic pH, suggested different roles in sucrose distribution of the two paralogs (Sauer and Stolz, 1994). Similar observations were found from yeast expressing two Arabidopsis SUT1 paralogs, AtSUC8 was hardly inhibited by PCMBS while AtSUC9 was highly sensitive to this compound (Sauer *et al.*, 2004). In addition, PsSUF1, being structurally related to SUT1, was functional in yeast but the activity was not energy and proton dependent. It also has a low affinity to sucrose and was not pH sensitive, the features correspond to facilitative carrier, implying that the sequence similarity only reflects the evolutionary development.

1.4.2 Sucrose transporter SUT2/SUC3 type

Similar to yeast hexose transporter (HXT)-like proteins which possesses the extended domains functioning as sensors, it has been hypothesized that SUT2 may serve as a sucrose sensor. In bacteria and yeast, many transport proteins display the sensing functions, transmitting information about the external concentration or metabolite flux to downstream signaling cascades (Lalonde *et al.*, 1999). Transporter-like sensors were also found in yeast which functions as measuring extracellular hexose levels and controlling the expression of HXT genes (Ozcan *et al.*, 1996; Ozcan *et al.*, 1998). The extended C-terminal domains of SNF3 and RGT2 contain conserved motifs that serve as signaling domains (Ozcan *et al.*, 1998). In addition, the transporter-like sensors typically show low expression and no detectable or only weak transport activity (Schulze *et al.*, 2000; Barth *et al.*, 2003). In plants, SUT2/SUC3 type possesses special features which contains the extended amino acids (about 30 amino acids) at the N-terminus and the central cytoplasmic loop (about 50 amino acids), thereby has the higher molecular mass (Barker *et al.*, 2000). Due to the structural similarity to yeast sugar sensors and their inability to compliment the phenotype of invertase deficient yeast mutant, LeSUT2 and AtSUT2 were proposed to be the sucrose sensor rather than transporter (Barker *et al.*, 2000). However, later experiment on yeast expressing AtSUT2 was able to prove the sucrose transport activity which showed low affinity for sucrose (11.5 mM (Schulze *et al.*, 2000)). The N-terminus of SUT2/SUC3 determined the substrate affinity which the chimeric protein of N-terminus of AtSUT2 and C-terminus of StSUT1 showed lower affinity

for sucrose than StSUT1 (Schulze *et al.*, 2000). Therefore, the sucrose sensor assumption has been modified to be the sucrose flux sensor, playing a role in measuring the rate of sucrose transport across the plasma membrane (Schulze *et al.*, 2000).

As relevance from the co-localization of LeSUT2 with other SUTs and the indirect evidence from split ubiquitin analysis, another model has been proposed that all sucrose transporters might interact with each other to form heterooligomers, and thus regulate the sucrose transport rate (Barker *et al.*, 2000; Reinders *et al.*, 2002). In contrast, the LeSUT2 ortholog, PmSUC3 was not co-localized with the high affinity PmSUC1 and might be functioned in retrieval of sucrose along the translocation path. Moreover and base on their localization, SUT2/SUC3 type was found to be expressed in phloem sieve element (SEs) as well as in sink tissues (Barth *et al.*, 2003; Meyer *et al.*, 2004; Hackel *et al.*, 2006), which might be a sucrose efflux carriers and involve in phloem unloading into apoplast. SUT2 type from monocots shows no extended central loop and may have a function equivalent to dicotyledon SUT1/SUC2 members. Expression profile, expression level, and transport properties suggest that the SUT2 type member OsSUT1 from rice, which lacks the extended loop, serves as an ortholog of SUT1/SUC2. Thus, additional work is required to determine whether SUT2 members serve as sensors or simply as a second set of low-affinity transporters. In case SUT2 does not act as a sucrose sensor, other membrane proteins must be responsible for this function because physiological analyses strongly suggest that plasma membrane–bound sugar-/sucrose-sensing pathways exist in plants (Martin *et al.*, 1997).

1.4.3 Sucrose transporter SUT4 type

Kinetic analysis of sucrose uptake revealed the presence of multiple kinetic components (Delrot and Bonnemain, 1981; Maynard and Lucas, 1982) with high-affinity/low-capacity (HALC) and low-affinity/high-capacity (LAHC) systems in plants. SUT1 type fits the HALC kinetics whereas the properties of most SUT4 subfamily members correspond to LAHC (Weise *et al.*, 2000). Although SUT4 possesses amino acid sequences with molecular weight similar to SUT1, it exhibits different biochemical properties and gene expression patterns. Members of the SUT4-

type typically exhibit K_m values varying between 6.0 and 11.7 mM (Weise *et al.*, 2000; Zhou *et al.*, 2007; Reinders *et al.*, 2008; Zhang *et al.*, 2008), and are characterized as low affinity / high-capacity (LAHC) transport systems that have a low affinity for sucrose but facilitate the movement of large quantities of sucrose when the sucrose concentrations are high. In tobacco, StSUT4 is localized in the plasma membrane of sieve elements in source leaves and has been shown to be a high capacity transporter (Weise *et al.*, 2000). The high expression levels of Arabidopsis *AtSUT4* and lotus *LjSUT4* in sink tissues, together with recent localization of AtSUT4 and barley HvSUT4 in the tonoplast, led to a suggestion that they were functionally related to sink capacity (Endler *et al.*, 2006). In Solanaceae, SUT4 colocalizes with SUT1 and SUT2 in SE (Barker *et al.*, 2000; Weise *et al.*, 2000), and analyses using the split ubiquitin system indicate that SUT4 can interact with SUT1 and SUT2 (Reinders *et al.*, 2002; Schulze *et al.*, 2003). However, the functional role of the potential heterodimer remains to be further investigated.

1.5 Structural analyses of plant sucrose transporters

The structures of sucrose transporters are predicted to be composed of 12 transmembrane helices as relevance from independent hydropathy analyses using different plant sucrose transporter protein sequences (e.g. (Riesmeier *et al.*, 1992; Sauer and Stolz, 1994)). Immunolocalization studies with site-specific antibodies revealed the predicted even number of transmembrane helices and showed that N- and C-termini of plasma membrane SUT proteins are on the cytoplasmic side of the membrane (Stolz *et al.*, 1999). The hypothesis stated that gene duplication and fusion of plant sucrose transporters evolved from one or more ancestral transporters which contain only six transmembrane helices was confirmed by alignments of available plant sucrose transporter sequences from all three types with sequences of other H^{+}-sugar symporters. This is also supported the membership of plant sucrose transporters that they belong to the major facilitator superfamily (Marger and Saier, 1993). This information was used to create and compare the structural models of sucrose transporters of the three different types (Figure 1.4). The obvious differences were observed in transmembrane helices VI and VII (central cytoplasmic loop) or VII and VIII.

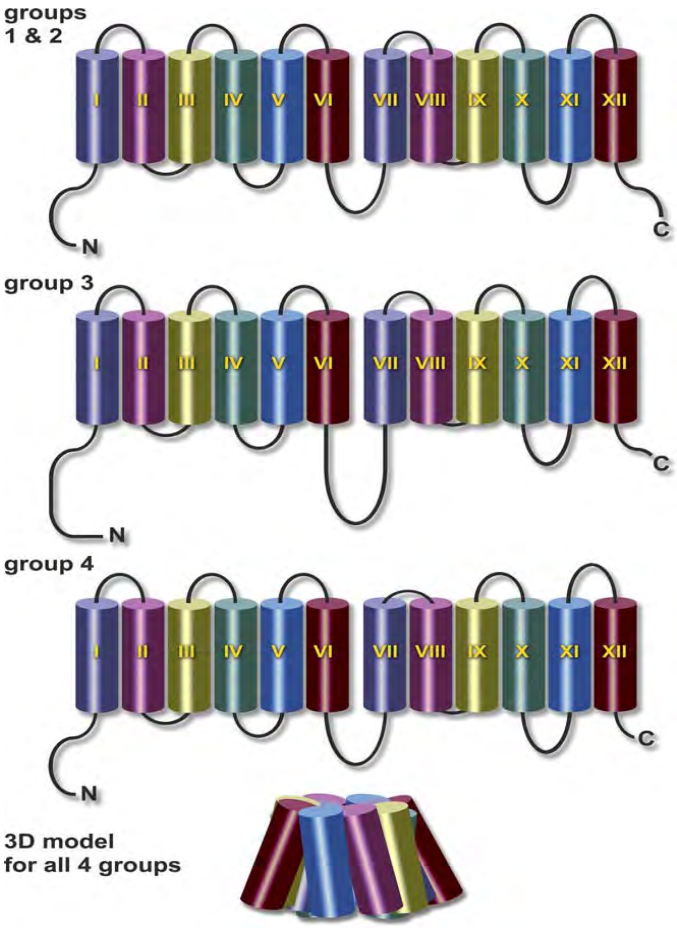


Figure 1.4 Predicted structures of plant sucrose transporters. Group 1 and group 2 are SUT1 type from monocots and dicots, group 3 is SUT2 type and group 4 is SUT4 type, respectively. The bottom model represents the hypothetical structure of SUT arranging in the plasma membrane (Sauer, 2007).

SUT2 type (group 3 in Figure 1.4) possesses the different structure from other SUTs that it contains longer amino acid sequence (15-20%) with elongated N-terminus (about 20 amino acids) and enlarged central cytoplasmic loop (about 60 amino acids). However, this type has shorter C-termini than SUT1 (groups 1 and 2). The predicted vacuolar transporter SUT4 contains shorter amino acid sequence than other two SUT types which possesses the shortest C-termini and also very short linker sequence between the transmembrane domain VII and VIII. The available crystal structures of

other transporters (LacY and GlpT) were used to predict the potential arrangements of sucrose transporters when they are in the plasma membrane or tonoplast membrane (Abramson *et al.*, 2003; Huang *et al.*, 2003). The sequence similarity between SUTs and the published LacY and GlpT structures can be used to predict the working structure that the transmembrane helices I, IV, VII and X form an hourglass like structure and the substrate pore involving mediation of sucrose transport is surrounded by the other eight helices (Figure 1.4 bottom).

1.6 Sucrose transport into vacuoles

Central vacuoles are large and can occupy more than 80% of the total cell volume (Winter *et al.*, 1993), separating from the surrounding cytosol by a single semi-permeable membrane (so called tonoplast). They are also important for storage of various compounds. Moreover, the large size allows central vacuoles to be responsible for other plant metabolic process such as management of cellular energy, accumulation of reserves and nutrients, regulation of cellular pressure, detoxification and ecological interactions. Sucrose is stored in the vacuoles at a high concentration but there are also high levels of the other monosaccharides glucose and fructose typically present in this organelle (ap Rees, 1994). In C3 and CAM plant, sucrose import into vacuoles uses the passive transport driven by the existing concentration gradient between cytosol and vacuolar lumen while the sugar beet taproot vacuole imports sucrose against its concentration using proton motive force to drive the sucrose movement via an H^+-antiport mechanism (Getz, 1987).

There are several lines of evidences investigating the sucrose transport into vacuoles of storage organs of various economically important plants such as red beet, sugar beet and sugarcanes. Although vacuoles are the important organelles for storage of sucrose, genes encoding for vacuolar sucrose transporters are still unidentified. In contrast to sucrose transport at plasma membrane mediated by sucrose/H^+-symporters which imports sucrose and proton with the stoichiometry of 1:1, sucrose transport at the vacuolar membrane appeared to process via sucrose/H^+ antiporter. In agreement with the acidic pH inside of the vacuoles, sucrose transport by red beet tonoplast membrane vesicles showed the optimum activity at pH 7 and the activity was stimulated by addition of ATP. The affinity for sucrose is high with the K_m for

sucrose of 1.7 mM (Getz, 1991). More recent and in contrast to the results from sucrose uptake in red beet tonoplast vesicles, sucrose uptake into tonoplast vesicles from sweet lime fruit was assigned to be facilitate diffusion as the addition of sucrose or hexoses did not interrupt the pH gradient (in the presence of ATP) generated over the tonoplast membrane (Echeverria *et al.*, 1997).

Sucrose transport into isolated tomato fruit tonoplast vesicles showed the sensitivity towards PCMBS but the activity was not stimulated by ATP. Furthermore, regulation of pH and sucrose gradient over tonoplast membrane as well as sucrose gradient between source and sink tissues appeared to be mediated by hydrolyzing enzyme activity rather than active sucrose uptake (Milner *et al.*, 1995). Moreover, the similar results were observed from sugarcane suspension cells that sucrose uptake was not energized by ATP and proton efflux could not be measured. Therefore, the passive but carrier mediated sucrose transport was postulated (Preisser and Komor, 1991). Further evidence on heterologous expression of putative vacuolar sucrose carrier from sugar beet in sucrose uptake deficient yeast mutant did not show an ability to complement yeast phenotype. The antibodies rose against the purified sucrose carrier peptide showed the migration corresponding to the marker from tonoplast fraction after isolation by floatation centrifugation. However, sucrose uptake activity was not observed in isolated sugar beet tonoplast membrane vesicles which have been shown to be transport competence (Chiou and Bush, 1996).

1.7 Regulation of sucrose transport

The sink organ's requirements are influenced by the communications between sources and sink which in turn affect the photosynthesis and transport of sucrose (Quick, 1998). Regulation of sucrose transport is thus an essential mechanism to regulate various whole-plant responses. Sucrose transport regulation is thought to be affected by several factors. Environmental factors and endogenous signals affect sugar export from leaves by controlling sucrose transport (Frommer *et al.*, 1996; Bush, 1999; Lalonde *et al.*, 1999). Overexpression of pyruvate decarboxylase in potato resulted in ten folds increases of sucrose export, emphasizing the capacity for upregulating transport (Tadege *et al.*, 1998). The induction of *StSUT1* and *AtSUC2* expression were observed in leaves during source to sink transition (Riesmeier *et al.*,

1993; Truernit *et al.*, 1995). Depending on their availability, the regulation by substrates is reasonable mechanism to control the transport activity. SUT2 is induced when sucrose is abundant whereas other SUTs are down-regulate (Chiou and Bush, 1998; Barker *et al.*, 2000; Vaughn *et al.*, 2002). The post-translational modifications of SUTs such as protein phosphorylation as well as modifications of the abundance of plasma membrane proteins also affect transport activity (Kühn *et al.*, 1996; Roblin *et al.*, 1998). Besides regulation at the transcriptional level, protein turnover can be influenced by synthesis, degradation, or cycling of transporters. It is also reliable that regulation occurs within the membrane because SUTs can form homo- and heteromeric complexes (Reinders *et al.*, 2002). This would also provide a direct evidence for regulation within enucleate SE. However, more detailed analyses are required to determine the mechanisms for sensing and signal transduction.

1.8 Sucrose transport in cassava

Cassava or manioc (*Manihot esculenta* Crantz, Euphorbiaceae) was originally a perennial shrub of the New World. It is an outbreeding species possessing $2n = 36$ chromosomes and is considered to be an amphidiploid or sequential allopolyploids. Mature cassava plant composes of leaves, stem, lateral and fibrous roots, storage tubers and fruits (Figure 1.5, Ekanayake *et al.*, 1997). Cassava is widely grown as a staple food and animal feed in countries of tropical and sub-tropical Africa, Asia and Latin America between 30◦ N and 30◦ S with a total cultivated area over 13 million hectares, more than 70% of it being in Africa and Asia (El-Sharkawy, 1993). It is currently the most important food source for carbohydrate, after rice, sugarcane and maize, for over 500 million people in the developing countries of the tropics and sub-tropics. Its main value is in its storage roots with dry mass containing more than 80% starch. In some areas where the crop is grown, particularly in Africa, young leaves are also harvested and processed for human consumption as a vegetable or as an ingredient in a form of sauce eaten along with main staple meals (Lancaster and Brooks, 1983). About 70% of world cassava root production (which is estimated to be over 45 million metric tons of dry roots annually) is used for human consumption either directly after cooking or in processed forms. The remaining 30% is used for animal feed and other industrial products such as starch, glucose, and alcohol.

In areas where cassava is commonly used directly for human consumption, particularly in Africa and Latin America, sweet cassava, a low cyanogens cultivar, is preferably used to avoid health hazards. In high cyanogens cultivars (bitter cassava), the hydrocyanic acid is removed from cassava roots and leaves by using a mix of complex traditional methods and modern technologies during food processing and preparation. Cassava can grow and tolerate to rigorous environmental factors, including abiotic stresses and low-fertility acidic soils under variable rain-fed conditions. Although cassava requires a warm climate (>20 ◦C mean day temperature) for optimum growth and production, and for maximum leaf photosynthesis, it is often cultivated in the high-altitude tropics (up to 1800 m above sea level) with a lower mean annual temperature where crop growth is slower (Irikura *et al.*, 1979), leaf photosynthetic activities are reduced and storage roots harvesting time are much delayed compared to what occurs in the warmer climates. The crop is vegetatively propagated by mature woody stem cuttings (or stakes, 15–30 cm long) planted horizontally, vertically, or inclined on flat or ridged soils at densities ranging from 5,000 to 20,000 cuttings per hectare, depending on the cropping system and purpose of production. Seeds are used mainly in breeding programs in order to obtain the better plant characteristics. Storage roots are generally harvested 7–24 months after planting, depending on cultivar, purpose of use and growing conditions. Due a rapid deterioration after harvest, fresh roots have to be used immediately after harvesting, either eaten on the farm, marketed for consumption, processed for starch extraction, dried for flour production, roasted for food products and/or used for animal feed.

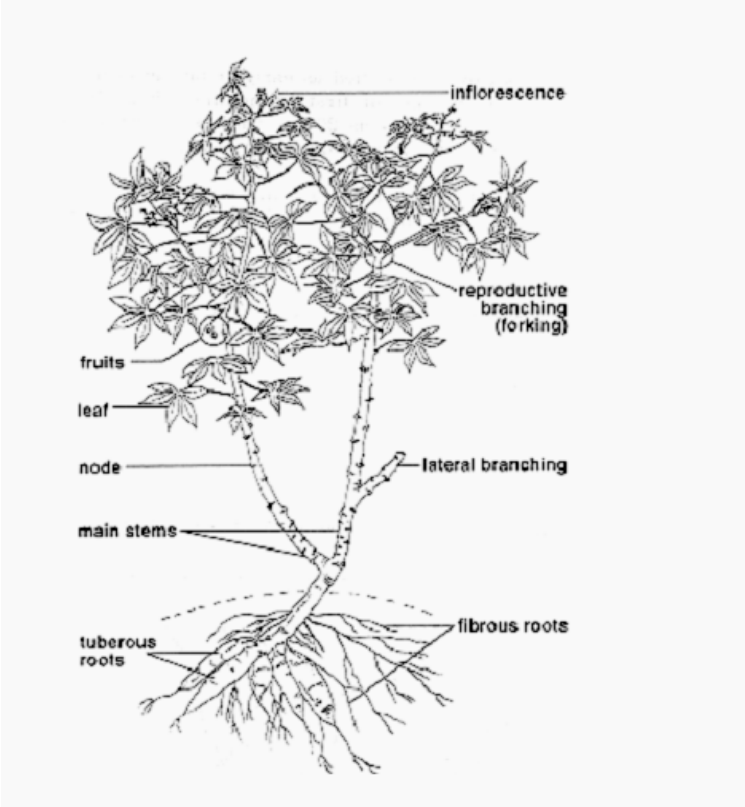


Figure 1.5 Morphology of cassava. Mature cassava plant composes of leaves, stem, lateral and fibrous roots, storage tubers and fruits (Ekanayake *et al.*, 1997).

Starch biosynthesis in cassava requires sucrose to be appropriately distributed from source (mature leaves) to storage root sinks. The preliminary study on sucrose transport in cassava has used the native systems for elucidating the transport characteristics. Sucrose transport in cassava leaves and root discs has been reported for the first time by Eksittikul *et al.* (2001). There were multiple kinetic components presented in cassava, which the high affinity component was observed in leaf whereas storage root possessed low affinity component. Moreover, their results suggested the presence of ATP and/or proton-dependent sucrose transport activities similar to those reported in other plants. However, the different responses between the leaf and root discs to linamarin, a substrate for cyanide synthesis in cassava implicated the distinct cassava SUT isoforms.

In 2005, there was a report on the identification of 4 cDNAs encoding cassava SUT designated MeSUT1, MeSUT2, MeSUT4 and MeSUT5 (Rattanakitti, 2005). All four cassava sucrose transporters exhibited characteristics of amino acid sequences similar to other reported SUTs and each of them can be categorized into corresponded SUT-type (Rattanakitti, 2005).The results also suggested that each MeSUT isoforms

were differently expressed in distinct organs. In developing leaves, the expressions of *MeSUT* genes were correlated to leaf functional characteristics. In different layers of storage roots, the expressions of *MeSUT1* and *MeSUT5* transcripts were higher in the cortex containing phloem than in the parenchyma layers accumulating starch. On the other hand the expression levels of *MeSUT2* and *MeSUT4* genes were equal in all layers. During cassava development, the expression of *MeSUT2* gene in storage roots was equally expressed, whereas the expression levels of *MeSUT1*, *MeSUT4* and *MeSUT5* genes varied depending on the time of harvesting.

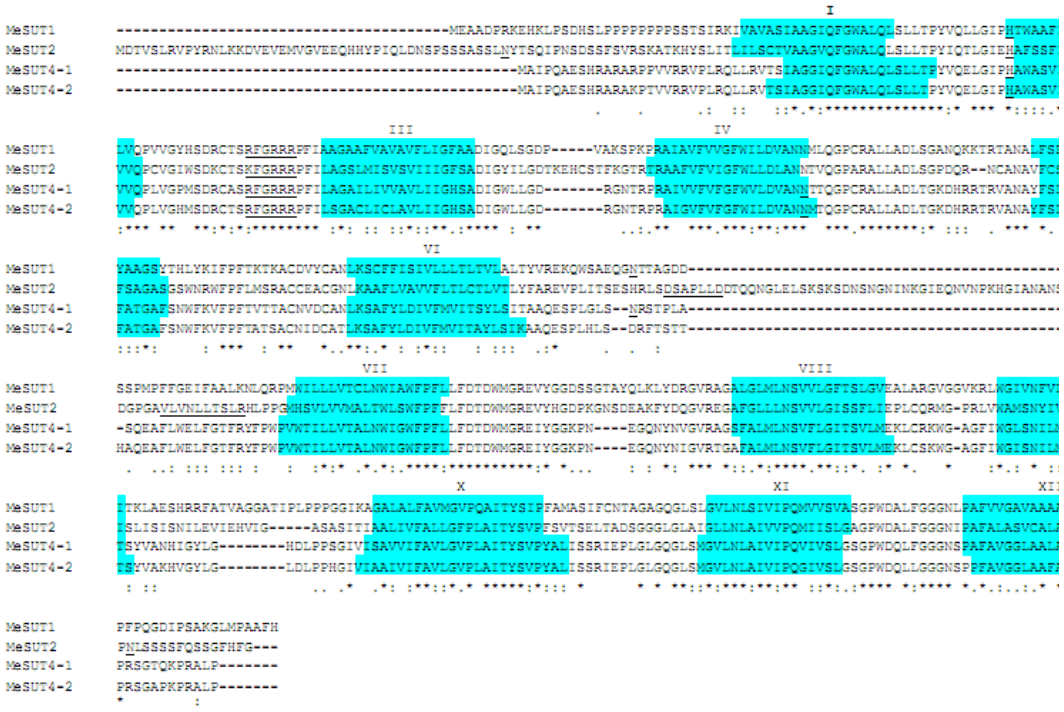


Figure 1.6 Amino acid sequence alignment of MeSUTs. The twelve putative transmembrane domains (blue highlights) were predicted using TMHMM program. MeSUT4-1 and MeSUT4-2 were changed to MeSUT4 and MeSUT5. Identical, strongly similar, and weakly similar amino acid residues are indicated by asterisks, colons, and periods, respectively (Rattanakitti, 2005).

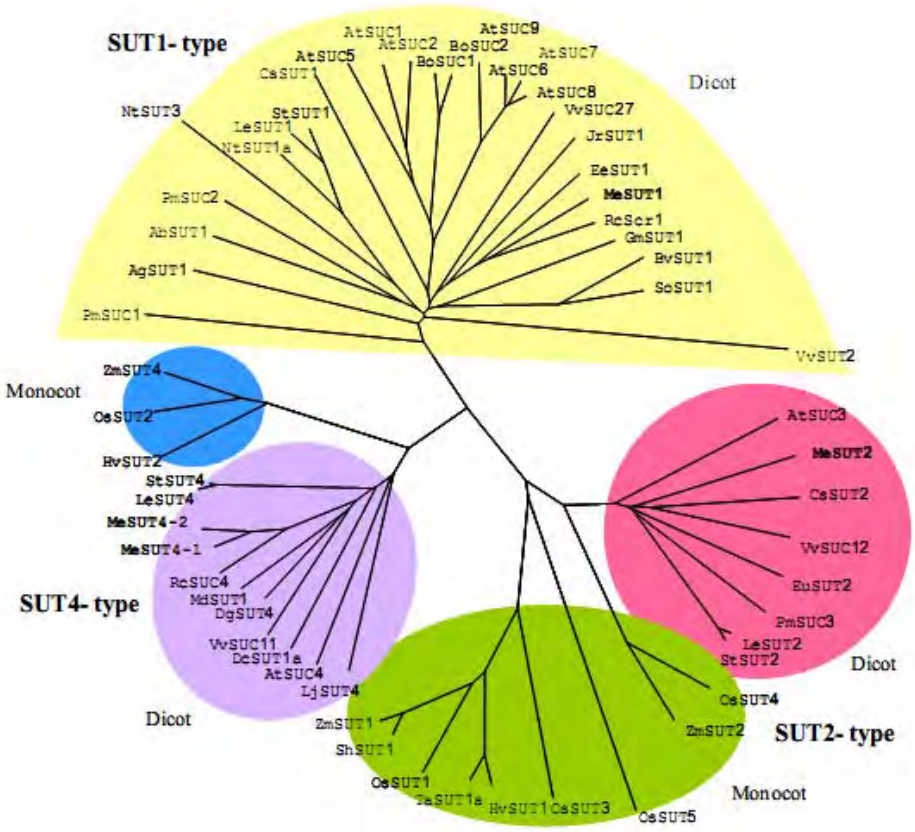


Figure 1.7 Phylogenetic analysis of MeSUTs amino acid sequences. MeSUT1 is categorized to be SUT1 type, while MeSUT2 is classified as SUT2 type. MeSUT4 (MeSUT4-1) and MeSUT5 (MeSUT4-2) were classified as SUT4 type (Rattanakitti, 2005).

1.9 The objectives of this study

The sucrose transport in cassava has been identified using native systems and the gene putatively encoding MeSUTs proteins have isolated from leaf and storage roots cDNA library However, the kinetic properties of these MeSUTs have never been reported. To obtain the biochemical characteristics of each MeSUTs, which might be potential way to improve the sucrose partitioning and the starch contents in cassava, each MeSUTs will be individually expressed using invertase deficient SUSY7/ura3 yeast mutant as heterologous expression system. The objectives of this study are:

1. To express MeSUTs proteins in the heterologous yeast expression systems.
2. To characterize ^{14}C-sucrose transport with respect to their kinetics into *MeSUT*s yeast transformants.
3. To evaluate effects of pH, various inhibitors, competitive sugars and cyanide precursors synthesized in cassava (linamarin) on sucrose uptake into MeSUTs expressed yeast cells.
4. To compare biochemical properties obtained from sucrose transport in native system (i.e. plasma membrane vesicles extracted from cassava leaves and roots) and heterologous systems (yeast and *Xenopus* oocytes) expressing *MeSUT*s.

CHAPTER II
MATERIALS AND METHODS

2.1 Plant meterials

The freshly harvested mature leaves and roots of 6 months old cassava (*Manihot esculenta* Crantz.), cultivar Kasetsart 50 (KU 50) grown at Rayong Field Crops Research Center were used for native membrane vesicle preparation.

2.2 Chemicals and reagents

Chemicals	Company
^{14}C-sucrose	Amershame Life Science
3-(Nmorpholino) propanesulfonic acid (MOPS)	Sigma, USA
Agar	Difco,USA
Agarose	Scharlau, Spain
Ammonium sulphate	Carlo Ebra, Italy
Amplicillin	Sigma, USA
Peptone	Difco,USA
Bovine serum albumin (BSA)	Sigma, USA
Deoxy nucleotide triphosphates (dNTPs)	Fermentas, USA
Dextrans T500	GE Healthcare, Sweden
Dimethylsulfoxide (DMSO)	Sigma, USA
Disodium ethylenediaminetetraacetic acid (EDTA)	Sigma, USA
Dithiothreitol (DTT)	USB, Canada
Ethidium bromide	Sigma, USA
Glass fiber filter (GF/C)	Whatman, England
Glycerol	Univar, Australia
Glucose	Sigma, USA
Lactose	Univar, Newzealand
Lithium acetate	Sigma, USA
Maltose	Difco,USA
Nitrocellulose millipore filter	Whatman,Germany
Phenol	Sigma, USA
Phosphoric acid	Merk, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, USA

Polyethylene glycol	Sigma, USA
Polyvinylpyrrolidone (PVP)	Sigma, USA
POPOP	Sigma, USA
Potassium acetate	Sigma, USA
PPO	Sigma, USA
PVPP	Sigma, USA
Sodium acetate	Sigma, USA
Sodium chloride	Carlo Ebra, Italy
Sodium dodecyl sulfate (SDS)	Sigma, USA
Sodium hydroxide	Carlo Ebra, Italy
Sodium phosphate	Carlo Ebra, Italy
Sucrose	Sigma, USA
Sulfuric acid	Merk,Germany
T4 DNA ligase	Fermentas, USA
Tag DNA polymerase	Fermentas, USA
Toluene	BDH, England
Tris base	Research Organics, USA
Triton X-100	Acros organics, Belgium
Yeast nitrogen base without amino acids	Difco,Germany

The other common chemicals were reagent or analytical grade from BDH, Merck, Carlo Ebra, Fluka and Sigma.

2.3 Equipments

Equipments/Model	**Company**
Centrifuge/J2-21	Beckman, USA
L8-70 Ultracentrifuge	Beckman, USA
Electrophoresis Unit/Powerpac Basic	BioRad, USA
Liquid Scintillation Counter/LS56000	Beckman, USA
Spectrophotometer/DU650	Beckman, USA
Current amplifier/ Dagan TEV 200A	Dagan Corp., USA
Electrode voltage clamp/ pClamp 5.5.1	Axon Instruments,USA
Thermal cycler/PTC-200	MJ research, USA

2.4 Bacterial culture and stock culture maintenance

The bacterium used in this study was *E. coli* strain DH5α (Table 2.1). The cells were cultured either on solid media or in liquid media depending on the purpose of experiments. To grow bacteria on solid media, cells were streaked on LB (Luria-Bertani) agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% bactoagar; pH 7.0) and incubated at 37˚C for 16 h. The cultures grown on agar plates could be kept at 4 ˚C for 2 or 3 weeks for routine use. To grow the bacterial cells as a suspension, the cells were inoculated in LB broth (1% tryptone, 0.5% yeast extract and 1% NaCl; pH 7.0) and incubated at 37˚C with shaking at 250 rpm for 16 h for cells to reach the exponential growth phase. For transformed cells, the culture medium was supplemented with appropriate antibiotics. The concentrations of antibiotics were 50 μg of amplicilin (Amp).

To prepare frozen stock cultures, bacteria were grown in LB broth, and then a 500 μl aliquot of culture was mixed with sterile glycerol to final concentration of 20% (v/v). The mixture was submerged in liquid nitrogen and then kept at -80 ˚C.

2.5 Yeast culture and stock culture maintenance

Yeast cells (*Saccharomyces cerevisiae*: SUSY7/ura3, Table 2.1), kindly provided by Prof. Wolf B. Frommer and Dr. Sylvie Lalonde, were grown at 30 ˚C either in liquid YPD medium (2% yeast extracts, 1% peptone and 2% glucose) or YPD (1.5%) agar plates. For transformed cells were grown on synthetic dropout minimal medium (SD medium: see appendix 2).

For frozen stock cultures, yeast cells were grown in YPD broth in rotary shaker at 30 ˚C, 250 rpm until the growth reached exponential phase. A 500 μl aliquot of the cells cultures were mixed with 80% (v/v) sterile glycerol to the concentration of 20% (v/v) and kept at -80 ˚C.

Table 2.1 Genotype of bacterial and yeast strains used in this research.

Organism	Strain	Genotype
E.coli	DH5α	*fhu*A2 Δ*(arg*F-*lac*Z*)*U169 *pho*A *gln*V44 *Φ*80 Δ*(lac*Z*)*M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi-1 hsd*R17
Yeast (*S. cerevisiae*)	SUSY7/ura3	*Mat*α, *suc*2Δ::*URA*3, *mal*0, *trp*1, *LEU*2::P *ADH*1-*SUSY* (YIP128 A2)

2.6 DNA cloning techniques

2.6.1 Preparation of competent *E.coli* cells

The competent *E. coli* cells were prepared using the $CaCl_2$ method (Sambrook and Russell, 2001). A single colony of *E. coli* strain DH5α was inoculated in 5 ml of LB medium with shaking at 37 ˚C for 16 h. The overnight culture (4 ml) was transfered into 400 ml of LB broth in 1 liter flask and incubated with shaking at 37 ˚C for 1-3 h or until OD_{600} reached 0.6. The culture was chilled on ice for 10 min and cells collected by centrifugation at 3,000 xg for 10-15 min at 4 ˚C. The pellets were re-suspended in 1/2 of the initial volume of sterile ice-cold NaCl solution (0.1 M NaCl, 5 mM Tris-HCl; pH 7.0, 5 mM $MgCl_2$) and cells were recovered by centrifugation at 3,000 xg for 10 min at 4 ˚C, and re-suspended in 1/2 of the initial volume of sterile ice-cold $CaCl_2$ solution (0.1 M $CaCl_2$, 5 mM Tris; pH 7.0; 5 mM $MgCl_2$). The mixture was placed on ice for 20 min. The pellet was then collected by centrifugation at 3,000 xg for 10 min at 4 ˚C and resuspended in 1/20 of the initial volume of sterile ice-cold $CaCl_2$ solution. The cell suspension was placed on ice for at least 1 h before use. Alternatively, to prepare a frozen stock competent cell, sterile glycerol was added to the final concentration of 20% (v/v). The cells were dispensed in a 100 µl aliquot into a chilled microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80 ˚C until use.

2.6.2 Transformation

Transformation was performed according to the method described by Sambrook and Russell (2001) with some modifications. The frozen competent cells were thawed on ice and DNA samples were added into competent cell aliquots. The contents were mixed by gentle swirling, and then incubated on ice for 30 min. The mixture was heated in 42 ˚C water bath for exactly 90 sec and rapidly placed on ice. After the addition of 700 µl of LB broth, the cells were horizontally shaken at 37 ˚C for 45 min. The appropriate volume of transformed competent cells was spreaded on LB agar plate containing selective antibiotic. The plate was incubated at 37 ˚C overnight to allow the recovery of the transformed cells.

2.6.3 Manipulation of plasmid DNA

2.6.3.1 Plasmid DNA extraction

2.6.3.1.1 Rapid alkaline lysis method

Plasmid DNA was extracted by alkaline lysis with SDS according to the method described by Sambrook and Russell (Sambrook *et al.*, 2001). A single colony of transformed bacteria was grown in 3 ml of LB broth containing the appropriate antibiotic with vigorous shaking at 37 °C for overnight. The cells were spun down at 12,000 x*g* for 1 min at 4 ºC and pellets was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl; pH 8.0, 10 mM EDTA) by vigorous vortexing. To lyse cells, 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added to the bacterial suspension, mixed by inverting the tube rapidly for five times and stored on ice for 5 min. Then 150 µl of ice-cold solution III (3 M potassium acetate, 5 M glacial acetic acid) was mixed with the lysate by gentle inversion for approximately 10 sec. The mixture was stored on ice for 3-5 min, followed by centrifugation at 12,000 x*g* for 5 min at 4ºC. The supernatant was transfered to a fresh tube and subsequently extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixture. The aqueous phase was recovered by centrifugation at 12,000 x*g* for 5 min at room temperature. To precipitate plasmid DNA, two volumes of absolute ethanol was added and the mixture was allowed to stand for 2 min at room temperature. The DNA was collected by centrifugation at 12,000 x*g* for 5 min at 4ºC, washed with 70% ethanol, air-dried, and

then dissolved in 50 µl TE buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA; pH 8.0) containing 20 µg/ml DNase-free RNase A and stored at -20ºC until use.

2.6.3.1.2 QIAprep Spin Miniprep Kit (Qiagen)

The bacterial cells were collected by centrifugation at 12,000 x*g* for 1 min at 4ºC. According to the instruction manual, the pellet was resuspended in 250 µl of buffer P1, and the cells were lysed by addition of 250 µl buffer P2. Then 350 µl of Buffer N3 was added to the lysate, followed by centrifugation at 12,000 x*g* for 10 min at room temperature. The supernatant was applied to the QIAprep column, and then the flow-through was removed by centrifugation at 12,000 x*g* for 30-60 sec. The column was washed with 700 µl of buffer PE and centrifuged for 30-60 sec. After the flow-through was discarded, the residual wash buffer was removed by centrifugation for an additional 1 min. The column was placed in a clean microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl HB2BO to the center of the column, followed by centrifugation at 12,000 x*g* for 1 min.

2.6.3.2 Restriction digestion of plasmid DNA

The restriction digestion of plasmid DNA was performed according to the protocol of Fermentas™. The following components were sequentially added into the reaction (total volume of 20 µl): 16-16.5 µl of nuclease-free water, 2 µl of 10X recommended buffer for restriction enzyme, 1 µl (~1 µg) of substrate DNA, and 0.5-1 µl (5-10 U) of restriction enzyme. The reaction mixture was mixed and spun down briefly. The reaction was incubated at the optimum temperature for 1-16 hour before analyzed by agarose gel electrophoresis.

2.6.3.3 DNA dephosphorylation by calf alkaline phosphatase

The dephosphorylation of plasmid DNA was performed according to the protocol of Fermentas™. The following components were sequentially added into the reaction: 1 µg (~1 pmol termini) of linear DNA (~3 kb plasmid), 2 µl of 10X FastAP™ reaction buffer, 1 µl (1 U) of FastAP™ thermosensitive alkaline phosphatase and nuclease free water to a final volume 20 µl. The reaction was mixed thoroughly and briefly spun down. Then, the mixture was incubated at 37 °C for 10 min and stopped by heating at 75 °C for 5 min.

2.6.3.4 DNA ligation

The ligation reaction was performed according to the protocol of Fermentas™. The following components were sequentially added into the reaction: 20-100 ng of linear vector DNA, 1:1 to 5:1 molar ratio over the vector DNA of insert DNA, 2 µl of 10X T4 DNA ligase buffer, 1 u of T4 DNA ligase and nuclease-free water to a final volume of 20 µl. The mixture was incubated at 22°C for 16 h and 5 µl of the reaction mixture were used for *E.coli* transformation.

2.6.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate, identify, and purify DNA fragments. The solution of agarose in 0.5X TBE buffer (45 mM Tris-borate, 1mM EDTA) was prepared at the concentration appropriate for separating particular fragments expected in the DNA sample. In this study, the concentration of agarose gel was varied from 0.8-1.5% (w/v) depending on the size of the desired DNA fragments. Before being loaded into a well of the submerged gel, five volumes of sample was mixed with 1 volume of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol). The standard marker of 100 bp (SibEnzyme) or 1-kb DNA ladder (Fermentas) was loaded to help estimate the size of DNA fragments. The gel was run at constant voltage (40-100 V). To visualize the DNA, the gel was stained with 0.5 µg/ml ethidium bromide for 10-15 min, placed on a UV transilluminator, and photographed using a gel documentation system (GENE GENIOUS Bio Imaging system, SYNGENE).

2.6.3.6 Recovery of DNA fragments from agarose gel

DNA fragment was extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen). The gel slice containing the desired DNA fragment was incubated in buffer QG (300µl buffer/100-mg gel slice) at 50°C for 10 min. After the gel was completely dissolved, one gel volume of isopropanol was added, and the mixture was applied to a QIAquick spin column, followed by centrifugation at 12,000 x*g* for 1 min. The column was washed with 750 µl of buffer PE, and centrifuged at 12,000 xg for 1 min. To elute the DNA, 50 µl of buffer EB (10 mM Tris-HCl; pH 8.5)

or H_2O was added to the center of the QIAquick membrane, followed by centrifugation at 12,000 x*g* for 1 min.

2.6.3.7 Cloning and construction of expression vector harboring *MeSUT* coding sequences

The coding sequences of *MeSUTs* were amplified using plasmid harboring the full length of each gene obtained from the study of Rattanakitti, 2005. The forward and reverse primers for the amplification were shown in Table 2.2. The following components were added in each reaction (total volume of 10 µl): 0.5 µM of each primers, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 1 unit of iProof Taq DNA polymerase (Bio-Rad) and amplifications were carried out according to the PCR conditions indicated in Table 2.3.

Table 2.2 PCR primers used for amplification of *MeSUT* coding sequences. Underlines are the restriction sites for cloning of the coding sequences into pDR195 vector.

Genes	Forward primer	Reverse primer
MeSUT1	GACCGCGGATGGAGGCTGCTGA	TGCCGCGGTCAATGGAAGGCG
MeSUT2	TACCGCGGATGGACACAGTGTCG	GCGCCGCGGTTAGCCAAAATGA
MeSUT4	CCAGGATCCATGGCAATCCCAC	TATGGATCCTCATGGAAGGGCCC
MeSUT5	CCAGGATCCATGGCAATCCCAC	TATGGATCCTCATGGAAGGGCCC
StSUT1	GAAACGTGGACGATGGTGG	AACGGAAACCA(CT)(CG)CAATCCA

Table 2.3 PCR conditions for amplification of *MeSUT1*, *MeSUT2*, *MeSUT4* and *MeSUT5* coding sequences.

PCR step	***MeSUT1***	***MeSUT2***	***MeSUT4***	***MeSUT5***
Initial denatureation	98 °C	98 °C	98 °C	98 °C
	1 min	1 min	1 min	1 min
Cycle number of	30	30	30	30
Denaturation	98 °C	98 °C	98 °C	98 °C
	10 sec	10 sec	10 sec	10 sec
Annealing	61°C	61°C	65°C	65°C
	30 sec	30 sec	30 sec	30 sec
Extension	72°C	72°C	72°C	72°C
	45 sec	45 sec	45 sec	45 sec
Final extension	72 °C	72 °C	72 °C	72 °C
	5 min	5 min	5 min	5 min

The amplified products were subjected to A-addition treatment to create A-overhangs, and ligated into the pTZ57R/T cloning vector (MBI Fermentas, Figure 2.1). Each ligation product was transformed into *E. coli* strain DH5α and plasmids were purified from selected transformants using Qiagen plasmid extraction kit. The plasmids were digested with *Sac*II for MeSUT1 and MeSUT2 and *Bam*HI for MeSUT4 and MeSUT5. The DNA fragments were separated by agarose gel electrophoresis. The excised DNA fragments purified from agarose gel were subcloned into the corresponding sites of a dephosphorylated yeast/*E. coli* shuttle vector pDR195 shown (Figure 2.2 (Rentsch *et al.*, 1995)).The pDR195 vector is a gift from Dr. Silvie Lalonde. Positive clones were confirmed by nucleotide sequencing analysis to ensure the in-frame insertion of coding sequences in pDR195. The pDR195 vectors harboring sense orientation of *MeSUT* were subsequently used for transformation into SUSY7/ura3 yeast cells. The derived plasmids were named pDRMeSUT1, pDRMeSUT2, pDRMeSUT4 and pDRMeSUT5.

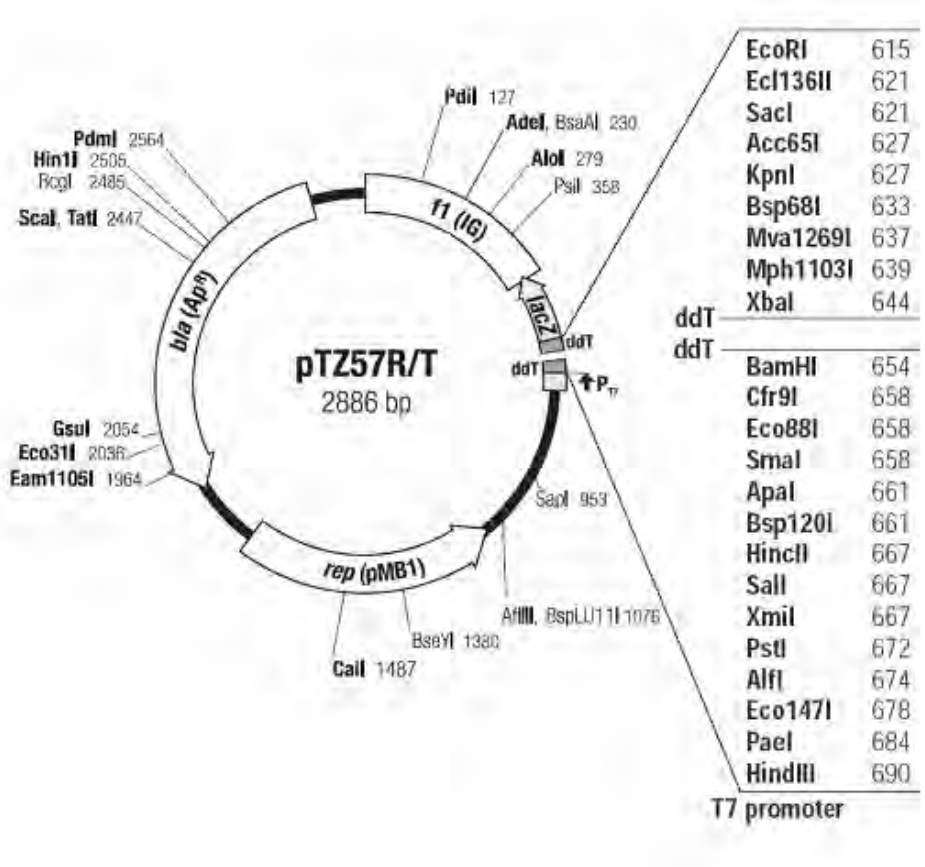


Figure 2.1 Restriction map of pTZ57RT vector.

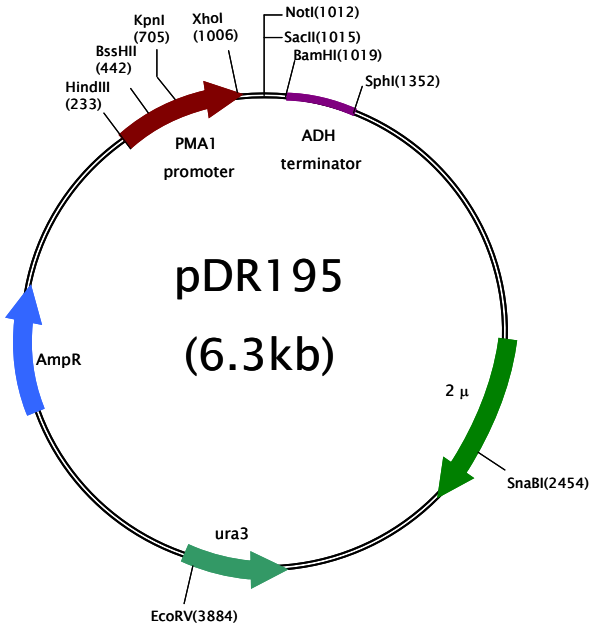


Figure 2.2 Restriction map of pDR 195 vector.

2.7 Expression of *MeSUT*s in yeast cells

2.7.1 Preparation of yeast competent cell

Saccharomyces cerevisiae strain SUSY7/ura3 yeast mutant, a modified version of SUSY7 strain (Riesmeier *et al.*, 1992) was used for competent preparation. This strain could not utilize sucrose as a sole carbon source and a part of URA3 gene was deleted for allowing selection on the media lacking uracil. According to the method described by Gietz *et al.* (1992), competent yeast cells were prepared by the following procedures; single colony was inoculated in 5 ml of LB medium and incubated at 37 °C for 16 h. One ml of the culture was transferred into 300 ml of YPD broth (1% yeast extract, 2% peptone, 2% glucose). Cells were grown at 30 °C and shaken at 250 rpm until the absorbance at 600 nm reached 0.5. Cells were harvested by centrifugation and subsequently washed in sterile water. The cell pellets were then resuspended in 1.5 ml of freshly prepared transformation buffer (1x Tris-EDTA, 100 mM lithium acetate).

2.7.2 Yeast transformation

The pDR195 vector harboring a *MeSUT* coding sequence (0.6–0.8 µg) was individually mixed with 100 µg of carrier DNA (salmon sperm DNA) in 1.5 ml microcentrifuge tubes. Yeast competent cell aliquots were added into each DNA samples followed by 600 µl of sterile 50% PEG 4000. Reactions were shaken at 30°C for 30 min and then heated at 42 °C for 15 min. Finally, the cells were harvested, re-suspended in 1 ml of 1x Tris-EDTA buffer and plated on minimal agar media containing glucose (SDG; 2% Glucose, 0.17% yeast nitrogen base without amino acids (Difco), 0.5% ammonium sulfate, and 0.13% tryptophane). The transformed yeast cells were incubated at 30 °C for 4-7 days to allow cell growth. Fast growing colonies appeared on the plates were picked and used for verifying the existence of *MeSUT*s in the transformed cells. Yeast transformants expressing StSUT1 cDNA was used as a positive control while non-transformed SUSY7/ura3 yeast cells and empty pDR195 yeast transformant were used as negative controls.

2.7.3 Yeast plasmid DNA extraction

Fast growing colonies appeared on agar plates after 4 days of incubation at 30 °C were picked and cultured in liquid minimal medium containing 2% glucose. The

yeast plasmid was extracted by using Zymoprep™ yeast plasmid minipreparation kit. According to the protocol, the full-grown yeast cells were aliquot to 0.5-1 ml into 1.5 microcentrifuge tubes. Cells were spun down at 600 xg for 2 minutes and the supernatant was discarded, Then 150 µl of solution 1 and 2 µl of Zymolase™ were added to the cell pellet. The cell suspension was incubated at 37 ^{o}C for 15-60 minutes. The solution was then mixed with 150 µl each of solution 2 and solution 3 by vortex. After centrifugation at 13,000 rpm, the supernatant was transferred to a new tube and 400 µl of isopropanol was added to the solution. DNA pellet was obtained by repeat centrifugation at 13,000 rpm for 8 minutes and allowed to air dry. The dried DNA pellet was dissolved in 35 µl of TE buffer.

2.7.4 Verification of *MeSUT*s genes in transformed yeast cells

The plasmid extracted from the transformed yeast cells was subjected to PCR amplification using *MeSUT* gene specific primers as shown in Table 2.2 in order to verify the existence of the targeted MeSUT sequences in the yeast transformants. The extracted plasmid DNA was re-transformed into *E.coli* and the positive clones were used for plasmid extraction. The purified plasmid was subjected to DNA sequencing in order to confirm the *MeSUT*s sequence identities.

2.7.5 Functional complementation of pDRMeSUT-transformed yeast

A single colony of the transformed yeast cells containing the pDR195 vector harboring *MeSUT* coding sequences were restreaked on minimal agar media (-uracil) containing either 2% glucose or sucrose as sole carbon sources. The cells were allowed to grow at 30°C for 5-10 days. In each plate, yeast cells transformed with pDRMeSUT was used to compare the growth rate with non-transformed SUSY7/ura3 and pDR195 transformant as negative controls and yeast transformed with *StSUT1* as a positive control.

2.8 Biochemical characterization of sucrose transporters expressed in yeast

2.8.1 Cell preparation for ^{14}C-sucrose uptake assays

Yeast transformants were grown at 30 °C in 250 ml of Erlenmeyer flasks containing 100 ml of liquid minimal medium with 2% glucose. The cultures were shaken at 200 rpm in a shaking incubator until OD_{600} reached 0.9-1.0 and cell pellets

were obtained by centrifugation at 3000 xg. The pellets were washed with 25 mM sodium phosphate buffers at optimum pH and re-suspended in the small volume of the same buffer. Equal fresh weight of cell aliquots were subsequently used for sucrose uptake assays.

2.8.2 ^{14}C-sucrose uptake experiments

The method for sucrose uptake assay was modified from Sauer and Stolz (1994). Each assay (total volume 200 µl) contained 1 mg of cell fresh weight. To perform sucrose uptake, [U- ^{14}C]-sucrose (specific activity =0.18 µCi/µmol) was added into the reactions and the samples were incubated at 30°C with 200 rpm shaking. The reactions were stopped at appropriate time by vacuum filtration with 8 ml of iced-cold water over a glass microfiber filter (GF/C;Whatman). Samples were rapidly rinsed three times with 3 ml of ice-cold water to eliminate non specific binding of ^{14}C-sucrose on the filters. The membrane filters were allowed to dry and transfered into vials filled with scintillation fluid which were subsequently subjected to radioactivity determination using liquid scintillation counter.

2.8.3 Determination of the radioactivity retained on the membrane filters

The dried membrane filters were put into scintillation vials and each vial was then added with 5 ml of scintillation fluid containing 0.03% POPOP (w/v), 0.5% PPO (w/v) and 50% Triton X-100 (v/v) in toluene. The radioactivity was measured as "cpm" using liquid scintillation counter (LS56000Beckman, USA). The total cpm of the incubation medium before staring the experiment is also counted. The uptake is calculated as dpm per reaction or nmol/mg FW otherwise indicated.

2.8.4 Effects of pH on ^{14}C-sucrose uptake into *MeSUT*s transformants

In order to identify optimum pH of each sucrose transporters, the assays of ^{14}C-sucrose uptake were performed in different pH. After the OD_{600} of the cell cultures reached 0.9-1.0, the cell pellets were collected and resuspended in 25 mM sodium phosphate buffer at pH 3, 4, 5, 6, 7 and 8. The sucrose uptake reactions were started by addition of ^{14}C-sucrose at a final concentration of 1 mM and the experiments were performed according to the methods described in section 2.8.2. The

cells were collected after 2 min of incubation which was in the linear range of sucrose uptake activity. All experiments were repeated three times.

2.8.5 Time course of ^{14}C-sucrose uptake

The time course experiments were performed at the optimum pH of each sucrose transporters according to the methods described in section 2.8.2.The reactions were collected at 0, 1, 2, 4, 6, 8 and 10 min. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and that for MeSUT4 and MeSUT5 was 100 mM. Sucrose uptake by pDR195 yeast transformant and non transformed SUSY7/ura3 were identified as non specific sucrose uptake (negative controls). The *StSUT1* yeast transformant was used as a positive control. All experiments were repeated three times.

2.8.6 Identification of kinetic parameters for sucrose transport

To obtain the kinetic parameters such as K_m and V_{max}, the experiments were performed as described in section 2.8.2 in various sucrose concentrations. ^{14}C-sucrose uptake into yeast expressing *MeSUT1* was carried out at a final concentration of 0, 0.1, 0.2, 0.5, 2, 5, 10 and 15 mM, respectively. For yeast expressing *MeSUT2*, the final concentrations of ^{14}C-sucrose were 0, 0.2, 0.5, 2, 5, 10, 15, 20, 25, 40, 80 and 120 mM, respectively. The final concentration of ^{14}C-sucrose for MeSUT4 and MeSUT5 were 0, 5, 10, 20, 40, 60, 80 and 100 mM, respectively. All experiments were repeated three times.

2.8.7 Effects of sulfhydryl modifying agents on ^{14}C-sucrose uptake

The effects of sulfhydryl modifying reagents on ^{14}C-sucrose uptake activity were performed using NEM and PCMBS as the compounds to be examined. The reagents were added at a final concentration of 50 µM, 30 sec prior to adding of ^{14}C-sucrose. The final concentrations of ^{14}C-sucrose in the reaction mixture for MeSUT1 and MeSUT2 were 1 mM and 10 mM for MeSUT4 and MeSUT5. The cells were collected after 2 min of incubation which was in the linear range of sucrose uptake activity. The experiments were carried out as described in section 2.8.2. All experiments were repeated three times.

2.8.8 Effect of vanadate on ^{14}C-sucrose uptake

The effects of vanadate, a P-type ATPase inhibitor, on ^{14}C-sucrose uptake activities of MeSUTs were examined. The compound was added to each reaction at a final concentration of 100 µM, 30 sec prior to adding of ^{14}C-sucrose. The experiments were performed using the same procedure in 2.8.7.

2.8.9 Effects of proton gradient disruptors and electron transport inhibitor on ^{14}C-sucrose uptake

The effects of CCCP and 2, 4-DNP, the proton gradient disruptors, and antimycin-A as an electron transport inhibitor on ^{14}C-sucrose uptake activity by MeSUTs were examined. The reagent was added to each reaction mixture at a final concentration of 50 µM, 30 sec prior to adding of ^{14}C-sucrose. The experiments were performed using the same procedure as described in 2.8.7.

2.8.10 Effects of various sugars on ^{14}C-sucrose uptake

Sugars used in this experiment were sucrose, glucose, fructose, maltose, lactose and mannose. They were added into each assay mixture at a final concentration of 10 mM, 30 sec prior to adding of ^{14}C-sucrose. The experiments were performed using the same procedure as described in 2.8.7.

2.8.11 Effects of linamarin on ^{14}C-sucrose uptake

The cyanogenic glucoside linamarin was examined for its effect on sucrose transport activity of all MeSUTs. Linamarin was added into the reaction mixture at final concentrations of 1, 5, and 10 mM, 30 sec prior to adding of ^{14}C-sucrose. The experiments were performed using the same procedure as described in 2.8.7.

2.9 Isolation of plasma membrane vesicles

Membrane fractions from cassava roots were isolated according to the following method:

2.9.1 Preparation of microsomal fraction

Mature cassava roots was homogenized four times for 30 seconds each with a Braun homogenizer in 450 ml of 50 mM MOPS/NaOH pH 7.5 contain 0.33 M

sucrose, 5 mM EDTA, 5 mM DTT, 5 mM ascorbic acid, 0.5 mM PMSF, 0.2 % casein (enzymatic hydrolyzate boiled for 10 min) and 0.2 % BSA (protease free) and 0.6 % PVPP (Larsson *et al.*, 1987; Gallet *et al.*, 1989). The homogenate was filtered through 240 μm nylon net, the supernatant was centrifuged at 10,000 xg for 10 min. The supernatant was further centrifuged at 100,000 xg for 30 min. The pellet or microsomal fraction was collected and suspended in 10 ml. of buffer A (5 mM potassium phosphate buffer pH 7.8 containing 0.33M sucrose, 5 mM KCl , 1 mM DTT and 0.1 mM EDTA). This microsomal fraction was used for further isolation of plasma membrane.

2.9.2 Isolation of plasma membrane by aqueous two phase system

The two phase system requires two water soluble polymers that are dissolved in water without phase separation at moderate concentration. In this study, Dextran T500 (Dx: predominantly poly (α-1, 6-glucose) and polyethylene glycol (PEG-3350) were used.

Phase system was made up by weighing from stock solution of 20% (w/w) Dextran T 500 and 40% (w/w) PEG 3350 and concentrated salt solution. The final weight of 4 g or 36 g phase system contains 6.5% (w/w) each of Dextran T500 and PEG3350 usually resulted in about 90% of plasma membranes partitioned to upper phase of PEG (Larsson *et al.*, 1994). The stock solution was added in decreasing order of their densities: Dx, PEG, salt and water respectively, 36 g final weight of phase system is used in the isolation of cassava leaves plasma membrane.

The microsomal fraction was added to the preweighed phase mixture then mixed and left to settle. The PEG upper phase containing plasma membrane fraction was collected and diluted at least 3 folds with buffer A and centrifuged at 100,000 xg for 1 h to collect the plasma membrane. The membrane was suspended in small volume of buffer and used in further study.

2.9.3 Measurement of the ^{14}C-sucrose binding activity of plasma membrane vesicles

Plasma membrane purified from section 2.9.2 was used to study sucrose binding under the non energized condition. Preliminary binding experiment was performed with 32 μg protein of plasma membrane in the reaction mixture containing

0.3 M sorbital, 0.5 mM $CaCl_2$, 0.25 mM and $MgCl_2$ in 50 mM potassium phosphate buffer pH 7.8 in the presence of ^{14}C-sucrose at 37 °C at various time points. The reaction was terminated by adding 1.75 ml of chilled buffer B containing 0.3 M sorbital, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$ in 50 mM potassium phosphate buffer pH 7.5 and 5 mM $HgCl_2$ with vigorous mixing. The mixture was rapidly filtered on Nitrocellulose Millipore filter (0.45 μ pore size, 25 mm diameter) pre-wetted with buffer B. The filter was further rinsed with 1.5 ml of rinsing buffer, then removed from the filter unit and placed in scintillation vial, oven-dried at 50 °C for 1 hr. added with 5 ml of scintillation fluid and measured the radioactivity .

The effect of pH on sucrose binding activity of root membrane vesicles was examined using the same procedures as described above but the assay buffers were varied between pH 3-8. The kinetic study at various ^{14}C-sucrose concentrations (0-120 mM) were performed at optimum pH. A control experiment was run for determination of non-specific binding of radioactivity on plasma membrane vesicle retained on the filter. The rinsing buffer was added to the reaction mixture in the presence of ^{14}C-sucrose which was incubated on ice then 32 μg protein was added and immediately filtered and processed as mentioned above, this reaction tube designated as zero time or blank.

2.9.4 Determination of protein concentration

Protein concentration was determined by the method of Bradford (Bollag and Edelstein, 1991) using bovine serum albumin as standard protein (See Appendix 4) One hundred μl of sample was mixed with 1 ml of coomassie blue reagent and left for 5 min before the absorbance was measured at 595 nm. One liter of coomassie blue reagent comprises of 100 mg of coomassie blue G 250, 50 ml of absolute ethanol, 100 of 85% phosphoric acid and 850 ml of distilled water.

2.10 Expression of *MeSUT* genes in *Xenopus laevis* oocytes

To gain more details of sucrose transport mechanisms mediated by four cassava sucrose transporters, it would be beneficial if they could be characterized in higher resolution expression system such as *Xenopus* oocyte. All four cassava sucrose transporter genes (*MeSUT1*, *MeSUT2*, *MeSUT4* and *MeSUT5*) were expressed in

Xenopus oocytes and their sucrose transport abilities were characterized. The experimental procedures were shown below.

2.10.1 Plasmid construction and cRNA transcription

The entire open reading frame of cassava sucrose transporters were amplified by PCR using primers as shown in Table 2.4. Each PCR products were cloned into pCR8/GW/TOPO vector (Invitrogen, Figure 2.3) and the correct sequences were confirmed. The resulting clones were recombined *in vitro* with the Gateway-compatible oocyte vector, p002/GW (Figure 2.4) to yield p002/GW-*MeSUT*. The constructs were linearized using either *Pma*CI or *Mlu*I (Takara) and 1 µg of each construct was used as a template for cRNA synthesis using the SP6 mMessage mMachine kit (Ambion,Austin, TX).

Table 2.4 Primer sequences for PCR amplification of *MeSUTs* cDNA**.**

cDNA	Forward primer	Reverse primer
MeSUT1	ATGGAGGCTGCTGATCCTAGGA	TCAATGGAAGGCGGCAGGCATC
MeSUT2	ATGGACACAGTGTCGTTACGGGTTC	TTAGCCAAAATGAAAACCAGATGATTG
MeSUT4	ATGGCAATCCCACAGGCGGAGT	TCATGGAAGGGCCCTGGGCTTC
MeSUT5	ATGGCGATCCCACAGGCGGAGT	TCATGGGAGAGCCCTGGGCTTT

2.10.2 Oocyte expression

Xenopus laevis oocytes, stages V and VI, were isolated by incubation in 10 mg/ml collagenase A (Roche Applied Science) in Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM Ca $(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.6, 100 µg/ml penicillin, and 100 µg/ml streptomycin) for 2–3 h until separated. The oocytes were then washed five times in 1 mg/ml bovine serum albumin in Barth's medium. The oocytes were injected with 50 nl (1 ng/nl) of MeSUTs cRNA and incubated at 15 ˚C in Barth's medium supplemented with 10 µg/ml gentamycin. Electrophysiological and sucrose uptake experiments were performed 4 days following the RNA injection.

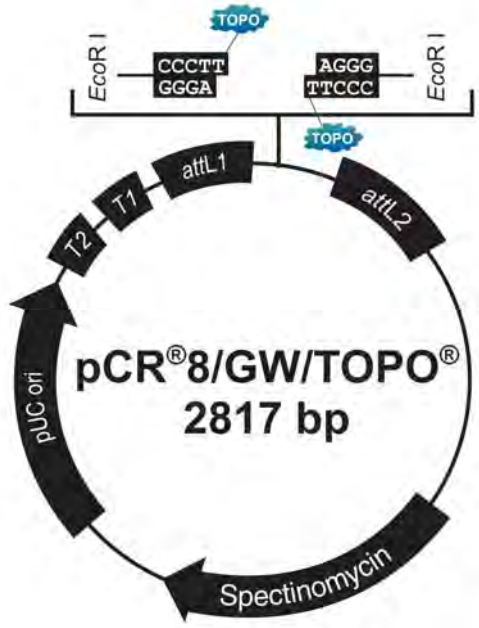


Figure 2.3 DNA map of pCR8/GW/TOPO cloning vector.

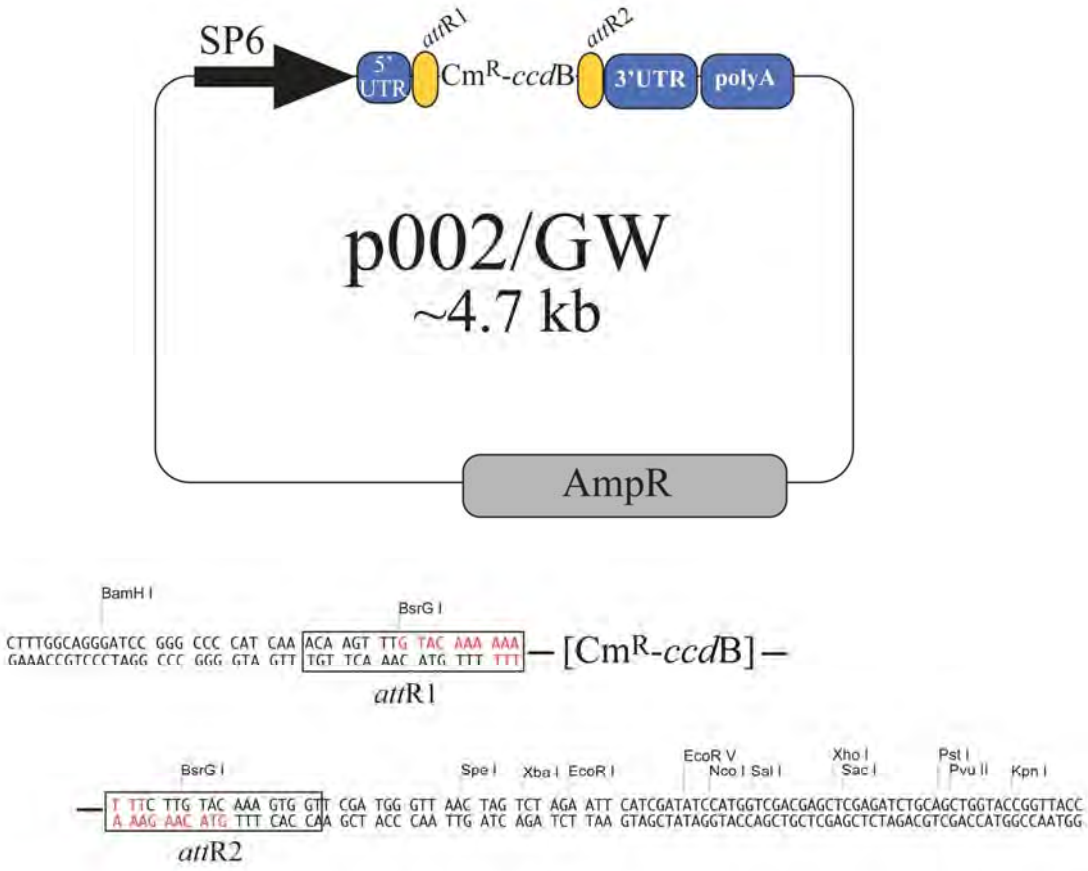


Figure 2.4 DNA map of pOO2/GW gateway vector. The recombination site (*att*R1 and *att*R2) and multiple cloning sites are shown below.

2.10.3 Electrophysiological measurement

Oocytes were bathed in modified Na Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM MES-Tris, at the appropriate pH with continuous perfusion at 1 ml min^{-1} . Recording pipets, filled with 1 M KCl with resistances between 1 and 3 MΩ were used. Currents were measured using the TEVC technique with a Dagan TEV 200A amplifier (Dagan Corp., Minneapolis, MN, USA). Currents were filtered on-line at 200 Hz and digitized at 2,000 Hz using pClamp 5.5.1 (Axon Instruments, Inc., Union City, CA, USA). The holding potential was −40 mV except where indicated and voltage pulses from −137.4 to 37.8 mV were applied for 203 ms. Steady-state currents were presented as the mean current between 150 and 200 ms following the onset of voltage pulses. Substrate-dependent currents were obtained by subtracting an average of background currents before and after substrate application. Substrates were added at the appropriate concentrations.

CHAPTER III
RESULTS

3.1 Construction of yeast expression vectors harboring *MeSUT* coding sequences

The coding sequences of *MeSUT1, MeSUT2* and *MeSUT4* were amplified from pTripExII plasmids containing *MeSUT* full-length cDNAs obtained from the preliminary study by Rattanakitti (2005). The amplified PCR products were individually ligated into pTZ57/RT cloning vector and the ligation products were transformed into *E.coli* strain DH5α. Positive clones were selected as white colonies based on blue-white colony screening. Plasmids purified from positive clones were digested using appropriate restriction enzymes (*Sac*II for *MeSUT1* and *MeSUT2* and *Bam*HI for *MeSUT4*) in order to release the coding sequences. DNA fragments containing the open reading frames of *MeSUT1, MeSUT2* and *MeSUT4* were cloned into the *E. coli/S. cerevisiae* shuttle vector pDR195 (Rentsch *et al.,* 1995), containing the *PMA1* promoter in front of the multiple cloning site and the *ADH* terminator. Since the SUT-encoding sequences were possibly cloned in either sense or antisense orientation with respect to the *PMA1* promoter, the recombinant plasmids purified from *E.coli* transformants were subjected to restriction enzyme digestion analysis. Figure 3.1 showed the results from restriction analysis of plasmids purified from selected bacterial transformants harboring pDR195 vector with *MeSUT1*coding sequence cloned in either sense or anti-sense orientation. The results showed that digestion of sense and anti-sense pDRMeSUT1 constructs with *Sac*II yielded two DNA fragments of 6.3 and 1.6 kb, which corresponded to pDR195 and *MeSUT1* coding sequence, respectively. Plasmids corresponding to sense and anti-sense pDRMeSUT1 constructs showed different restriction patterns when digested with *Hind*III. Digestion of the sense pDRMeSUT1 plasmid yielded three DNA fragments with the size of 5.5, 1.4 and 0.9 kb, while that of the antisense pDRMeSUT1 plasmid gave 6.2, 0.9 and 0.8 kb DNA fragments. The results suggested that the pDRMeSUT1 expression vector was successfully constructed.

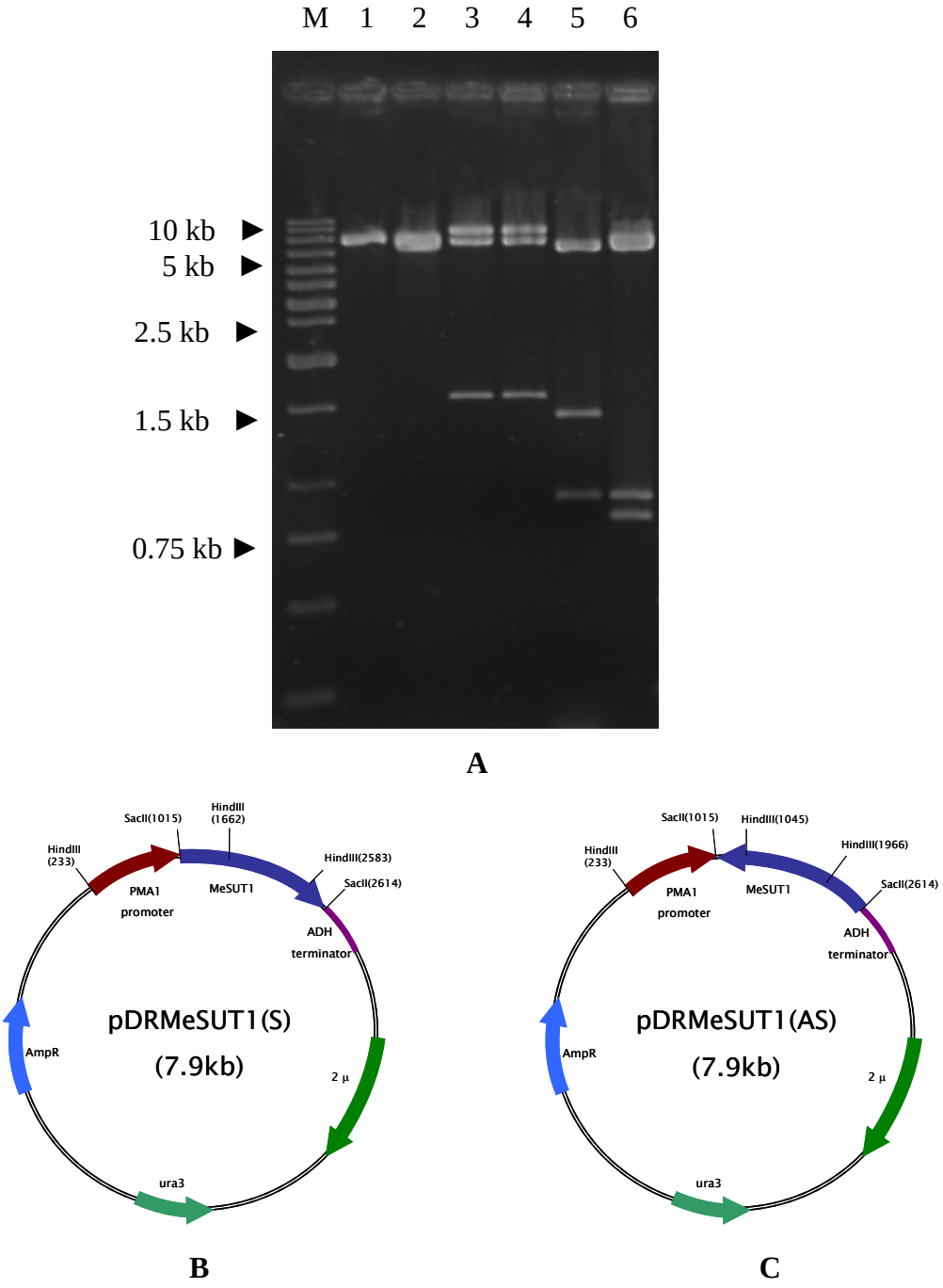


Figure 3.1 Confirmation of sense and anti-sense pDRMeSUT1 constructs.

(A) Digested pDRMeSUT1 expression vectors were separated by agarose gel electrophoresis. Lane 1, 3 and 4 show the digestion of pDR195, sense and antisense pDRMeSUT1 plasmids with *Sac*II. Lane 2, 5 and 6 display the *Hind*III-digestion pattern of pDR195, sense and antisense pDRMeSUT1. The sizes of DNA marker are indicated on the left (lane M). **(B)** and **(C)** are restriction maps of sense and anti-sense pDRMeSUT1 constructs.

Restriction analysis of plasmids purified from bacterial transformants harboring pDR195 vector with *MeSUT2* coding sequence cloned in either sense or antisense orientation were performed as shown in Figure 3.2 and compared with restriction maps predicted from the corresponding nucleotide sequences. Digestion of sense and antisense pDRMeSUT2 constructs with *Sac*II yielded two DNA fragments of 6.3 and 1.8 kb, which corresponded to the pDR195 vector and *MeSUT*2 coding sequence, respectively. Sense and antisense pDRMeSUT2 showed different restriction patterns when digested with *EcoR*V. Digestion of the sense pDRMeSUT2 plasmid yielded two DNA fragments of 4.8 and 3.3 kb, while that of the anti-sense pDRMeSUT2 yielded 4.3 and 3.8 kb DNA fragments. Figure 3.3 showed the result from digestion analysis of plasmids obtained from selected bacterial transformants for pDRMeSUT4. Digestion of sense and anti-sense pDRMeSUT4 constructs with *Bam*HI yielded two DNA fragments of 6.3 and 1.5 kb corresponding to the pDR195 vector and *MeSUT4* coding sequence, respectively. Digestion with *Xho*I distinguished sense pDRMeSUT4 from antisense pDRMeSUT4. *Xho*I-digestion of the sense pDRMeSUT4 gave two DNA fragments of 7.3 and 0.5 kb whereas the digestion of the anti-sense construct yielded 6.8 and 1.0 kb fragments. These results suggested the yeast expression vectors containing *MeSUT2* and *MeSUT4* were successfully constructed. Construction of pDRMeSUT5 plasmid was performed by Dr. Malinee Suksangpanomrung.

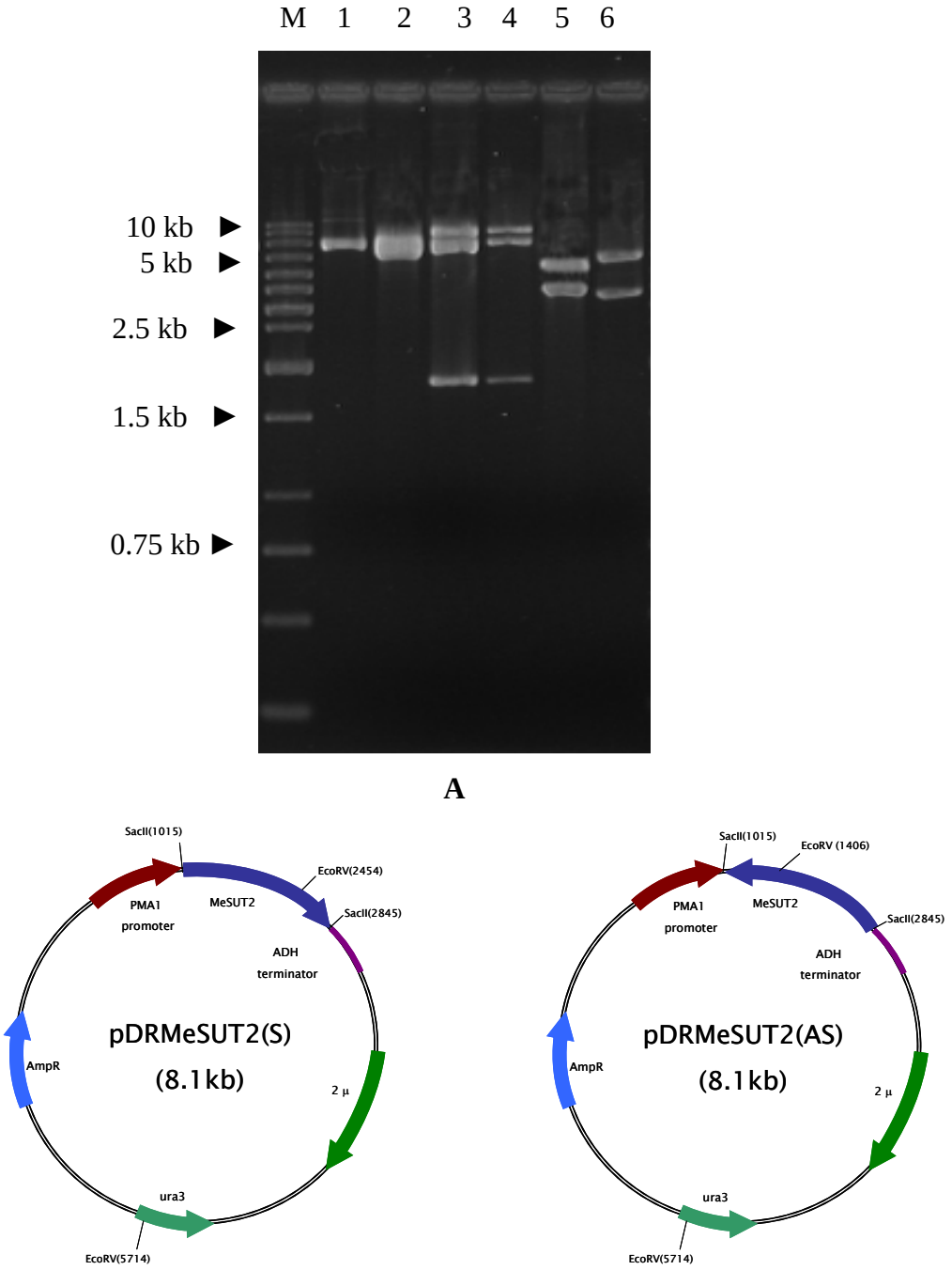


Figure 3.2 Confirmation of sense and antisense pDRMeSUT2 constructs.

(A) Digested pDRMeSUT2 expression vectors were separated by agarose gel electrophoresis. Lane 1, 3 and 4 show the digestion of pDR195, sense and antisense pDRMeSUT2 plasmids with *Sac*II. Lane 2, 5 and 6 display the *EcoR*V-digestion pattern of pDR195, sense and antisense pDRMeSUT2. The sizes of DNA marker are indicated on the left (lane M). **(B)** and **(C)** are restriction maps of sense and antisense pDRMeSUT2 constructs.

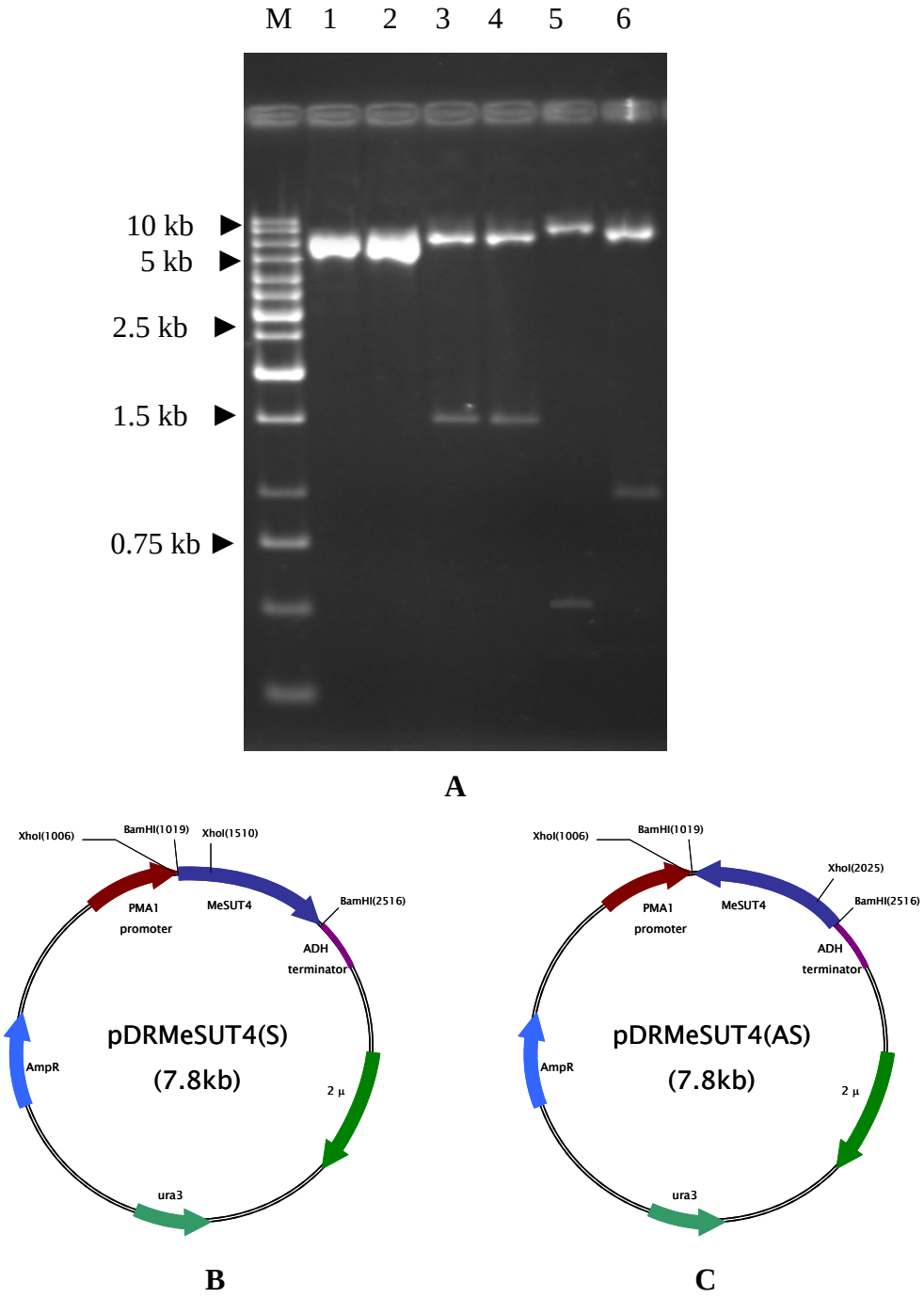


Figure 3.3 Confirmation of sense and antisense pDRMeSUT4 constructs.

(A) Digested pDRMeSUT4 expression vectors were separated by agarose gel electrophoresis. Lane 1, 3 and 4 show the digestion of pDR195, sense and antisense pDRMeSUT4 plasmids with *Bam*HI. Lane 2, 5 and 6 display the *Xho*I-digestion pattern of pDR195, sense and antisense pDRMeSUT4. The sizes of DNA marker are indicated on the left (lane M). **(B)** and **(C)** are restriction maps of sense and antisense pDRMeSUT2 constructs.

3.2 Verification of *MeSUT*s in transformed yeast cells

The SUSY7/ura3 yeast cells were transformed with the shuttle vector pDR195 containing each type of *MeSUT* coding sequences cloned in sense orientation (pDRMeSUT1, pDRMeSUT2, pDRMeSUT4 and pDRMeSUT5) by the method of Gietz *et al*. (1992). Isolated colonies of transformed yeast cells were selected and cultured in SD-medium containing glucose for plasmid extraction. The purified plasmid DNA was subsequently subjected to PCR amplification with gene-specific primers to ensure the presence of correct *MeSUT*-expressing cassettes in yeast transformants. Figure 3.4 showed the presence of 1.6 kb amplified product representing the *MeSUT1* coding sequence obtained from PCR analysis of pDRMeSUT1 as a positive control and plasmids extracted from three independent yeast transformants. The result indicated that these yeast transformants carried the expected pDRMeSUT1 and could be used for further biochemical characterization of MeSUT1 in yeast cells. Likewise, yeast transformants recovered from SD-medium lacking uracil following the transformation with pDRMeSUT2, pDRMeSUT4 and pDRMeSUT5 were obtained and confirmed by PCR analyses according to the results shown in figure 3.5, 3.6 (A) and 3.6 (B), respectively.

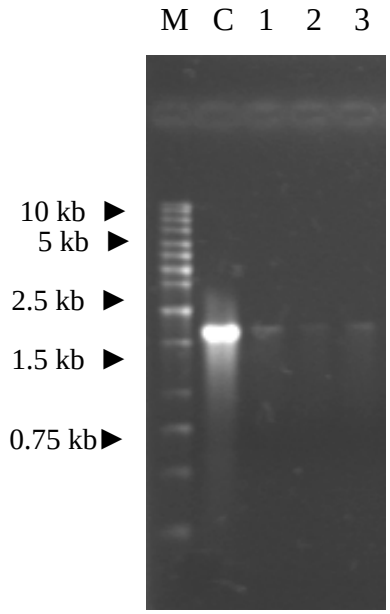


Figure 3.4 Confirmation of the presence of pDRMeSUT1 in transformed yeast cells. Plasmid DNA extracted from three independent clones of yeast cells transformed with pDRMeSUT1 sense construct were amplified by PCR technique. M is DNA marker, and C is positive control (pDRMeSUT1 vector). Lane 1, 2 and 3 are selected clone 1, 2 and 3.

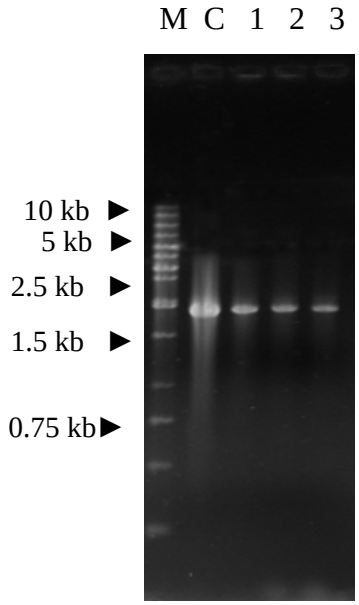


Figure 3.5 Confirmation of the presence of pDRMeSUT2 in transformed yeast cells. Plasmid DNA extracted from three selected clones of yeast cells transformed with pDRMeSUT2 sense constructs were amplified by PCR technique. M is DNA marker, and C is positive control (pDRMeSUT2 vector). Lane 1, 2 and 3 are selected clone 1, 2 and 3.

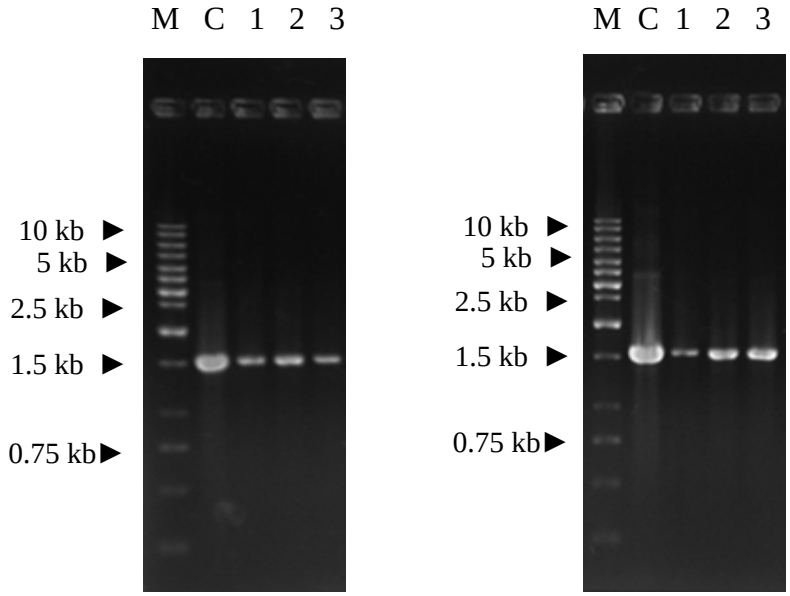


Figure 3.6 Confirmation of the presence of pDRMeSUT4 and pDRMeSUT5 in transformed yeast cells. Plasmid DNA extracted from three independent clones of yeast cells transformed with pDRMeSUT4 sense construct (A) and pDRMeSUT5 sense construct (B) were amplified by PCR technique. M is marker DNA, C is positive control (pDRMeSUT4 and pDRMeSUT5 vectors), lane 1, 2 and 3 are selected clones 1, 2 and 3 respectively.

The empty pDR195 vector and plasmid vector containing *StSUT1* gene the pDR195 expressing SUT1 of potato (pStSUT1) were also introduced into the SUSY/ura3 yeast mutants and the derived transformants were used as negative and positive controls, respectively. In order to confirm the presence of pDR195 in the transformed yeast, plasmid DNA extracted from pDR195 yeast transformant was used for retransformation into *E.coli* (DH5α) cells. Plasmid DNA extracted from *E.coli* transformant was digested with *Nco*I and analyzed by agarose gel electrophoresis. Figure 3.7 showed that the plasmid DNA digested with *Nco*I gave a single DNA band at 6.3 kb of pDR195. Yeast transformants expressing pStSUT1 were confirmed by plasmid PCR analysis using SUTR3 and 5'PMA as primers. The 0.9-kb PCR product corresponded to partial StSUT1 sequence as shown in figure 3.8.

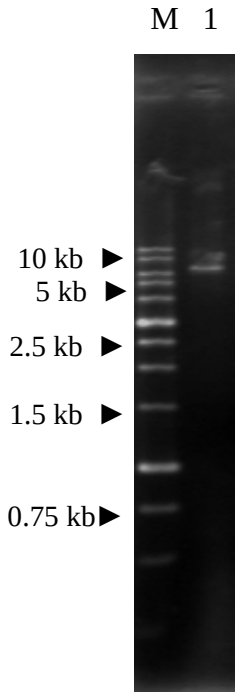


Figure 3.7 Confirmation of the presence of pDR195 vector in transformed yeast cells. Plasmid DNA extracted from *E.coli* cells transformed with yeast plasmid pDR195 was digested with *Nco*I. M is DNA marker and lane1 is digested pDR195.

All pDRMeSUTs vectors extracted from transformed yeast cells were retransformed into *E.coli* DH5α and the individual constructs were subjected to DNA sequencing. The sequencing results were proved for the sequence accuracy by pairwise alignment with the original *MeSUTs* sequences used for PCR cloning in the first step. The results revealed that all of *MeSUT*s sequences obtained after yeast transformation did not show any mutations and the sequences were identical to the original *MeSUT* cDNA sequences.

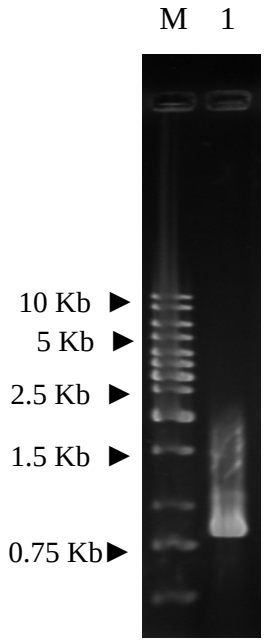


Figure 3.8 Confirmation of the presence of pStSUT1 in transformed yeast cells. Plasmid DNA extracted from a selected pStSUT1 yeast transformant was amplified by PCR technique (lane 1). M is DNA marker.

3.3 Functional complementation of yeast transformed with *MeSUT*s

To demonstrate that the putative *MeSUT* sequences encoded functional sucrose transporters, coding sequences of *MeSUT1, MeSUT2, MeSUT4* and *MeSUT5* cDNAs were expressed in the yeast strain SUSY7/ura3. This yeast strain is unable to hydrolyze exogenous sucrose and unable to grow on selective media lacking uracil. However, it can import sucrose and hydrolyze it internally when transformed with a functional SUT (Barker *et al.*, 2000), allowing it to grow on medium containing sucrose as the sole carbon source. It can also grow on media lacking uracil when complemented with *URA3* gene in expression vector. It is expected that if cassava SUTs are functional, SUSY7/ura3 yeast transformed with *MeSUTs* sense constructs should be able to grow on minimal media (-uracil) containing sucrose as a sole carbon source better than those of negative controls (pDR195 transformant and non-transformed SUSY7/ura3 yeast).

Yeast transformed with *MeSUT* coding sequences, empty pDR195 vector, pStSUT1 and non-transformed SUSY7/ura3 yeast were streaked on minimal agar media containing either 2% glucose or sucrose as sole carbon sources. The plates

were incubated for 3 days at 30 °C and the cell growths were observed. All SUSY7/ura3 yeast transformed with empty vector, *MeSUT* coding sequences and pStSUT1 grew well on minimal media (-uracil) containing 2% glucose as shown in Figures 3.9 respectively. All four pDRMeSUT and pStSUT1 (positive control) yeast transformants grew better on minimal media (-uracil) containing 2% sucrose while yeast transformed with empty pDR195 vector (negative control) showed less growth (Figure 3.10). On the other hand, non-transformed SUSY/ura3 cells (negative control) could not grow on uracil lacking agar plate containing either 2% glucose or 2% sucrose as sole carbon sources.

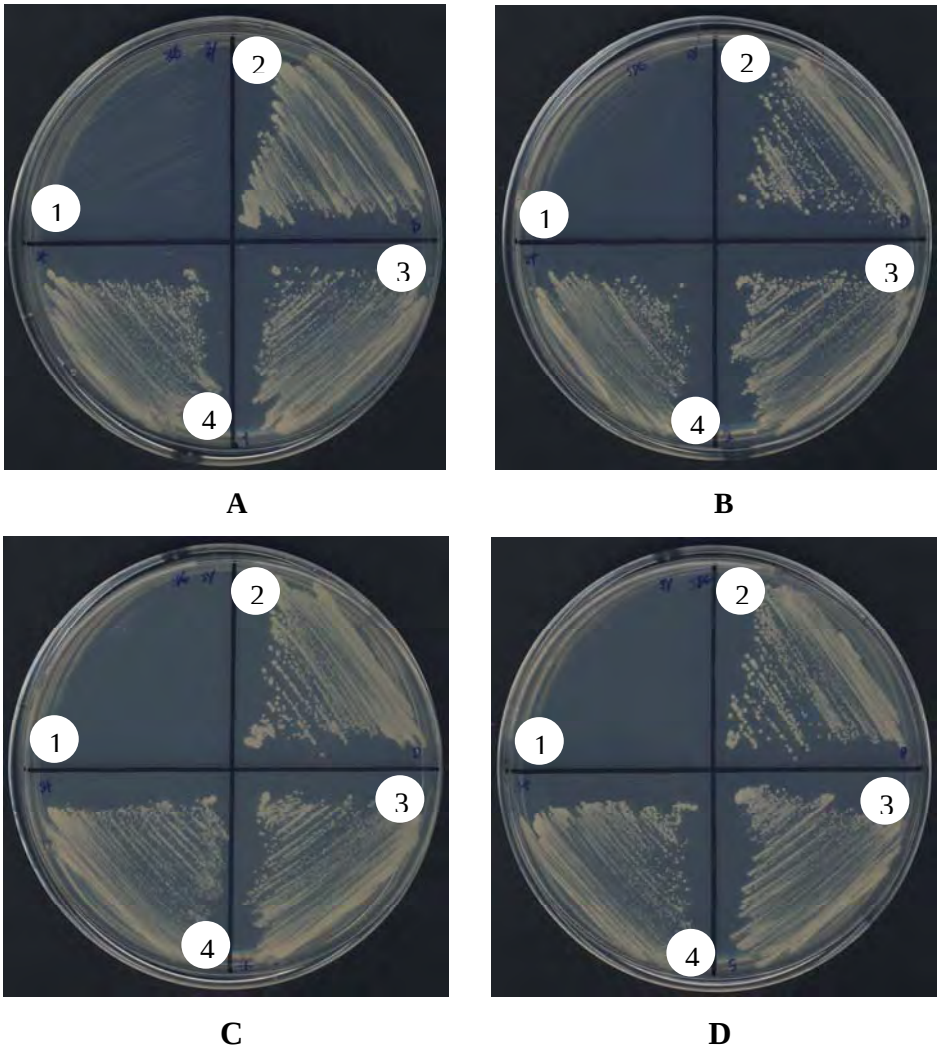


Figure 3.9 Growth of yeast transformants in minimal medium (-uracil) containing 2% glucose as a sole carbon source. The yeast transformants were streaked on agar plates and incubated at 30 ˚C for 3 days. The numbers in the figures are as follow: non-transformed SUSY7/ura3 (1), yeast transformed with empty pDR195 vector (2), *MeSUTs* in pDR195 (3) and *StSUT1* in pDR195 (4). The number 3 in each figure are yeast transformed with pDRMeSUT1 (A), pDRMeSUT2 (B) pDRMeSUT4 (C) and pDRMeSUT5 (D), respectively.

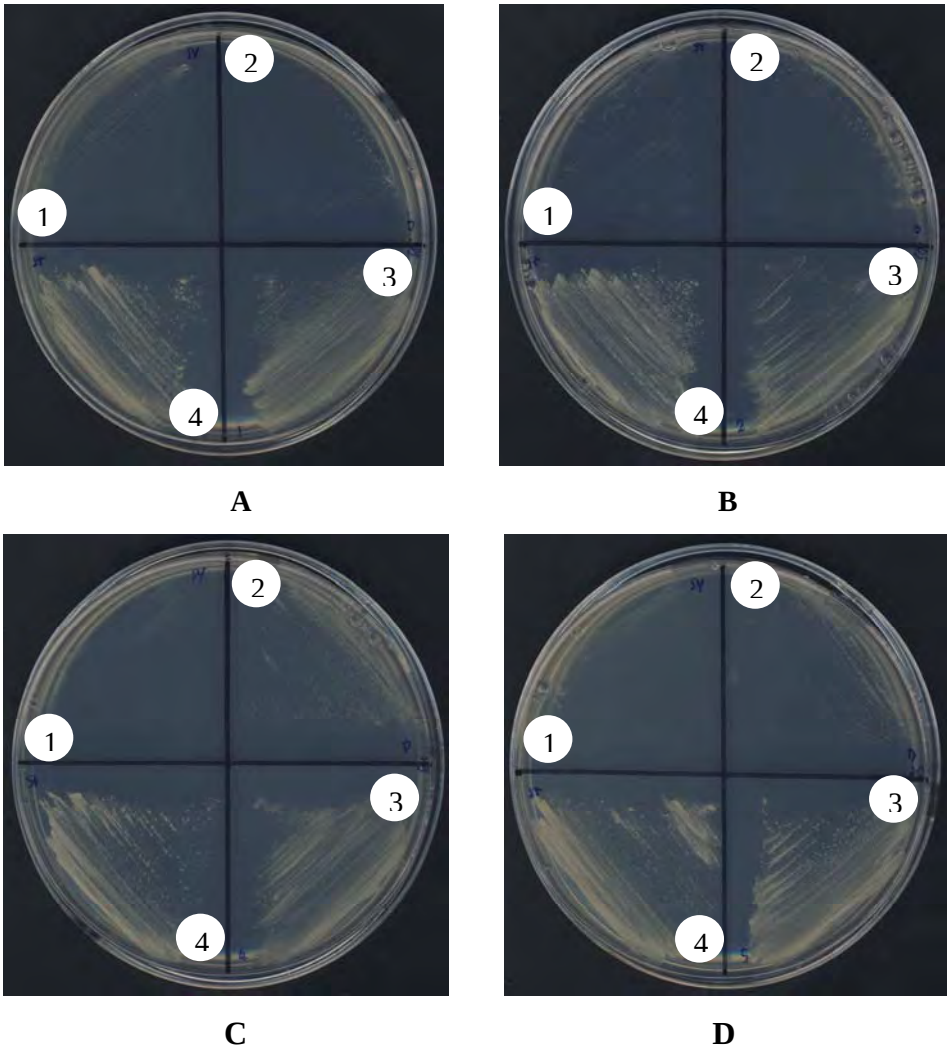


Figure 3.10 Growth of yeast transformants in minimal medium (-uracil) containing 2% sucrose as a sole carbon source. The yeast transformants were streaked on agar plates and incubated at 30 ˚C for 3 days. The numbers in the figures are as follows; non-transformed SUSY7/ura3 (1), yeast transformed with empty pDR 195 vector (2), *MeSUTs* in pDR195 (3) and *StSUT1* in pDR195 (4). The number 3 in each figure are yeast transformed with pDRMeSUT1 (A), pDRMeSUT2 (B) pDRMeSUT4 (C) and pDRMeSUT5 (D), respectively.

3.4 Biochemical characterizations of sucrose transporters expressed in yeast

3.4.1 Effects of pH on ^{14}C-sucrose uptake into yeast expressing MeSUTs

In order to identify the optimum pH for sucrose transport activity, *MeSUTs* yeast transformants were cultured in selective media containing glucose as a carbon source until the OD_{600} reach 0.8-1.0. Cells were collected and washed in sodium phosphate buffer at different pH. Sucrose uptake assays were performed using the same buffer as washing buffer with ^{14}C-sucrose at a final concentration of 1 mM.

The results showed the activities of all MeSUTs were pH dependent. MeSUT1 exhibited low sucrose transport activity at pH 3-5 but the highest activity was noticed at slightly acidic pH (Figure 3.11 A). The uptake rate substantially dropped when the assays were performed in alkaline pH (pH 7-8). MeSUT2, MeSUT4 and MeSUT5 exhibited similar characteristics of response to external pH. Their activities were relatively low at acidic pH range and increasing activities were observed at more alkaline pH. MeSUT2 and MeSUT4 showed steep optimum sucrose uptake at pH 7 and the activities decreased when the pH was increased up to pH 8 (Figure 3.11B, C). MeSUT5 showed slightly different response to pH with broader optimum pH observed at pH 7-8 (Figure 3.11 D).

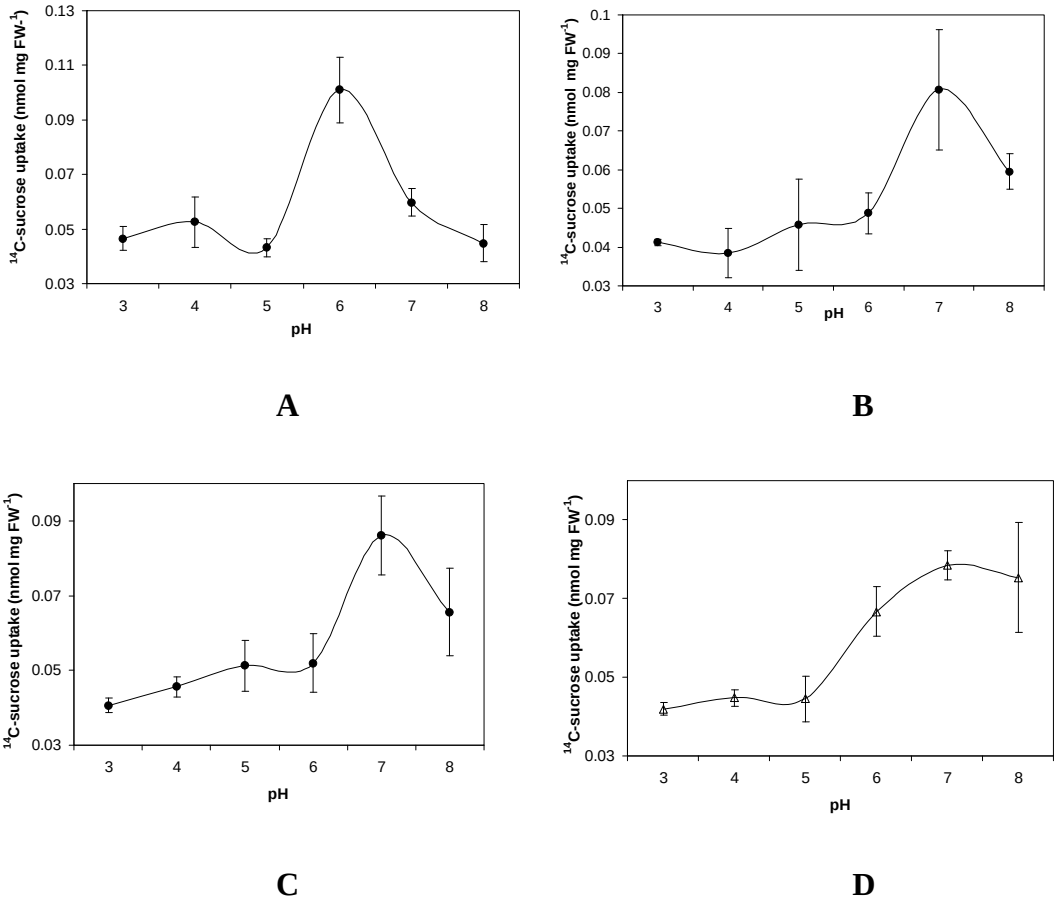


Figure 3.11 Effects of pH on ^{14}C-sucrose transport of yeast expressing MeSUT1 (A), MeSUT2 (B), MeSUT4 (C) and MeSUT5 (D). Assays were performed in various pH of 25 mM sodium phosphate buffer at 30 °C with 200 rpm shaking in the presence of 1 mM ^{14}C-sucrose. Data presented as means ± S.E. (n=3).

3.4.2 Time course of ^{14}C-sucrose uptake

Time course experiments were performed with all MeSUTs at the optimum pH determined with the presence of ^{14}C-sucrose at a final concentration of 1 mM. Sucrose uptake into *MeSUT1* and *MeSUT2* yeast transformants were similar that the sucrose accumulations were constantly increased with time and higher than those of non transformed yeast cells and yeast transformed with empty vector (negative controls), indicating that both *MeSUT1* and *MeSUT2* encode functional sucrose transporters (Figure 3.12 A and B).

Time course of sucrose uptake into yeast cells transformed with pDRMeSUT4 and pDRMeSUT5 were carried out at pH 7, the observed optimum pH, with 100 mM final concentration of ^{14}C-sucrose in each reaction (Figure 3.13 A and B). Yeast cells transformed with *MeSUT4* and *MeSUT5* displayed ability of sucrose uptake and the transport rates increased constantly with time. Residual sucrose uptake was observed in non-transformed SUSY/ura3 yeast cells or the empty vector pDR195 yeast transformant, which were used as negative controls.

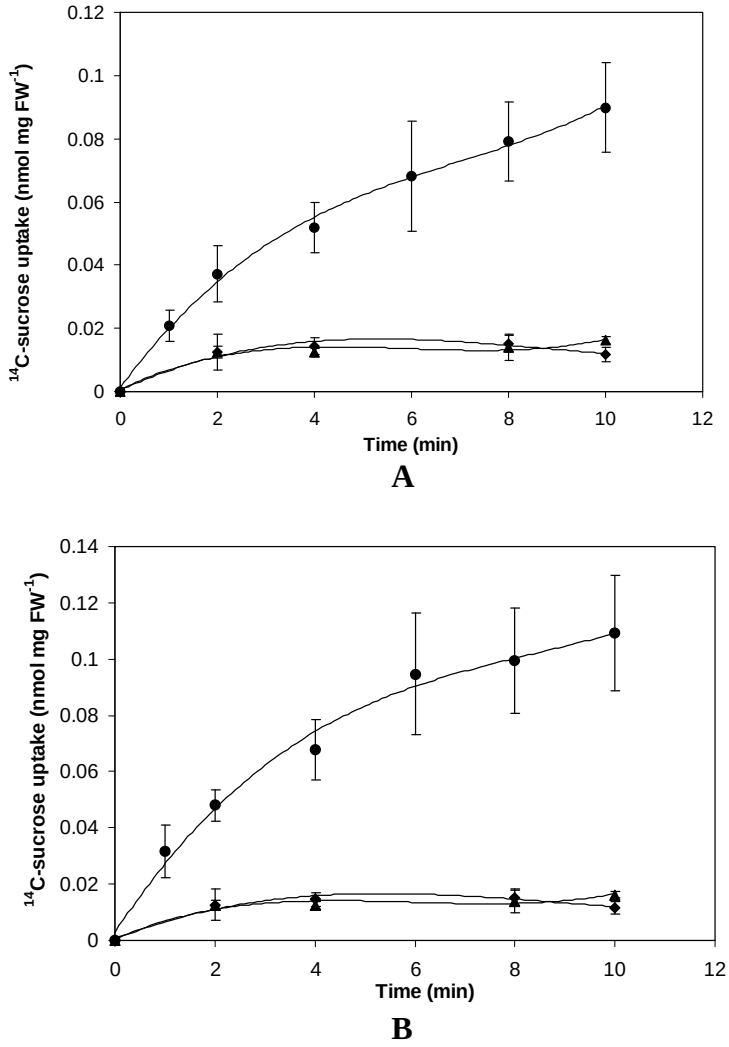
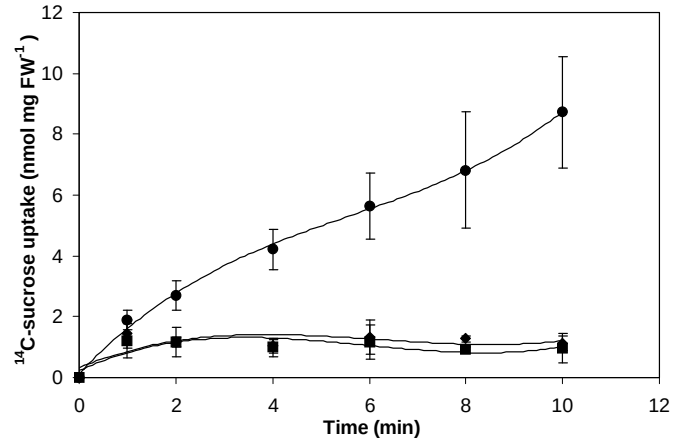
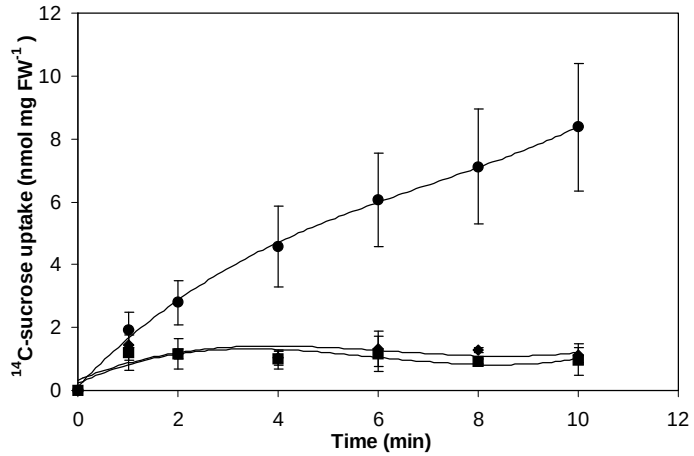


Fig. 3.12 Time course of ^{14}C-sucrose uptake into yeast expressing MeSUT1 (A) and MeSUT2 (B) (●). Non-transformed SUSY7/ura3 (♦) and yeast cell transformed with empty pDR195 vector (▲) were used as negative controls. Assays were performed in 25 mM sodium phosphate buffer pH 6 for MeSUT1 and pH 7 for MeSUT2 at 30 °C with 200 rpm shaking in the presence of 1 mM ^{14}C-sucrose. Data presented as means ± S.E. (n=3).



A



B

Figure 3.13 Time course of ^{14}C-sucrose uptake into yeast expressing MeSUT4 (A) and MeSUT5 (B) (●). Non-transformed SUSY7/ura3 (♦) and yeast cell transformed with empty pDR195 vector (▲) were used as negative controls. Assays were performed in 25 mM sodium phosphate pH 7 at 30 °C with 200 rpm shaking in the presence of 100 mM ^{14}C-sucrose. Data presented as means ± S.E. (n=3).

Yeast transformed with *StSUT1* was used as the positive control. The final concentration of ^{14}C-sucrose in the assays was 1 mM. ^{14}C-sucrose uptake of into *StSUT1* yeast transformant increased constantly with time and the uptake rates were higher than those of MeSUT1 and MeSUT2 indicated the different transport properties among SUTs from various plant species (Figure 3.14).

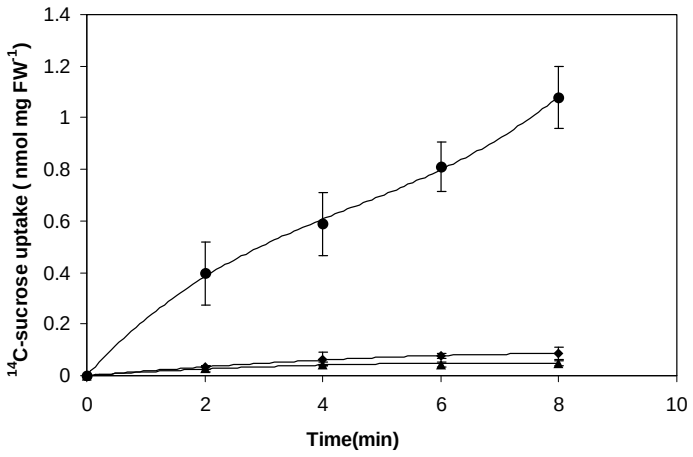
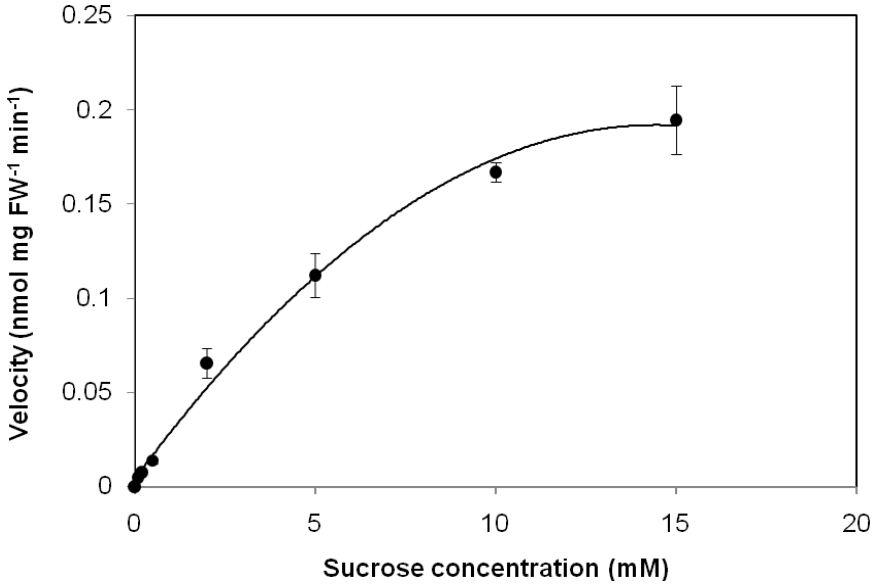


Figure 3.14 Time course of ^{14}C-sucrose uptake into yeast expressing StSUT1 (●). Non-transformed SUSY7/ura3 (♦) and yeast cell transformed with empty pDR195 vector (▲) were used as negative controls. Assays were performed in 25 mM sodium phosphate pH 5.5 at 30 °C with 200 rpm shaking in the presence of 1 mM ^{14}C-sucrose. Data presented as means ± S.E. (n=3).

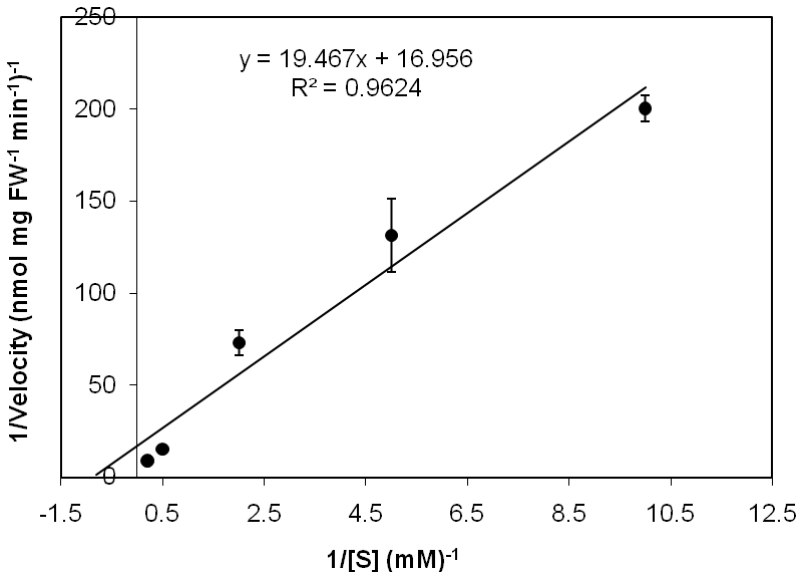
3.4.3 Identification of kinetic parameters for sucrose transport

To study kinetic of sucrose uptake, sucrose concentrations in the reactions were varied. Figure 3.15 (A) showed the ^{14}C-sucrose uptake activity of *MeSUT1* yeast transformant at various sucrose concentrations and the Lineweaver-Burk plot was shown in Figure 3.15 (B). The K_m and V_{max} of sucrose uptake activity from *MeSUT1* yeast transformant were 1.15±0.11 mM and 0.06 nmol/mg FW/min, respectively. The uptake characteristics of *MeSUT1* yeast transformants followed the Michaelis-Menten kinetic suggested the carrier mediated sucrose transport processes. MeSUT1 has low K_m value and can be classified into high affinity sucrose transport system (K_m 0.3-2 mM; (Riesmeier *et al.*, 1993; Manning *et al.*, 2001; Knop *et al.*, 2004))

The experiments on determination of kinetic parameters of *MeSUT2* yeast transformant were shown in Figure 3.16 (A) and (B). The apparent K_m and V_{max} values of MeSUT2 were 12.55±3.58 mM and 0.36±0.10 nmol/mg FW/min, respectively. The high K_m value of MeSUT2 indicated a low affinity sucrose transport component similar to previous reports (Schulze *et al.*, 2000; Barth *et al.*, 2003).

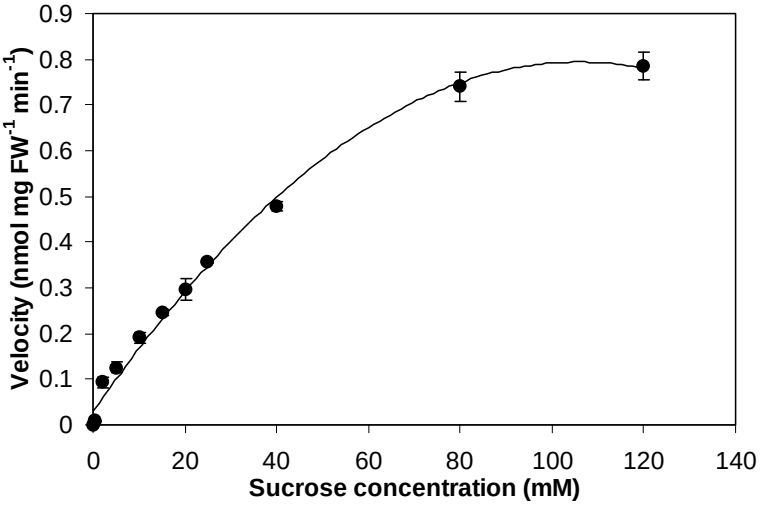


A

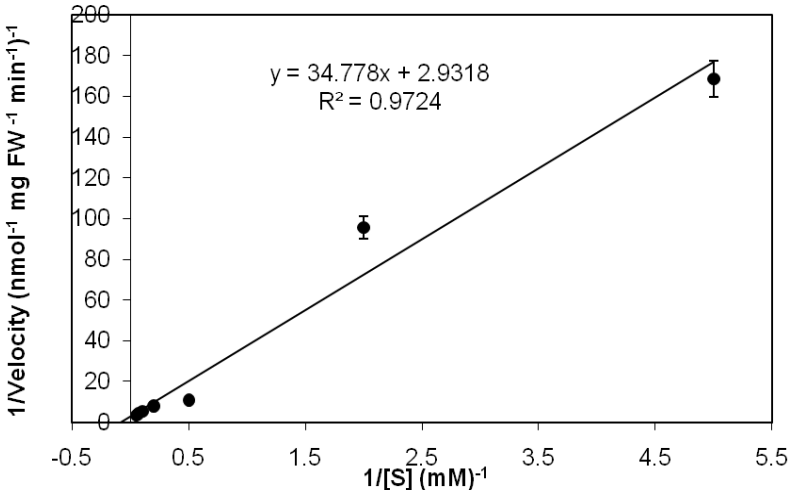


B

Figure 3.15 Concentration dependence of ^{14}C-sucrose uptake into yeast expressing MeSUT1 (A). Assays were performed in 25 mM sodium phosphate buffer pH 6 at 30 °C with 200 rpm shaking. Lineweaver Burk transformation was shown in (B) and the apparent K_m and V_{max} were 1.15±0.11 mM and 0.06 nmol/mg FW/min, respectively. Data presented as means ± S.E. (n=3).



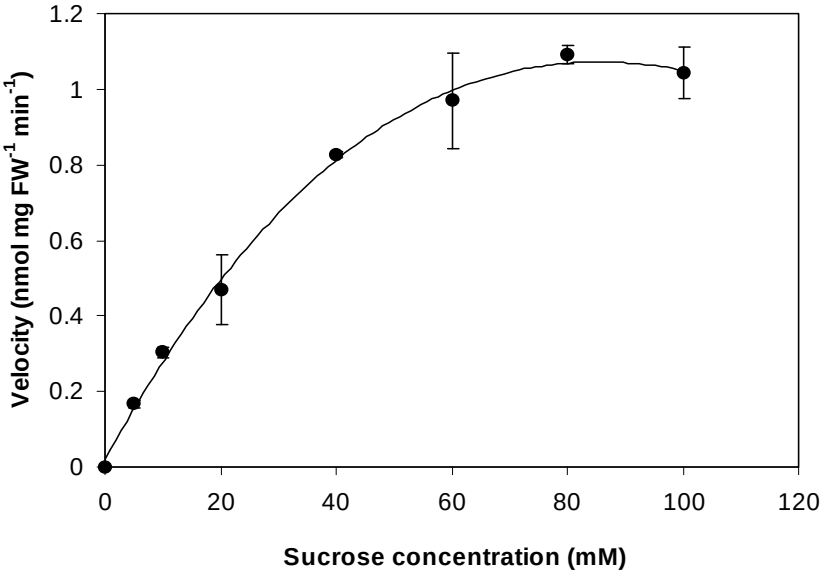
A



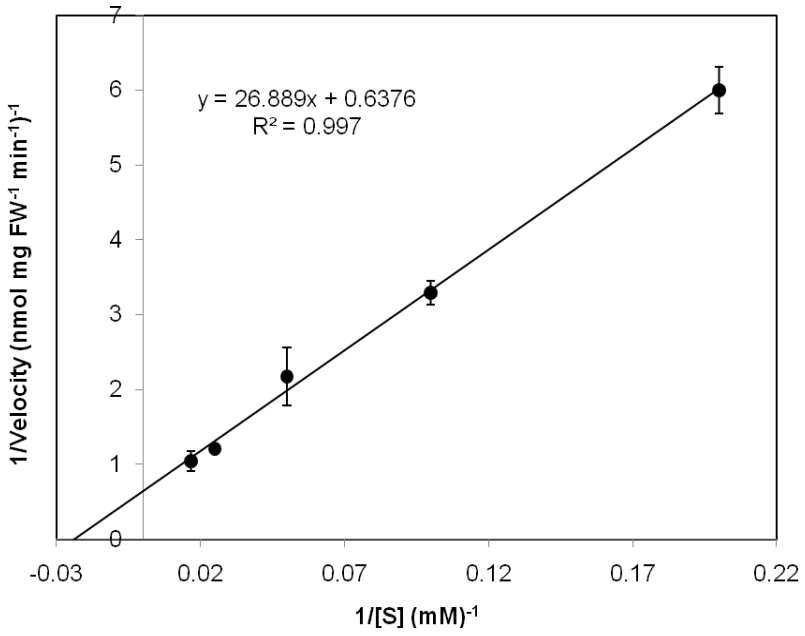
B

Figure 3.16 Concentration dependence of ^{14}C-sucrose uptake into yeast expressing MeSUT2 (A). Assays were performed in 25 mM sodium phosphate buffer pH 7 at 30 °C with 200 rpm shaking. Lineweaver Burk transformation was shown in (B) and the apparent K_m and V_{max} were 12.55±3.58 mM and 0.36±0.10 nmol/mg FW/min, respectively. Data presented as means ± S.E. (n=3).

Sucrose uptake mediated by MeSUT4 and MeSUT5 showed a characteristic of saturable kinetics. The K_m and V_{max} values of sucrose uptake activity from *MeSUT4* yeast transformant were calculated from sucrose uptake results and revealed that K_m= 42.25±1.54 mM and V_{max}= 1.58±0.13 nmol/mg FW/min, respectively (Figure 3.17 (A) and (B)). The experiments were done in the same pattern for *MeSUT5* yeast transformant, and the results were shown in Figure 3.18 (A) and (B). The apparent K_m and V_{max} values of MeSUT5 were 51.37±5.30 mM and 1.10±0.12 nmol/mg FW/min, respectively. These K_m values were higher than those of previously characterized high affinity sucrose transporter (SUT1) and the transport properties of MeSUT4 and MeSUT5 showed the similarity to the previously characterized low affinity/ high capacity (LAHC) sucrose transporter type (Weise *et al.*, 2000; Zhang *et al.*, 2008), suggesting that these two sucrose transporters belong to the low affinity class**.**

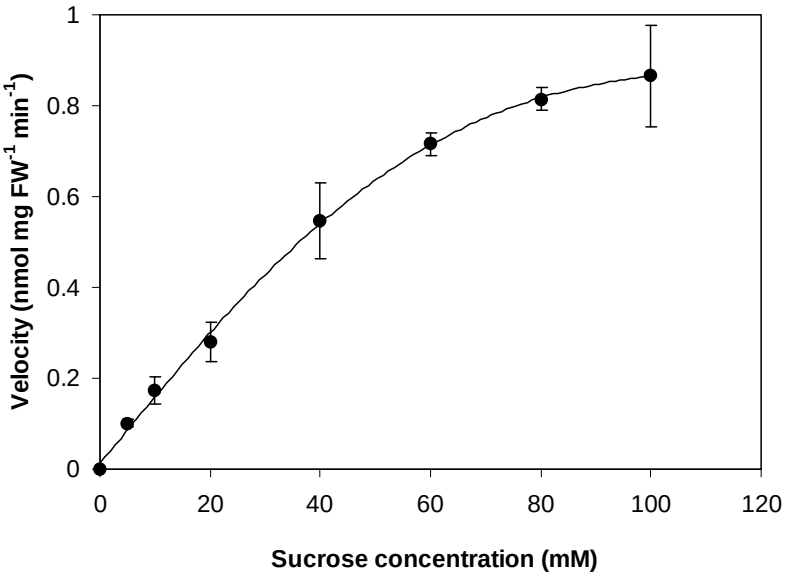


A

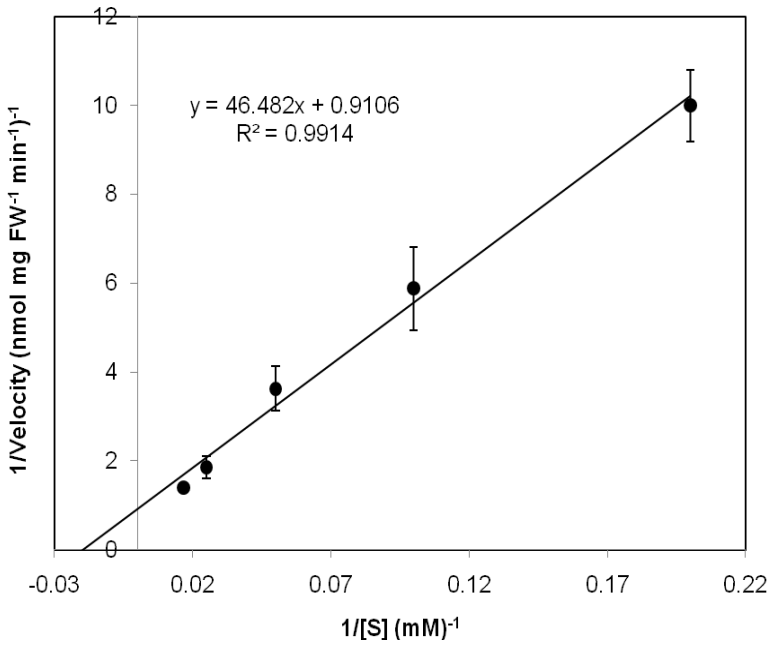


B

Figure 3.17 Concentration dependence of ^{14}C-sucrose uptake into yeast expressing MeSUT4 (A). Assays were performed in 25 mM sodium phosphate buffer pH 7 at 30 °C with 200 rpm shaking. Lineweaver Burk transformation was shown in (B) and the apparent K_m and V_{max} were 42.25±1.54 mM and 1.58±0.13nmol/mg FW/min, respectively. Data presented as means ± S.E. (n=3).



A



B

Figure 3.18 Concentration dependence of ^{14}C-sucrose uptake into yeast expressing MeSUT5 (A). Assays were performed in 25 mM sodium phosphate buffer pH 7 at 30 °C with 200 rpm shaking. Lineweaver Burk transformation was shown in (B) and the apparent K_m and V_{max} were 51.37±5.30 mM and 1.10±0.12nmol/mg FW/min, respectively. Data presented as means ± S.E. (n=3).

3.5 Effects of several inhibitors on ^{14}C-sucrose uptake activity

3.5.1 Effects of sulfhydryl modifying reagents on ^{14}C-sucrose uptake

The effects of various inhibitors on ^{14}C-sucrose uptake were assayed in order to obtain basic information on the sucrose transport mechanisms. The effects of sulfhydryl modifying reagents on ^{14}C-sucrose uptake activity were performed for evaluation of the influences of amino acids containing thiol groups on transport activity by using NEM and PCMBS as the compound to be examined. The reagents were added at a final concentration of 50 µM. MeSUT1 activity was most sensitive to NEM at 27 % inhibition whereas the remaining three MeSUTs were almost unaffected by this compound. Only MeSUT1 showed a strong reduction on the sucrose uptake inhibited by PCMBS at 30% inhibition. MeSUT4 was slightly inhibited by this compound at 19% inhibition whereas MeSUT2 and MeSUT5 were not affected (Figure 3.19).

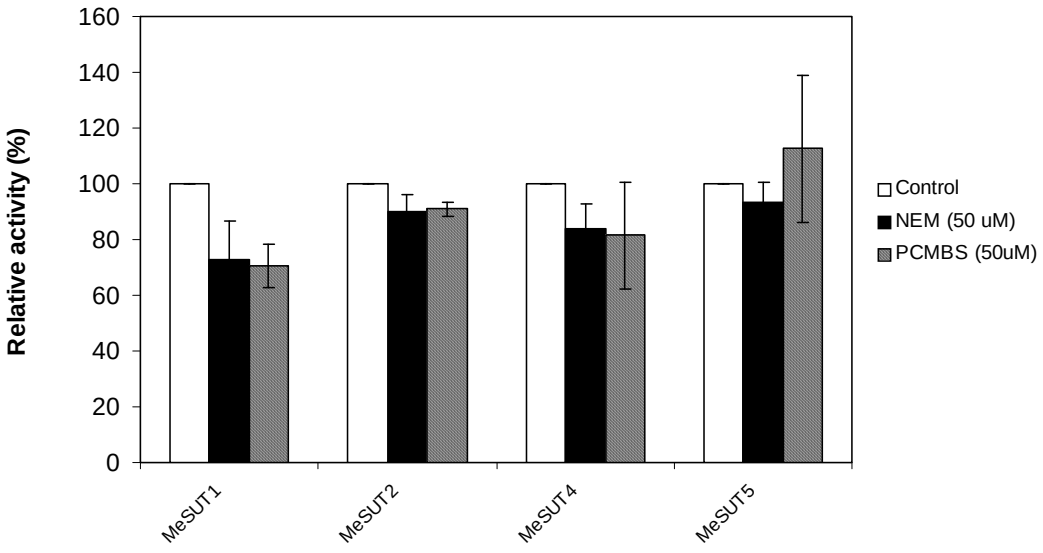


Figure 3.19 Effects of sulfhydryl modifying reagents on ^{14}C-sucrose uptake into yeast expressing MeSUTs. Assays were performed in 25 mM sodium phosphate buffer at the optimum pH of each sucrose transporters and incubated at 30 °C with 200 rpm shaking. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and for MeSUT4 and MeSUT5 was 10 mM. Inhibitors were individually added into the reactions at the final concentration of 50 µM. All experiments were repeated three times and data presented as means ± S.E. (n=3).

3.5.2 Effects of P-type ATPase inhibitor on ^{14}C-sucrose uptake

A plasma membrane ATPase inhibitor, vanadate, was added into the assays in order to examine the effect of this ATPase on sucrose uptake by MeSUTs. The reagents were added at a final concentration of 100 µM. From the results in Figure 3.20, MeSUT1 was most affected by vanadate at 28% inhibition indicating it was dependent on P-type ATPase. This compound had less effect on MeSUT4 which reduced the activity to 84% while MeSUT2 and MeSUT5 were insensitive.

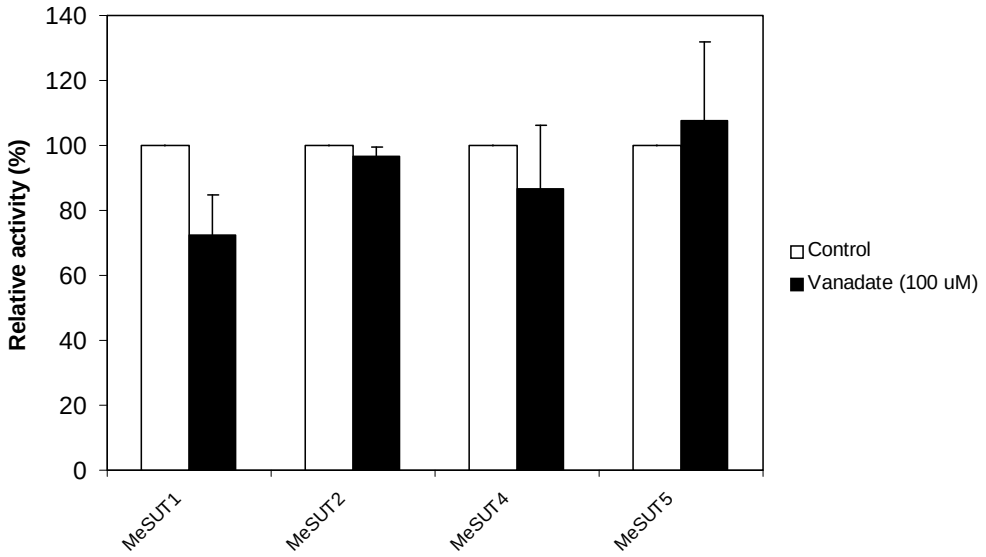


Figure 3.20 Effects of P-type ATPase inhibitor (vanadate) on ^{14}C-sucrose uptake into yeast expressing MeSUTs. Assays were performed in 25 mM sodium phosphate buffer at the optimum pH of each sucrose transporters and incubated at 30 °C with 200 rpm shaking. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and for MeSUT4 and MeSUT5 was 10 mM. Inhibitors were individually added into the reactions at the final concentration of 100 µM. All experiments were repeated three times and data presented as means ± S.E. (n=3).

3.5.3 Effects of proton gradients disruptors and electron transport inhibitor on ^{14}C-sucrose uptake

The sucrose transport mediated by sucrose transporters is an active transport which cooperates with proton gradients and uses energy as driving force. The protonophore CCCP and 2, 4-DNP were employed to investigate the role of proton on the sucrose uptake activity and Antimycin A was used for inhibiting the election transport which generate ATP. The reagents were added at a final concentration of 50 μM. From Figure 3.21, all MeSUTs were strongly inhibited by CCCP and antimycin A, which clearly identified that these transporters were driven by proton gradient and were energy dependent. Different effect was observed on another protonophore 2, 4-DNP which reduced the activity of MeSUT1 (55%) and MeSUT4 (30%) but not of MeSUT2 and MeSUT5, indicating that this compound reacted at different site from CCCP.

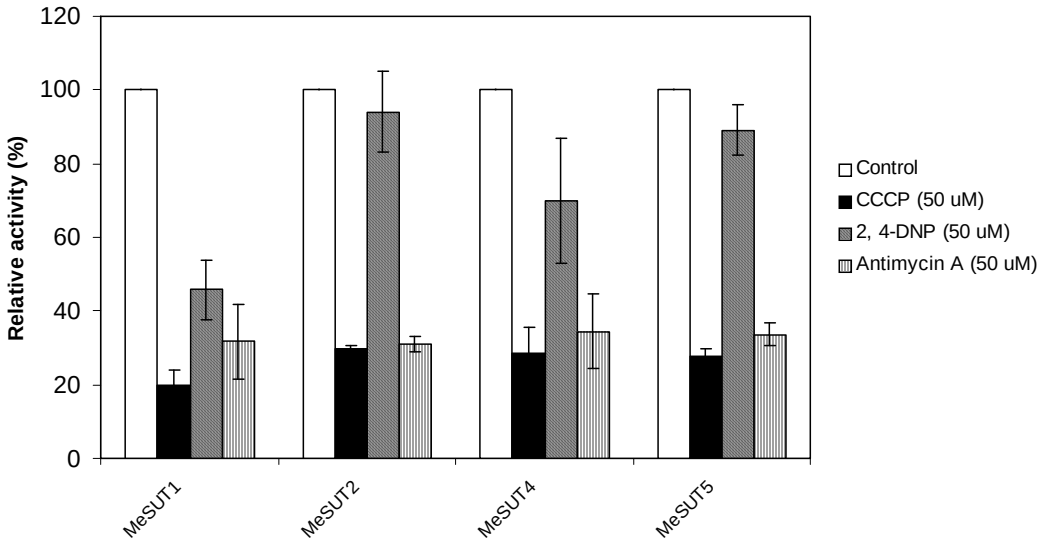


Figure 3.21 Effects of proton gradient disruptors and electron transport inhibitor on ^{14}C-sucrose uptake into yeast expressing MeSUTs. Assays were performed in 25 mM sodium phosphate buffer at the optimum pH of each sucrose transporters and incubated at 30 °C with 200 rpm shaking. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and for MeSUT4 and MeSUT5 was 10 mM. Inhibitors were individually added into the reactions at the final concentration of 50 μM. All experiments were repeated three times and data presented as means ± S.E. (n=3).

3.6 Effects of various sugars on ^{14}C-sucrose uptake

Unlabelled glycosides those might act as potential substrates for MeSUTs were added into the reactions at 10 folds higher than ^{14}C-sucrose in order to identify the substrate specificities. As shown in Table 3.1, unlabelled sucrose strongly inhibited the activity of MeSUT1, MeSUT4 and MeSUT5 but slight inhibition was observed in MeSUT2. Surprisingly, glucose and fructose, which are sucrose hydrolyzed products, strongly inhibit all MeSUTs activity that differed from other plant SUTs. Maltose, a typical disaccharide that inhibits other SUTs activity, only slightly reduced sucrose uptake by MeSUT1 and MeSUT2 but strong inhibition was observed in MeSUT4 and MeSUT5. Sucrose uptake was also drastically decreased when mannose, a glucose structurally related monosaccharide, was presented in the reaction whereas lactose showed no effect on all MeSUTs activity.

Table 3.1 Effects of various sugars on ^{14}C-sucrose uptake into yeast expressing MeSUTs.

Sugars	**Residual transport rate (%)**			
	MeSUT1	**MeSUT2**	**MeSUT4**	**MeSUT5**
None	100	100	100	100
Sucrose	71.72 ± 4.56	83.31 ± 4.22	36.78 ± 2.57	34.91 ± 2.40
Glucose	40.68 ± 2.32	44.42 ± 5.42	7.69 ±1.01	10.21 ± 0.75
Fructose	50.62 ± 2.84	46.22 ± 2.63	15.75 ± 1.10	18.18 ± 2.53
Maltose	88.12 ± 4.22	88.62 ± 4.98	37.05 ± 4.11	35.76 ± 1.93
Lactose	102.05 ± 8.37	91.14 ± 2.03	73.35 ± 7.61	81.34 ± 9.94
Mannose	55.35 ± 7.70	45.38 ± 1.67	16.90 ± 1.42	39.45 ± 14.60

Assays were performed in 25 mM sodium phosphate buffer at the optimum pH of each sucrose transporters and incubated at 30 °C with 200 rpm shaking. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and for MeSUT4 and MeSUT5 was 10 mM. Unlabelled sugars were individually added into the reactions at 10 folds higher than ^{14}C-sucrose. All experiments were repeated three times and data presented as means ± S.E. (n=3).

3.7 Effects of linamarin on ^{14}C-sucrose uptake

Linamarin, a cyanogenic glucoside synthesized and stored in several cassava organs, was used for determining its roles whether it might influence the activity of MeSUTs. The final concentrations used in the experiments were 1 and 5 mM which closed to the concentration *in situ* (~2mM, (McMahon *et al.*, 1995; White *et al.*, 1998)). MeSUT1 and MeSUT2 seem to be unaffected by linamarin since no strong inhibition or stimulation was observed while MeSUT4 and MeSUT5 were inhibited by this compound and more reductions on the activity were observed at higher linamarin concentrain (Table 3.2)

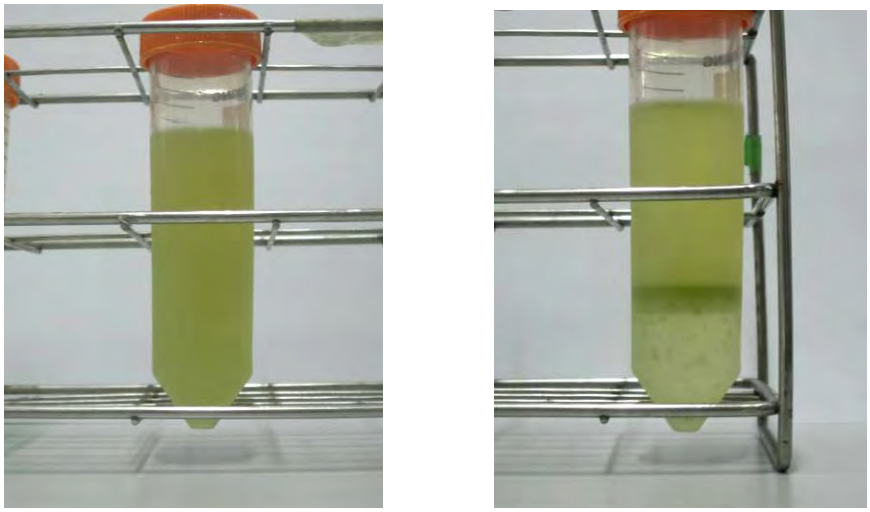
Table 3.2 Effects of linamarin on ^{14}C-sucrose uptake into yeast expressing MeSUTs.

Concentration	**Residual transport rate (%)**			
	MeSUT1	**MeSUT2**	**MeSUT4**	**MeSUT5**
None	100	100	100	100
1 mM	104.99 ± 8.50	99.07 ± 16.31	81.77 ± 25.64	71.68 ± 11.69
5 mM	100.90 ± 11.00	86.17 ± 6.81	63.53 ± 11.16	62.12 ± 2.37

Assays were performed in 25 mM sodium phosphate buffer at the optimum pH of each sucrose transporters and incubated at 30 °C with 200 rpm shaking. The final concentration of ^{14}C-sucrose for MeSUT1 and MeSUT2 was 1 mM and for MeSUT4 and MeSUT5 was 10 mM. All experiments were repeated three times and data presented as means ± S.E. (n=3).

3.8 Isolation of plasma membrane vesicles from cassava tubers

The native plasma membrane was extracted and purified from cassava tubers (5 kg fresh weight) using aqueous two phase system. Cassava tuber homogenate was centrifuged at 10,000 xg for 10 min. The supernatant was further centrifuged at 100,000xg for 30 min to obtain the microsomal fraction. This fraction contained mixture of plasma membrane, tonoplast, internal membranes of the endoplasmic reticulum and membranes of cytoplasmic organelles and was be used for separation of plasma membrane vesicles by aqueous two phase partition (Flowers and Yao, 1992). The plasma membrane was fractionated into the upper phase (PEG, Figure 3.22). The estimated protein concentration was about 92 mg.



A **B**

Figure 3.22 Separation of plasma membrane vesicles from microsomal fraction using aqueous two phase system. The microsomal fraction was added on the top of the separating phase and mixed thoroughly (A). The phase was let to stand for 3-5 hours at 4 °C in order to isolate the plasma membrane from other cellular components (B). The upper phase contains the plasma membrane which is used for characterizations.

3.9 Measurements of the ^{14}C-sucrose binding activity of plasma membrane vesicles

3.9.1 Effects of pH on ^{14}C-sucrose binding activity of root plasma membrane vesicles

To investigate the effects of pH on sucrose binding activity of root plasma membrane vesicles, the pH of the assay buffers were varied. Plasma membrane vesicles purified from cassava roots showed the low ^{14}C-sucrose binding activity at the acidic pH range (Figure 3.23). The activity was increased at more alkaline pH and the highest sucrose binding activity was observed at pH 7.8. The activity was slightly decreased when the pH was increased to pH 9.

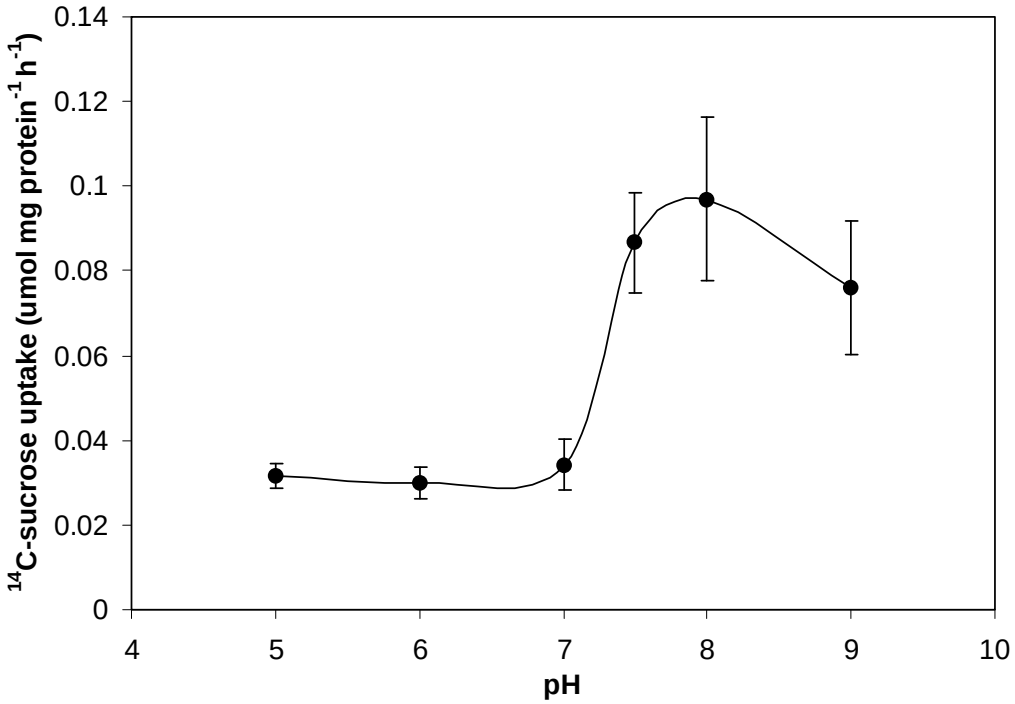


Figure 3.23 Effects of pH on ^{14}C-sucrose binding of cassava root membrane vesicles. Assays were performed in various pH of assay buffer in the presence of 1 mM ^{14}C-sucrose. Data presented as means ± S.E. (n=3).

3.9.2 Time course of ^{14}C-sucrose binding of cassava root plasma membrane vesicles

The time course of sucrose binding of root plasma membrane vesicles were performed at the assay buffer pH 7.8 with the final concentration of ^{14}C-sucrose of 1 mM. Sucrose binding of root plasma membrane vesicle increases with time up to 180 min and started to decline (Figure 3.24).

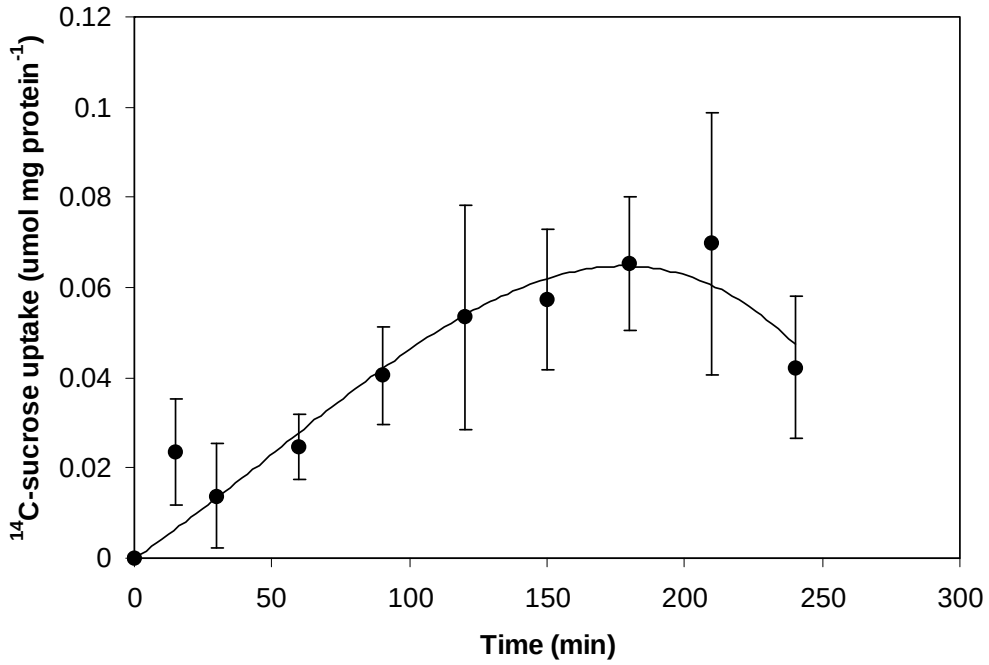
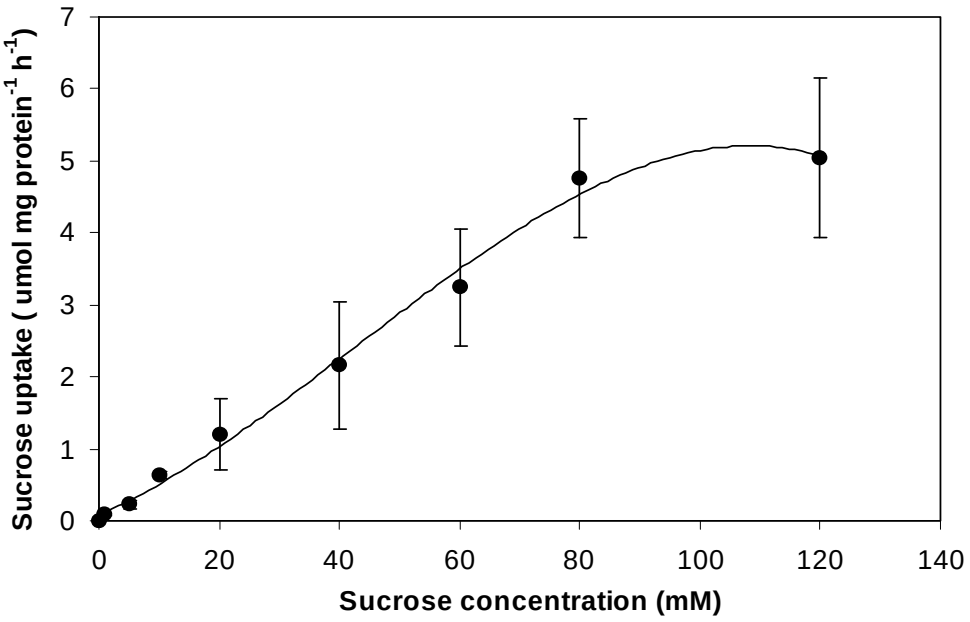


Figure 3.24 Time course of ^{14}C-sucrose binding activity of root plasma membrane vesicles. The assays were performed in the buffer pH 7.8 with the final concentration of 1 mM ^{14}C-sucrose.

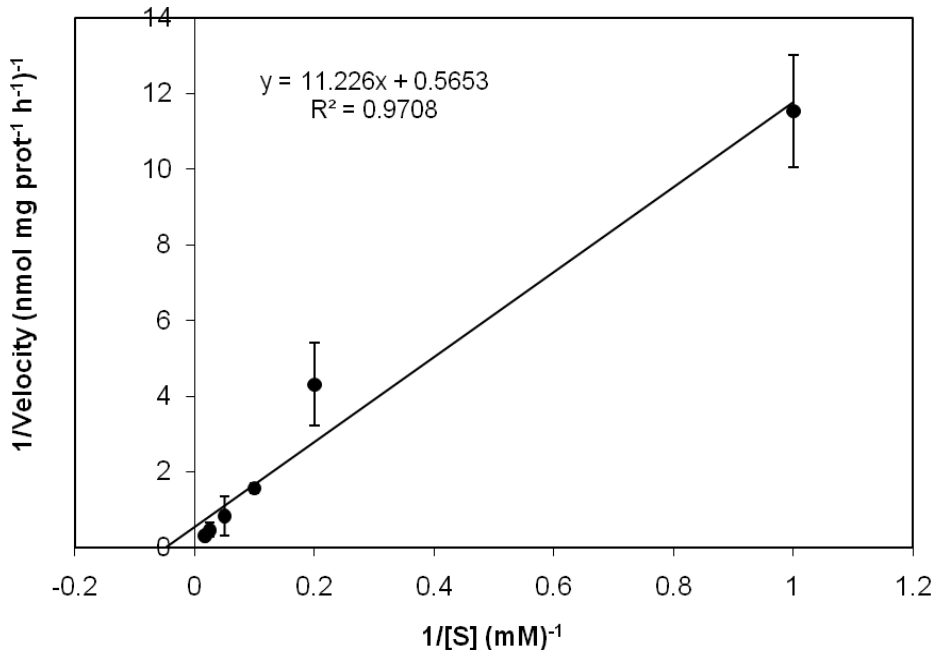
3.9.3 The kinetic of sucrose binding on membrane

The kinetic studies of sucrose binding of root plasma membrane vesicles were shown in Figure 3.25. Sucrose binding of plasma membrane vesicles from root increased at higher concentration of ^{14}C-sucrose concentration, approaching maximal rate at 30 mM. The sucrose uptake saturation curve appeared to follow the Michaelis-Menten kinetics, indicating that the uptake of sucrose was carrier-mediated process. The double reciprocal plot of saturation curve revealed that the apparent K_m and V_{max}

of root membrane vesicles were 19.16 ± 6.44 mM and 1.47 ± 0.54 μmol/mg protein/h, respectively.



A



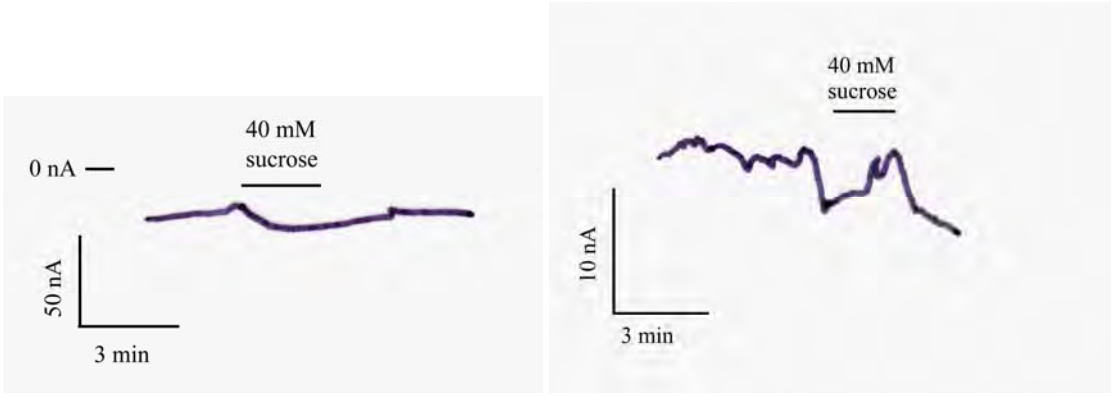
B

Figure 3.25 Concentration dependence of ^{14}C-sucrose binding of root plasma membrane vesicles. Assays were performed in buffer pH 7.8 Lineweaver Burk transformation was shown in (B) Data presented as mean ± S.E. (n=3).

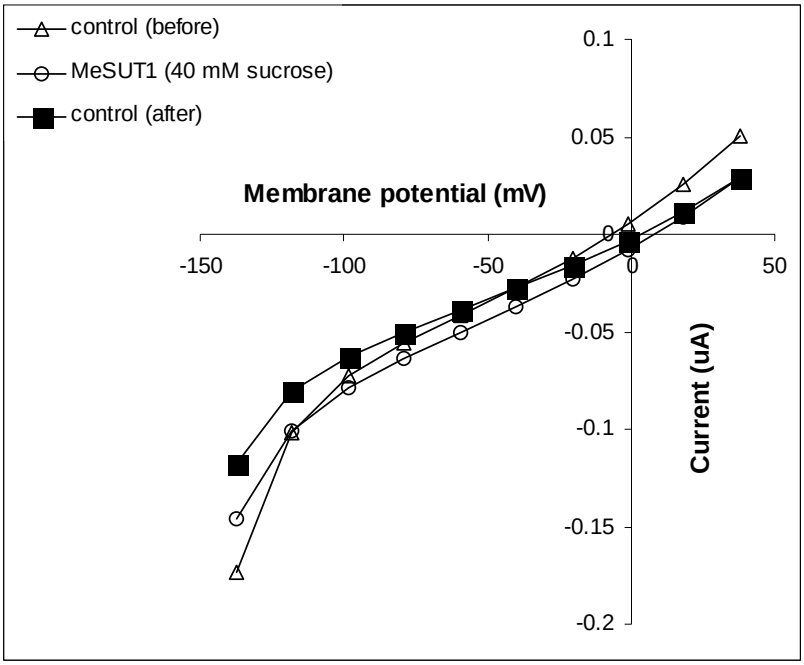
3.10 Expression of MeSUTs in *Xenopus laevis* oocytes

All four cassava sucrose transporter genes were expressed in *Xenopus* oocytes and their sucrose transport abilities were characterized in order to gain more details of sucrose transport mechanisms mediated by these transporters.

Oocytes injected with MeSUT1, MeSUT4 and MeSUT5 cRNAs can survive and the properties of the proteins were assayed. However, all of oocytes injected with MeSUT2 cRNAs died after 3 days of incubation, thus, there was no sucrose transport result for MeSUT2. Characterization of oocytes expressing MeSUT1 and MeSUT5 yielded similar results. The oocytes were clamped at -40 mV and both of them showed sucrose transport activities as there were inward currents induced after 40-50 mM sucrose were applied into the assays (Figure 3.26 A and 3.27A). The currents were measured at the range of membrane potentials between -137.4 mV and 37.8 mV in the presence or absence of 40 mM sucrose for MeSUT1 and 50 mM sucrose for MeSUT5. Although increases in currents were observed when sucrose was applied in the assays, those currents were very small and not clearly different compared to the background before or after addition of sucrose. In order to see the inward current clearly, the oocytes expressing MeSUT1 or MeSUT5 were clamped at -70 mV and the currents were amplified for 5 times (Figure 3.26 A and B) . Oocytes expressing either MeSUT1 or MeSUT5 induced small inward currents but MeSUT1 expressed oocytes were unstable and tend to died easily when using these oocytes for sucrose transport assays. Therefore, oocytes expressing MeSUT1 and MeSUT5 cannot be used for further characterization of the kinetic parameters. Unlike MeSUT1 and MeSUT5, OsSUT1-gfp-D329N which was the positive control expressed in oocytes induced a large inward current and more currents were observed at more negative membrane potentials when 50 mM sucrose was presented in the assay (Figure 3.28 B and D). There was no induced current when applying sucrose at the same concentration on uninjected oocyte which was used as negative control, confirming that the sucrose transport was mediated by the expressed proteins (Figure 3.28 A and C).

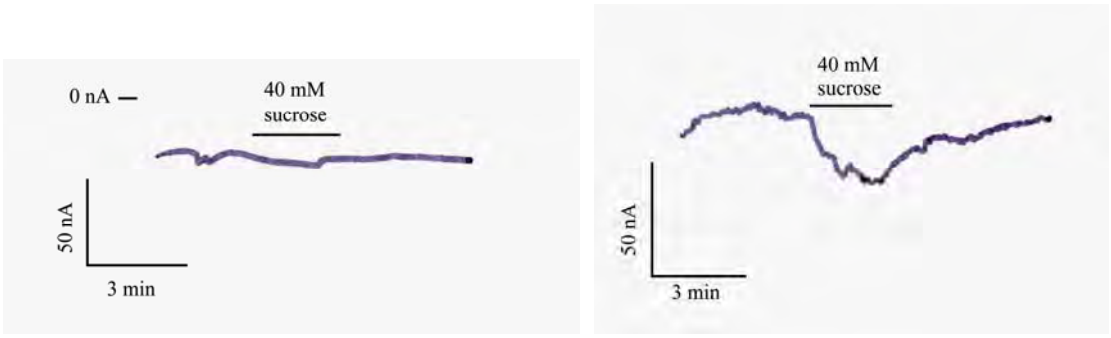


A **B**



C

Figure 3.26 MeSUT1 mediated sucrose transport activity. (A) *Xenopus* oocytes injected with MeSUT1 cRNA were perfused with sodium Ringer solution, pH 5.6 and clamped at a membrane potential of -40 mV. 40 mM sucrose was added and removed at the times indicated by the lines above the trace. A downward deflection of the trace indicates an influx of currents. (B) Oocyte was clamped at -70 mV and current was amplified 5 times. (C) Voltage dependence of sucrose transport activity of MeSUT1. Currents were recorded over a range of membrane potentials before, during and after addition of 40 mM sucrose in sodium Ringer solution, pH 5.6.



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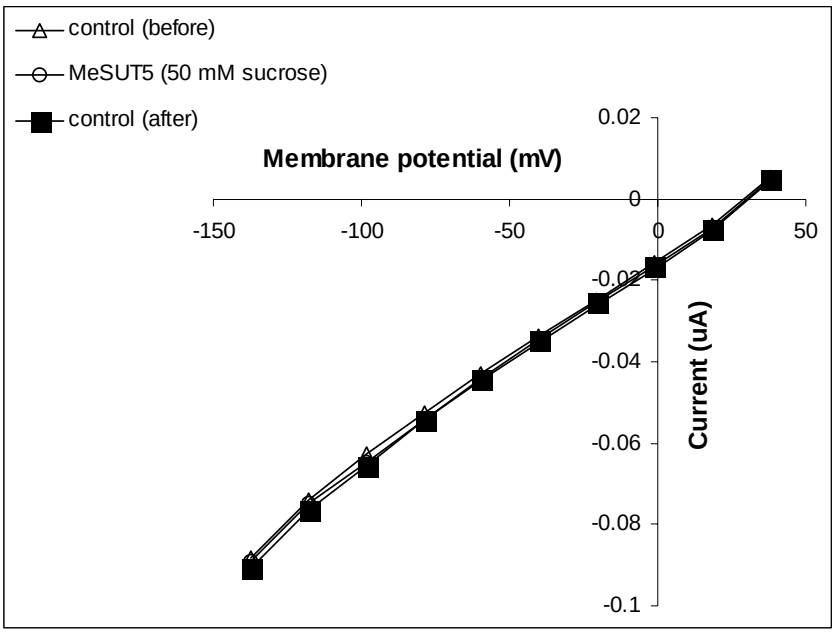
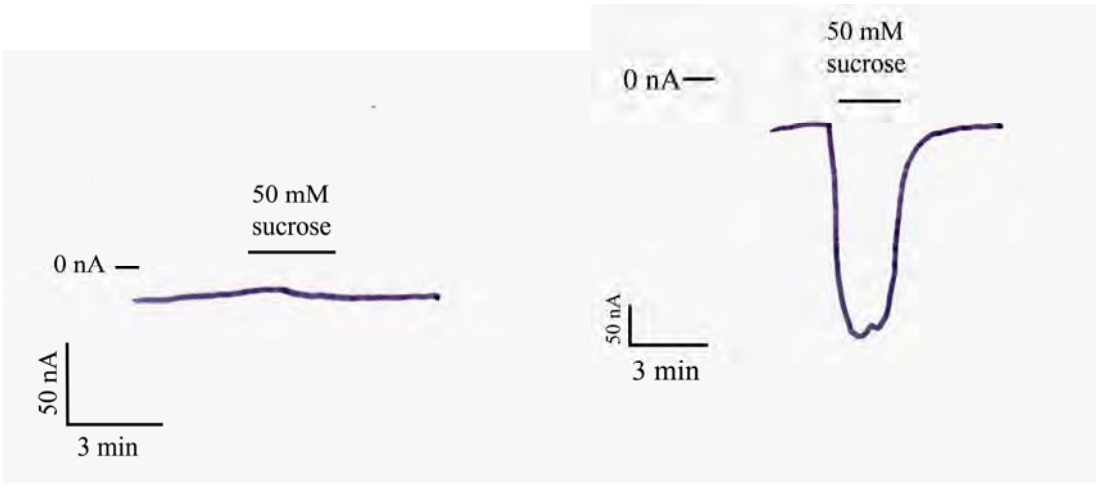


C

Figure 3.27 MeSUT5 mediated sucrose transport activity. (A) *Xenopus* oocytes injected with MeSUT5 cRNA were perfused with sodium Ringer solution, pH 5.6 and clamped at a membrane potential of -40 mV. 50 mM sucrose was added and removed at the times indicated by the lines above the trace. A downward deflection of the trace indicates an influx of currents. (B) Oocyte was clamped at -70 mV and current was amplified 5 times. (C) Voltage dependence of sucrose transport activity of MeSUT5. Currents were recorded over a range of membrane potentials before, during and after addition of 40 mM sucrose in sodium Ringer solution, pH 5.6.



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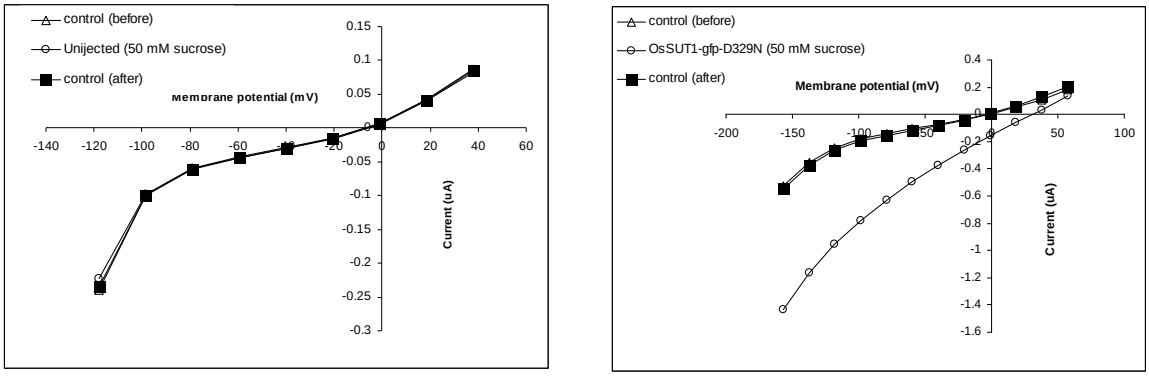
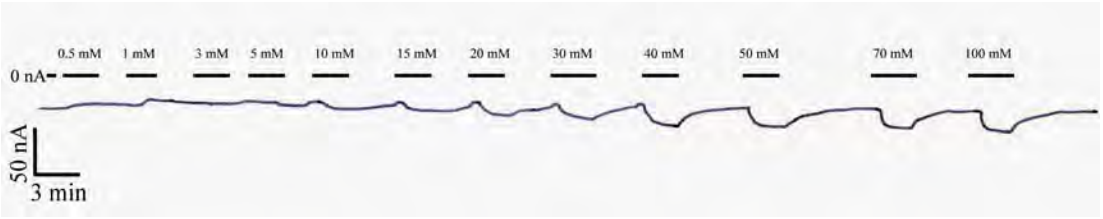


C **D**

Figure 3.28 Sucrose transport activity of uninjected oocytes and oocyte expressing OsSUT1-gfp-D329N. Either uninjected *Xenopus* oocytes (negative control; A) or oocyte injected with OsSUT1-gfp-D329N cRNA (positive control; B) were perfused with sodium Ringer solution, pH 5.6 and clamped at a membrane potential of -40 mV. 50 mM sucrose was added and removed at the times indicated by the lines above the trace. A downward deflection of the trace indicates an influx of currents. Voltage dependence of sucrose transport activity of uninjected oocyte (C) and oocyte expressing OsSUT1-gfp-D329N (D). Currents were recorded over a range of membrane potentials before, during and after addition of 50 mM sucrose in sodium Ringer solution, pH 5.6.

Oocyte expressing MeSUT4 induced more inward currents than the induced currents from MeSUT1 and MeSUT5 but still smaller than the positive control. There was more induced currents when external sucrose concentrations was increased (Figure 3.29 A). The currents were measured at a range of membrane potentials the same as performed in MeSUT1 and MeSUT5 (Figure 3.29 B). $K_{0.5}$, the concentration of sucrose at half the induced current observed, of MeSUT4 was determined at pH 5.6 and the membrane potential of -94 mV (Figure 3.29 C). The analyzed $K_{0.5}$ was 12.35 mM and I_{max} was -0.02 µA/min, suggesting that MeSUT4 had low affinity for sucrose. However, these data might not be reliable as the great variations of $K_{0.5}$ at various membrane potentials were observed (Figure 3.29 D). This did not happen with other characterized sucrose transporters reported to date. Normally, the currents used for $K_{0.5}$ analysis were measured at the highest negative membrane potential but this was not the case for MeSUT4 because of the errors observed. Moreover, there were increments of data errors when higher sucrose concentrations were applied. Therefore, the data achieved from characterizing of MeSUT4 expressed in oocytes might only be indicative but could not be used as reliable results.



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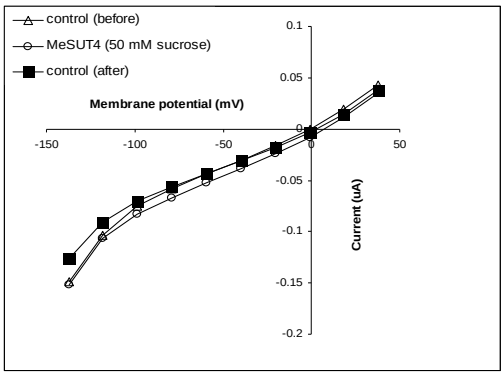

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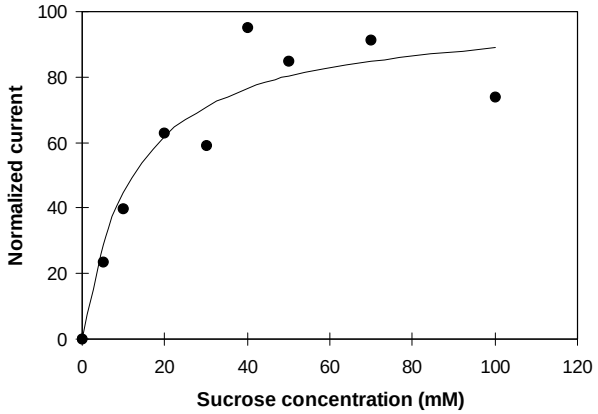

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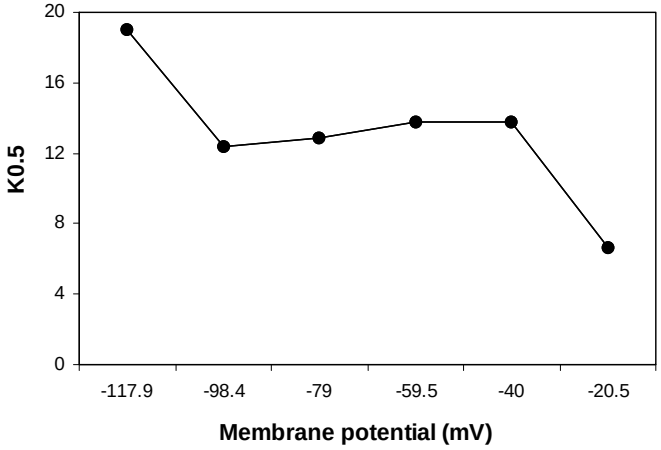

D

Figure 3.29 Kinetic analysis of MeSUT4 mediated sucrose transport in *Xenopus* oocytes. Oocytes injected with MeSUT4 cRNA were clamped at a membrane potential of -40 mV. Sucrose were added and removed at the times indicated by the lines above the trace (A). Currents recorded before, during, and after application of 50 mM sucrose (B). Sucrose-dependent currents recorded at different sucrose concentrations and currents were plotted against sucrose concentrations (C). Various $K_{0.5}$ were plotted against different membrane potentials to analyze the data accuracy (D).

CHAPTER IV
DISCUSSIONS

The photoassimilated carbons produced during CO_2 fixation in photosynthesis are transported throughout plant organs mainly in the form of sucrose. Sucrose is predominantly synthesized in the source tissues, usually mesophyll layer of mature leaves, and distributed through phloem to heterotrophic organs where photosynthesis does not take place (sink organs), such as developing leaves, meristematic tissues, storage organs and reproductive organs for supporting developments and maturations. The multiple kinetic components of sucrose transport in plants were preliminary identified in *V. faba*, suggesting that high and low affinity sucrose transport systems were present in leaves (Delrot *et al.*, 1981). To refine the roles of distinct gene responsible for the regulation of each transport system, genes encoding sucrose transporters (SUT) or sucrose carriers (SUC) were first identified in spinach and potato (Riesmeier *et al.*, 1992; Riesmeier *et al.*, 1993) and their functions were characterized in heterologous yeast system. To date, *SUT* genes have been widely identified in various plants. Based on their sequence similarity, sucrose transporters can be categorized into three subfamilies designated SUT1/SUC2, SUT4 and SUT2/SUC3 (Lalonde *et al.*, 2004). Functional characterization of SUT1 indicates its kinetic properties fit with high affinity class whereas SUT4 corresponds to low affinity class. The functions of SUT2/SUC3 type are still under debate whether it might act as a sucrose sensor that regulates the sucrose transport in plants.

In cassava, the first evidence for sucrose transport has been investigated in leaves and root discs, suggesting the presence of high affinity sucrose transport system in leaves and low affinity component in roots (Eksittikul, 2001). Sucrose uptake in cassava leaf discs showed a high affinity to sucrose with the K_m value of 1.3 mM while that of roots discs showed a lower affinity with the K_m value of 11 mM. In addition, sucrose transport mediated by both systems exhibited the energy dependent transport properties similar to those reported in other plants (Giaquinta, 1976). Another research on cassava sucrose transporters identified cDNA sequences encoding 4 putative SUTs in cassava leaf and storage root cDNA library which were designated as *MeSUT1, MeSUT2, MeSUT4* and *MeSUT5* (Rattanakitti, 2005; Suksanapanomrung, personal communication). They were categorized into three

subfamilies due to their high amino acid sequence homology with SUTs from other plants. Interestingly, two novel isoforms of SUT4 from cassava (MeSUT4 and MeSUT5) were identified and showed to be distinguishable from other plants (Rattanakitti, 2005). In this study, the kinetic properties of each MeSUTs were characterized using heterologous yeast (*Saccharomyces cerevisiae*) expression system. Yeast offers many advantages for characterizing plant transporters such as it is a eukaryotic cell whose characteristics are closely related to those of plant cells and can support the expression of foreign proteins from other eukaryotic organisms. The conditions for yeast to grow and handle are not complicated, similar to those of bacteria, and the time for cell growth is relatively short. Therefore, yeast is the promising expression system for biochemical characterization of SUTs. The mutant strain of yeast namely SUSY7/ura3 has been widely used for expression of plant sucrose transporters (Weise *et al.*, 2000; Knop *et al.*, 2004; Sauer *et al.*, 2004). Generally, the wild type yeast utilizes sucrose through the activity of extracellular invertase and monosaccharide transporter. The extracellular invertase breaks sucrose into its hydrolyzed products, glucose and fructose, and the hydrolyzed monosaccharide is then import into the cell by monosaccharide transporter. Unlike wild type, SUSY7/ura3 yeast mutant is unable to secrete invertase due to the gene is genetically knocked out and so affect the sucrose import into the cell (Figure 4.1). The sucrose sythase gene (*SUSY*) is expressed in cytosol and this strain can utilize sucrose only when the plant sucrose transporter is functionally expressed at the plasma membrane (Lalonde *et al.*, 1999). Some part of *URA3* gene is also deleted to allow selection of the transformed cells on the media lacking uracil (-uracil).

In our experiments, SUSY7/ura3 yeast mutant transformed with pDR195 vector containing *MeSUTs* coding sequences and pStSUT1 (positive control) were able to grow on the selective media (-uracil) containing sucrose as a sole carbon source while the non-transformed cells and empty pDR195 vector transformant were slightlyor unable to grow, indicating that MeSUTs were functional in yeast. The transformed cells were also streaked on selective media (-uracil) containing glucose as a sole carbon source in order to check the uracil lacking phenotype of SUSY7/ura3 mutant. Yeast transformed with empty pDR195 and pDR195 harboring MeSUTs were able to grow on this medium whereas no growth was observed in the non-transformed SUSY/ura3, suggested the ability to compliment SUSY/7ura3 phenotype of pDR195.

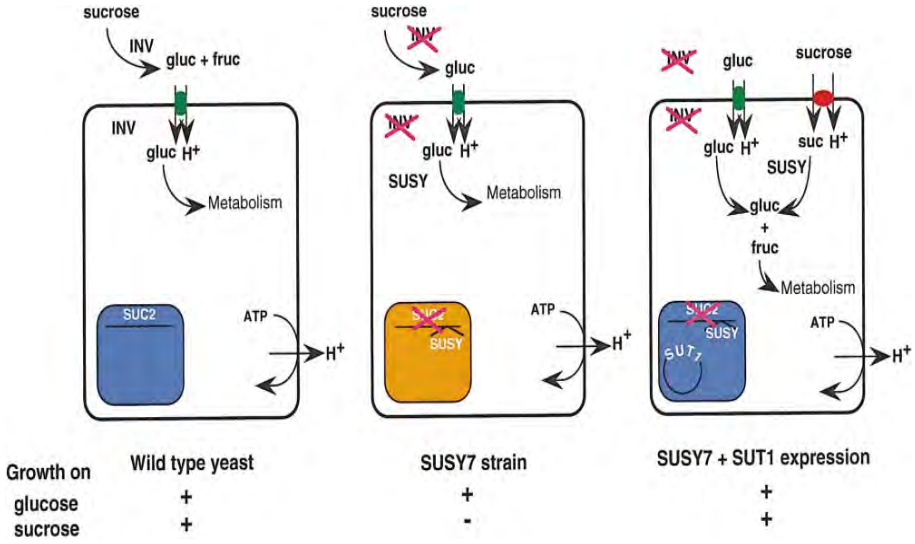

Figure 4.1 Diagram showing sucrose uptake mechanism compared between wild type yeast and SUSY7 yeast mutant. The wild type yeast (*Saccharomyces cerevisiae*) utilizes sucrose through the activity of extracellular invertase and monosaccharide transporter (left). The extracellular invertase breaks sucrose into its hydrolyzed products, glucose and fructose, and the hydrolyzed monosaccharide is then import into the cell by monosaccharide transporter. SUSY7 yeast mutant is unable to secrete invertase due to the gene was genetically knock out and so affect the sucrose import into the cell (middle). The sucrose sythase gene (*SUSY*) is expressed in cytosol and this strain can utilize sucrose only when the plant sucrose transporter is functionally expressed at the plasma membrane (right) (Lalonde *et al.*, 1999).

To investigate the biochemical properties of each cassava sucrose transporters, ^{14}C-sucrose uptake experiments were performed. Each type of MeSUTs exhibited different and distinct characteristics of sucrose transport either in the same plant species or from the other plants. The characteristics for each MeSUTs are individually discussed.

4.1 The similarities between MeSUT1 and sucrose/H^+ symporter

Yeast transformed with *MeSUT1* cDNA was able to accumulate ^{14}C-sucrose and the uptake rate increase constantly with time compared to those of negative controls which only residual uptakes were observed, indicating the expression of the protein at the yeast plasma membrane. Moreover, sucrose transport mediated by MeSUT1 was pH dependent similar to other characterized SUT1 but some distinct characteristics were observed. This transporter differently responded to change in pH compared with other reported SUT1 which showed highest activities at highly acidic pH (Shakya *et al.*, 1998; Knop *et al.*, 2004; Zhou *et al.*, 2007). MeSUT1 did not showed sucrose uptake at pH5 or lower, with increasing activity observed at slightly acidic pH (pH 6). The activity decreased subsequently when the external pH were changed to alkaline and this characteristic resembles the neutral sucrose transporter AtSUC1 (Sauer *et al.*, 2004). Even in the same plant species, sucrose uptake by yeast expressing *AtSUC1* from *Arabidopsis thaliana* showed the highest rate at pH 5-6 whereas the increasing activity of AtSUC2 was observed at pH below 6 (Sauer and Stolz, 1994). In addition, there are several evidences suggesting the differences of pH optimum of sucrose transporters studied in various plants. Regardless of the system used for study, sucrose carrier from sugar beet membrane vesicles displayed the highest activity at pH between 5 and 6 and the activity decreased about 60% at pH 7 (Bush, 1990). This might implicate the importance of pH on the regulation of sucrose transporter. Most of 'acid' sucrose transporters were active at highly acidic environment and their main function was loading of sucrose from apoplast into the specialized cells of phloem (Sauer, 2007). Under the same environmental condition, another type of sucrose transporter such as neutral transporter may play a role in efflux of sucrose out of the cell. The different functions of both carriers might be regulated by the variation of sucrose concentrations and pH differences between

inside and outside of the plasma membrane. However, the study on the membrane reconstitution of the purified protein and protein localization should be carried out to resolve this hypothesis.

^{14}C-sucrose uptake into yeast expressing MeSUT1 was saturable and followed Michaelis Menten kinetics, suggested that the process was carrier mediated and can be distinguished from facilitate diffusion by sucrose facilitators PsSUF1, PsSUF4 or PvSUF1 (Zhou *et al.*, 2007) and sucrose binding proteins (Overvoorde *et al.*, 1996). The low apparent K_m value of MeSUT1 indicated that this transporter can be classified into high affinity sucrose transport system with K_m values less than 2 mM (Lalonde *et al.*, 2004). MeSUT1 depended on proton gradient as its activity was strongly inhibited by proton gradient uncouplers, CCCP and 2, 4-DNP. Sucrose uptake by MeSUT1 was also influenced by SH-group inhibitors, PCMBS and NEM, similar to other characterized SUT1, indicating that the activity involved amino acids containing thiol groups. Vanadate, the plasma membrane ATPase (P-type ATPase) inhibitor, was able to reduce sucrose transport activity, suggesting that the ATP used by MeSUT1 may be associated with P-type ATPase. Antimycin A, the electron transport inhibitor, drastically reduced the activity, indicated energy dependent mechanism. These data implied the biochemical properties of MeSUT1 in agreement with characteristics of sucrose/H^+ symporter.

The potential substrates for MeSUT1 were verified in order to determine the substrate specificity. MeSUT1 displayed unusual responses to sugars compared with other characterized SUT1. Usually, addition of glucose provides the energy for yeast cell expressing SUT and subsequently stimulates sucrose uptake activity (Sauer and Stolz, 1994). On the contrary, ^{14}C-sucrose uptake in MeSUT1 expressing yeast cells was effectively competed by monosaccharides such as glucose, mannose or fructose which caused reduction on transport activity. Furthermore, maltose, an effective sugar competitor for several sucrose transporters (Meyer *et al.*, 2000; Barth *et al.*, 2003; Sauer *et al.*, 2004; Zhang *et al.*, 2008), exhibited small effect on MeSUT1 activity. This unusual response raised the question that the difference may involve the sucrose binding site of MeSUT1 and other sucrose/H^+ symporters. The His65 in loop I/II, which has been proposed to be important for sucrose binding (Lu *et al.*, 1998), was conserved across members of SUTs including MeSUT1 (Rattanakitti, 2005). Of more

significance, this residue was also found in sucrose facilitator PsSUF1 which was inhibited by glucose and fructose (Zhou *et al.*, 2007) similar to the observation in MeSUT1. However, the differences between the sucrose facilitator and MeSUT1 were obvious in the sensitivity towards CCCP and PCMBS. Thus, the dissimilar responses to sugars of various SUT might indicate the influence of other nearby residues for sucrose binding. Cassava is the cyanogen producing plant and accumulation of cyanogenic glucosides was found in leaves and tubers there might be some relationships between cyanogenic glucoside such as linamarin on sucrose transport activity. Eksittikul *et al*. (2001) reported the inhibition of ^{14}C-sucrose uptake in leaf discs by linamarin while stimulatory effect was observed in root discs. Our results suggested that there was no alteration of sucrose uptake into yeast expressing *MeSUT1* when linamarin was present in the reactions. It was possible that the effect of linamarin observed in native cassava leaf and root discs was either on other molecular mechanisms related to sucrose uptake that was not presence in yeast system or on other type of sucrose transporters presence in the tissues. However, the investigation of the effects of linamarin on transcriptional level of MeSUT1 might be beneficial tools to clarify the roles of this compound on the regulation of MeSUT1 gene expression.

4.2 The similarities between sucrose/H^+-antiporters and MeSUT2, MeSUT4 and MeSUT5

Yeast expressing AtSUT2 showed the sucrose transport activity with low affinity for sucrose (Schulze *et al.*, 2000). The N-terminus of SUT2/SUC3 determined the substrate affinity as evidenced from the experiment on yeast expressing chimeric protein of N-terminus of AtSUT2 and C-terminus of StSUT1. The chimeric protein had higher K_m for sucrose than that of StSUT1, suggested the low affinity of AtSUC2 was regulated by N-terminus region (Schulze *et al.*, 2000). In the same study, SUT2 was proposed to be sucrose flux sensor playing a role in measuring the rate of sucrose transport across the plasma membrane. SUT2/SUC3 was found to be expressed in phloem sieve element (SEs) as well as in sink tissues (Barth *et al.*, 2003; Meyer *et al.*, 2004; Hackel *et al.*, 2006), which might act as sucrose efflux carrier involved in phloem unloading into apoplast. SUT4 is another type of low affinity class but the role in sucrose partitioning is different from SUT2. SUT4 has a low affinity for

sucrose with the K_m values varying between 6.0 and 11.7 mM and found to be expressed in minor veins of source leaves and sink tissues where high capacity sucrose transport is required (Weise *et al.*, 2000). In this study, MeSUT2 and two isoforms of cassava SUT4, MeSUT4 and MeSUT5 exhibited similar ^{14}C-sucrose uptake characteristics. ^{14}C-sucrose uptake into yeast cells expressing *MeSUT2*, *MeSUT4* and *MeSUT5* showed distinct optimum pH which differed from other sucrose/H^+ symporters. All reported SUTs have been demonstrated to be a sucrose/H^+-symporters which exhibited highest activity at acidic environment which favored the generation of proton gradient required for driving the sucrose transport in the same direction as protons. Although their sucrose transport activities were dependent on pH, MeSUT2 as well as MeSUT4 exhibited highest sucrose uptake at pH 7 while optimum activity of MetSUT5 was found at broader pH range (pH 7-8). These results implicated that these MeSUTs might function as sucrose/H^+-antiporters, which usually found to be functional at tonoplast membrane (Neuhaus, 2007). Sucrose influx into the vacuole assumed to be driven by a proton antiport mechanism as observed in sucrose transport into tonoplast membrane vesicles of red beet roots (*Beta vulgaris* L., (Getz, 1991)) and Japanese artichoke (*Stachys sieboldii*, (Greutert *et al.*, 1993)). Sucrose uptake into red beet tonoplast vesicles exhibited pH optimum at neutral pH, similar to those of MeSUT2, MeSUT4 and MeSUT5. These might be the result of sucrose transport at the vacuoles (tonoplast) by sucrose/H^+ antiporters whose activity depends on pH gradient (ΔpH) between inside and outside of the vacuoles. The pH within vacuoles is acidic (pH~ 5) while the cytosolic pH was maintained to be neutral in order to preserve most enzyme activities (Martinoia *et al.*, 2006). Thus, the higher proton concentration within the vacuoles generated ΔpH between inside and outside of vacuoles which drive the export of the proton while sucrose is imported into a tonoplast via sucrose/H^+-antiporter (Figure 4.2).

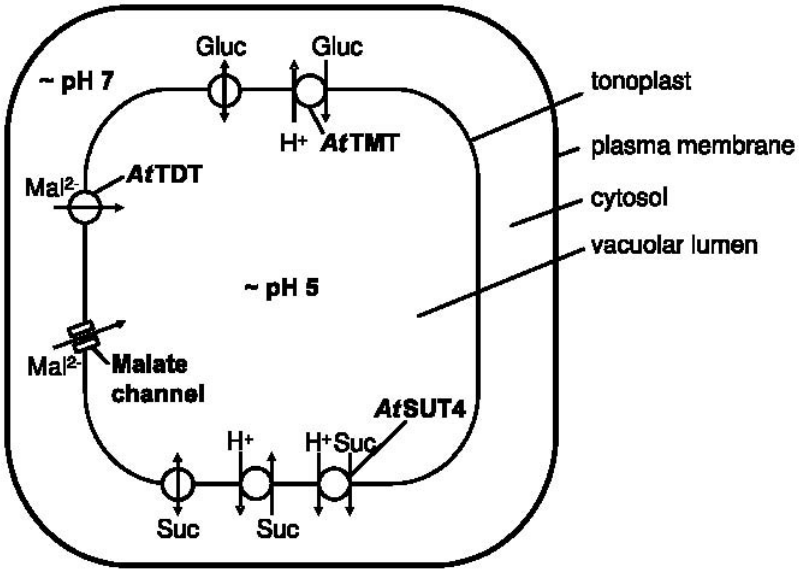

Figure 4.2 Sugars and malate transport via various channels across the tonoplast of *Arabidopsis* vacuole. Sucrose/H^+ antiporter is shown in the figure (from Neuhaus, 2007).

The pH inside of yeast cells was reported to be pH~5 (Conway and Downy, 1949), while the optimum pH of each MeSUTs was around 7, therefore ΔpH can be generated and these MeSUTs may operate the sucrose uptake via antiport mechanisms. Moreover, under the ATP energized condition or the ΔpH (inside acidic) established, sucrose uptake mediated by sucrose/H^+ antiporter in red beet tonoplast membrane vesicles was strongly inhibited by CCCP (Getz, 1991), indicating that sucrose/H^+-antiport mechanism depends on proton gradient. In agreement with this result, sucrose uptake by MeSUT2, MeSUT4 and MeSUT5 were substantially reduced in the presence of CCCP as well as electron transport inhibitor antimycin A suggesting similar involvement of proton gradient and ATP. However, another protonophore, 2, 4-DNP seemed to be less effective on these three MeSUTs, which might result from different uncoupling mechanisms and site specificities. Active transport of sucrose in yeast expressing MeSUTs was assumed to be at the yeast plasma membrane which operated through proton gradient between inside and outside of the cells. The more effective inhibition by CCCP was probably due to its direct disruption of the proton gradient at the plasma membrane whereas 2, 4-DNP

uncoupled the proton gradient at mitochondrial membrane where sucrose transport activity was not reported. Vanadate, a plasma membrane ATPase (P-type ATPase) inhibitor, slightly inhibited MeSUT4 but no effect on MeSUT2 and MeSUT5, similar to the sucrose uptake by red beet tonoplast vesicles. It was possible that MeSUT2, MeSUT4 and MeSUT5, when expressed in yeast, were not associated with P-type ATPase. Alternatively, their sucrose uptakes were associated with vacuolar ATPase (V-type ATPase). One may speculate that these MeSUTs functioned in association with H^+-translocating inorganic pyrophosphatase (V-PPase) since it can be found at plant vacuolar membrane. However, V-PPase exists only in plants but has not been found in heterogous yeast system (Martinoia *et al.*, 2006).

Table 4.1 summarized the K_m values of several sucrose transporters previously reported, including those reported in th SUT2 and SUT4 were proposed as low affinity sucrose transporter (Schulze *et al.*, 2000; Weise *et al.*, 2000). MeSUT2 catalyzed the saturable low affinity sucrose transport resembled other reported SUT2. MeSUT4 and MeSUT5 sucrose uptake also followed the Michaelis-Menten's kinetics, suggesting that sucrose transports mediated by these three MeSUTs were carrier-mediated processes. Typically, K_m values for sucrose uptake activities of SUT4-type transporters are in the range of 6-11.7 mM and thus proposed to be the LAHC class (Sauer, 2007). The K_m values for sucrose of both SUT4 isoforms of cassava were approximately five folds higher than those of previously characterized SUT4, but comparable to the K_m values of the sucrose facilitators (SUF) from pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) which possessed K_m values ranging from 37.8 to 99.8 mM (Zhou *et al.*, 2007). Although the predicted amino acid sequences of PsSUF1, PsSUF4 and PvSUF1 were related to SUTs, these facilitators were unaffected by changes in the external pH, CCCP or antimycin A as observed for MeSUT2, MeSUT4 and MeSUT 5. Although amino acid sequences were correlated to classification of high affinity SUT1, exceptions have been reported for GmSUT1 and PvSUT1 (Aldape *et al.*, 2003; Zhou *et al.*, 2007). These two transporters were phylogenetically classified into high affinity SUT1 type but their K_m values exceeded that reported for the class (5.4 mM for GmSUT1 and 8.5 mM for PvSUT1). Therefore, phylogenetic analyses may only reflect the evolutionary relationship among SUTs.

Table 4.1 Comparison of kinetic parameter of functional characterized SUTs.

SUT type	K_m (mM)	Accession No.	Reference
SUT1/SUC2			
AtSUC1	0.50	X75365	Sauer and Stolz (1994)
AtSUC2	0.77	X75382	Sauer and Stolz (1994)
AtSUC8	0.15	NM127031	Sauer *et al*., (2004)
AtSUC9	0.5	NM120699	Sauer *et al*., (2004)
DcSUT2	0.5	Y16768	Shakya and Sturm (1998)
GmSUT1	5.6	BM733362	Aldape *et al*., (2003)
MeSUT1	1.15	DQ138374	This study
PsSUT1	8.5	AF109922	Zhou *et al*., (2007)
StSUT1	1	X69165	Riesmeier *et al*., (1993)
SUT2/SUC3			
AtSUT2	11.5	AJ289165	Schulze *et al*., (2000)
MeSUT2	12.55	DQ138373	This study
PmSUC3	5.5	AJ534442	Barth *et al*., (2003)
SUT4			
AtSUT4	11.6	AF175321	Weise *et al*., (2000)
DcSUT1	0.5	Y16766	Shakya and Sturm (1998)
LjSUT4	16	AJ538041	Reinders *et al*., (2008)
MeSUT4	42.25	DQ138371	This study
MeSUT5	51.37	DQ138372	This study
StSUT4	6	AF23778	Weise *et al*., (2000)

The responses to sulfhydryl modifying reagents of MeSUT2, MeSUT4 and MeSUT5 were different. Only MeSUT4 was sensitive to NEM and PCMBS at small level, while there were no significant inhibitory effects on MeSUT2 and MeSUT5. The presence of PCMBS or NEM strongly reduced the sucrose transport since these reagents directly modified the active cysteine residues, which was important for control of sucrose transport activity in other SUTs (Meyer *et al.*, 2000; Barth *et al.*, 2003). However, this results was not unusual since similar observations were reported in yeast cell expressing *AtSUC8* of which sucrose uptake was unaffected by PCMBS while another isoform AtSUC9 exhibited sensitivity to this inhibitor (Sauer *et al.*, 2004). It may be hypothesized that cysteine residues in the structures of MeSUT2 and MeSUT5 were arranged in the positions differed from other sulfhydryl sensitive SUTs which had no important role in regulating sucrose uptake by these sucrose transporters.

Effects of several sugars were tested in order to identify the potential substrates those can be transported. Unlike SUT2 from other plants as well as SUT1 and SUT4, MeSUT2 was not highly specific to sucrose as ^{14}C-sucrose uptake was hardly reduced when unlabelled sucrose was added in the reaction. Furthermore, maltose, a typical effective competitor disaccharide for SUTs, showed almost no effect on MeSUT2. In contrast, MeSUT2 seemed to prefer the monosaccharide glucose, fructose and mannose because the presence of these sugars in the assays significantly reduced the ^{14}C-sucrose uptake. This is the first observation that SUT2 type showed high selectivity toward monosaccharide rather than sucrose. MeSUT4 and MeSUT5 showed broad selectivity towards various sugars. Adding of unlabelled disaccharides, sucrose and maltose caused significant reductions on ^{14}C-sucrose uptake as typical characteristics of reported SUTs. However, similar to MeSUT1 and MeSUT2, glucose and fructose which are hydrolysis products of sucrose as well as glucose analog mannose, drastically competed and reduced sucrose uptake activities of both cassava SUT4 isoforms. The presence of glucose in the reaction is likely to provide energy for sucrose/H^+ symporter and subsequently stimulated sucrose transport in yeast cell expressing *SUT* genes. Several sucrose transporters also displayed variation of substrate selectivity other than sucrose such as AtSUC9 and

LjSUT4 which transported wide ranges of glucosides (Sivitz *et al.*, 2007; Reinders *et al.*, 2008)

Similar to the results from MeSUT1, linamarin did not affect the transport activity of MeSUT2 at low concentration (1 mM) but slight inhibition was observed at higher limamarin concentration (5 mM), suggesting that it may not directly interact with MeSUT2 protein. In contrast, there were slight reduction of sucrose uptake of MeSUT4 and MeSUT5 at low linamarin with stronger inhibitions observed at higher concentration. These results were contrast to the effects of linamarin observed on sucrose uptake in leaf and root discs (Eksittikul *et al.*, 2001) that this compound inhibited sucrose uptake in leaf discs but activated the transport activity in root discs. This cyanogenic glucoside was found to accumulate at relatively high concentration (~ 2 mM) in cassava leaves and tubers (McMahon et al. 1995; White et al. 1998). The inhibitory effect of several β-glucosides on some sucrose transporters have been reported (Chandran *et al.*, 2003) but the roles of these glucosides on regulation of sucrose transport have not yet been postulated. In order to compare the results from sucrose uptake into native leaf and root discs to cassava SUT4 isoforms, many additional factors should be taken into account such as the source or sink status of the leaf used. In this study, individual gene was characterized in yeast cells where reponses may not be similar to in leaves or tubers. On the other hand, the effect of linamarin may not be directly on MeSUT proteins. The stronger effect of linamarin on native cassava tissues may be due to its action on other regulatory factors beside sucrose transporters which subsequently affect sucrose transport activity in natural conditions. These regulators are not present in heterologous yeast system and thus, less effect was observed.

4.3 Hypothetical roles of MeSUTs on sucrose partitioning in cassava

The possible roles of each MeSUTs on sucrose partitioning in cassava could be drawn from the correlation between expression of *MeSUTs* (previous study) and data obtained from biochemical characterization in this study. MeSUT1 was found to be highly expressed in both source and sink tissues during the light period (Rattanakitti, 2005) and its biochemical characteristics resemble high affinity sucrose/H^+-symporter. It is possible that MeSUT1 might be responsible for exporting sucrose from source leaves during the time of high rate of photosynthesis, and also

unloading sucrose into storage tubers since the transcripts were detected in tubers at high levels. Furthermore, the selectivity toward monosaccharides of MeSUT1 could be hypothesized that this protein is able to import monosaccharide for starch synthesis in cassava tubers. The function of SUT2/SUT3 type in previous report is still under debate. PmSUC3 was proposed to have a function in retrieval of sucrose along the phloem (Barth *et al.*, 2003), while CsSUT2 was considered to control the sink activity (Li *et al.*, 2003). In this study, the tentative function of MeSUT2 could be raised in comparison with the expression study. The low affinity MeSUT2, which was equally expressed in all tissues but was not dependent on developmental stages, might play a role in sucrose sensing which subsequently modulate the other protein's activity as proposed by Reinders *et al* (2002). *MeSUT4* was found to be highly expressed in mature leaves and storage tubers during the light period, whereas MeSUT5 transcripts were highly accumulated at night. Both MeSUT4 and MeSUT5 exhibited the characteristics similar to sucrose/H^+-antiporters which usually expressed at sink vacuolar membrane (Neuhaus, 2007). It is possible that these two proteins are essential for regulation of sucrose partitioning within cassava tubers. MeSUT4 might be responsible for importing sucrose and/or other sugars into vacuoles during the day, while MeSUT5 might export saccharides stored in the vacuoles to phloem in the night period. However, the localization studies as well as the reconstituted membrane of purified MeSUTs proteins would help in confirming the site specificity and their transport mechanisms.

4.4 Sucrose binding activity of purified native cassava root membrane vesicles

To extend the knowledge of sucrose transport characteristics from sucrose transporter expressed in heterologous system, the native cassava membrane vesicles were employed for characterizing the kinetic properties. The crude membrane from cassava tubers was extracted and purified using aqueous two phase separation. This method allowed the membrane vesicles to be purified up to 90% (Larsson *et al.*, 1994) by partitioning between two aqueous soluble polymers (PEG3360 and Dextran T500) according to the differences between surface properties of all organelles present in the microsomal fraction. The extracted membrane was formed into vesicles visualized under the light microscope (data not shown). ^{14}C-sucrose uptake experiments of purified plasma membrane vesicles were performed under non energized condition as

described by Lemoine *et al*. (1991) thus, the results obtained were designated as sucrose binding activity. Under the variation of pH, sucrose binding activity of cassava root membrane vesicles was pH dependent and showed the highest rate at pH 8 which is an alkaline pH. This result was contrast to the observation on sucrose uptake in cassava leaf discs which possessed the optimum activity at pH 5 (Eksittikul, 2001). However, the findings from sucrose uptake of red beet membrane vesicles suggested the shift of pH from 7 (energized condition) to 8 (non energized condition, (Getz, 1991)). Active sucrose uptake required the acidic conditions to generate the proton gradient for sucrose to be translocated against its concentration, thus, sucrose uptake in cassava leaf discs which was suggested as an active transport or the ATP energized red beet membrane vesicles were preferable at more acidic pH. On the contrary, the non energized condition does not require ATP or pH gradient to be associated with the binding, thus, the binding of sucrose was higher at more alkaline pH. Both leave and root membrane vesicles exhibited Michaelis Menten kinetics which indicated the carriers associated mechanisms. Kinetic data of sucrose binding of cassava leaf and root membrane vesicles were different but resembled those of leaf and root discs (Table 4.2).

Table 4.2 Comparative results of sucrose binding/uptake kinetic parameters obtained from various systems.

System studied	K_m	V_{max}	Reference
Leaf discs	1.13 mM	3.7 nmol/h/cm^2	Eksittikul *et al.*, 2001
Root discs	20 mM	0.018 nmol/h/cm^2	Eksittikul, 2001
Leaf plasma membrane	0.62 mM	3.01 nmol/mg protein/h	Yaiyen, personal communication
Root plasma membrane	26.79 mM	2.34 μmol/mg protein/h	This study

^{14}C-sucrose binding in leaf membrane vesicles showed high affinity characteristics while root membrane vesicles bound sucrose with lower affinity, suggested the multiple kinetic components present in source and sink tissues. The low K_m values may implicate that sucrose transport in leaves were mainly of high affinity type whereas in root the sucrose transport may involve bulk transport or low affinity/ high capacity type which correlated well with their functions.

Although the binding activity of leaf membrane vesicles was similar to the sucrose transport by MeSUT1 and the binding constant of root membrane fell into low affinity class like MeSUT2, MeSUT4 and MeSUT5, several aspects should be concerned. First, the systems used for study were totally different. The native membrane system and the yeast membrane may contain different components which may affect the transport/binding of sucrose differently. Second, sucrose bindings of membrane vesicles were assayed under non energized condition which did not include the involvement of energy system whereas sucrose uptake in yeast expressing *MeSUTs* utilized energy since the assays were performed using intact living cells.

4.5 Expression of MeSUTs in *Xenopus laevis* oocytes

Beside yeasts, the oocytes from frog (*Xenopus laevis*) were widely used as a heterologous system for characterizing plant solute transporters due to many advantages for the transmembrane protein activity to be measured (Miller and Zhou, 2000). The time for protein expression in oocytes is relatively short (3-5 days) comparable with the time for yeast to grow. Characterization of carriers expressed in oocytes is usually detected by physiological methods with the technique called two electrodes voltage clamp. This technique allows determination of the stoichiometry between substrate and cotransported ions. Several substrates can be tested using the same reaction without changing new oocyte. The assay procedures are safe with no necessity to deal with radioactive chemicals like sucrose uptake in yeast. The currents at several membrane potentials can be measured simultaneously when the command program linked to the computer was run. Therefore, it is beneficial to obtain more biochemical characteristics of the cassava sucrose transporters expressed in *Xenopus* oocytes.

The cRNA of all MeSUTs were individually synthesized and injected into the stage V and VI oocytes. The injected oocytes were incubated in Barth’s medium at 15

°C for 3-4 days for allowing expression of the proteins. The electrophysiological measurements were performed with the clamping voltage at -40 mV. Two batches of all oocytes injected with MeSUT2 cRNA died after 3 days of incubation (about 12-13 oocytes/batch) compared to those of uninjected control and oocytes injected with MeSUT1, MeSUT4 and MeSUT5 cRNA which were still alive. It was assumed that MeSUT2 proteins were expressed but they were toxic to cells and subsequently caused cell death. When the results were compared with those of oocytes injected with OsSUT1-gfp-D321N cRNA (a positive control), MeSUT1 and MeSUT5 expressed oocytes could induce inward currents but the currents were very small (~10 nA) even at high concentration of sucrose (40-50 mM).Therefore, electrophysiological experiments was not continued due to the low accuracy. An attempt to see the clear induced currents were performed by setting the voltage and amplification fold to be higher (-70 mV, 5X). At higher amplification, the induced currents by MeSUT1 and MeSUT5 could be clearly seen. However, the currents were still small and there was instability of oocytes membrane, leading to cell death in MeSUT1 cRNA injected oocytes. According to the expression of LjSUT4 in oocytes, this protein was naturally expressed on the *Lotus japonicus* root vacuolar membrane (Reinders *et al.*, 2008). The mistargeting of the proteins leading them to be functioned at oocyte plasma membrane and thus the activity was effectively measurable. It could be that the small induced currents from oocytes expressing MeSUT1 and MeSUT5 caused from trace of proteins were targeted to the oocyte plasma membrane.

MeSUT4 was the only protein whose activity could be clearly detectable at the clamping voltage of -40 mV. MeSUT4 induced current increased at higher sucrose concentration and reached the maximum currents at 40 mM with the apparent $K_{0.5}$ of 12.35 mM ($K_{0.5}$ resembles K_m that they are the concentration of substrate at half of V_{max}, however the parameters used for calculation are different), suggested the low affinity sucrose transport similar to the results observed from the characterization in yeast. However, the K_m observed from yeast expressing MeSUT4 were about 4 folds higher than those obtained from oocytes. The significant variation in K_m between these two expression systems might be caused from the differences in characterization methods. Assaying sucrose uptake in yeast was performed by using the radioactive labeled, an experiment which the yeast membrane potential was not controlled. In electrophysiology measurements, the oocyte membrane potential must be controlled

and variation of membrane potential can cause K_m changes in characteristics of plant transporters which transport substrate in association with ions movement (Boorer *et al.*, 1996).

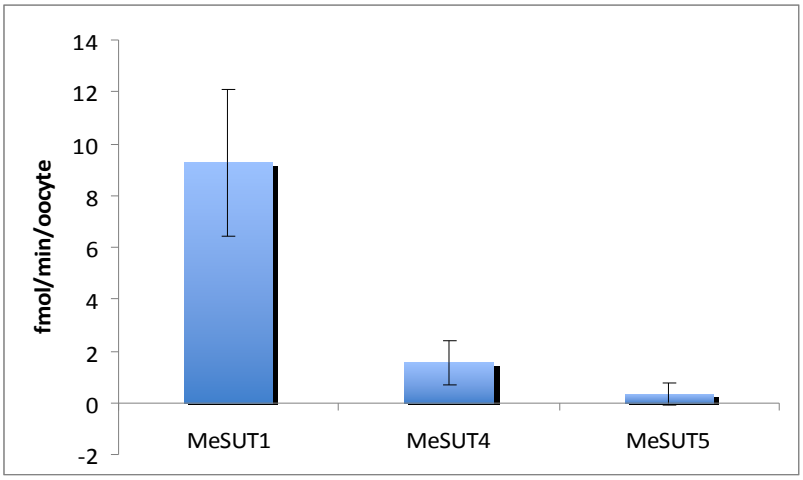

Figure 4.3 Oocytes expressing *MeSUT1*, *MeSUT4* or *MeSUT5* were incubated with ^{14}C-labeled sucrose. Background uptake into uninjected oocytes was subtracted from each transporter after 30 min of incubation.

^{14}C-sucrose uptake into MeSUTs expressing oocytes were kindly performed by Dr. Anke Reinders (Figure 4.3, personnal communication), yielding different results from electrophysiological studies. High ^{14}C-sucrose transport of *MeSUT1* expressed oocytes was observed while the activity was low when using electrophysiological measurements. To prove the reliability of the data obtained, various $K_{0.5}$ were plotted against their membrane potential. As shown in Figure 3.29, fluctuations of the $K_{0.5}$ were observed when membrane potential was increased, characteristics not usually observed in reliable data. It is possible that expression levels of MeSUT4 on the oocyte plasma membrane was low ,thus, not sufficient amount of the proteins to cause current induction compared to other SUTs expressed in the same system.

CHAPTER V
CONCLUSIONS

1. Cassava sucrose transporter genes were successfully transformed into SUSY7/ura3 yeast cells and expressed as MeSUT1, MeSUT2, MeSUT4 and MeSUT5. They were able to complement the yeast SUSY7/ura3 phenotypes, suggested the proteins were functional in yeast.

2. Sucrose uptake by MeSUT1 was pH dependent and possessed the optimum activity at pH 6 with K_m and V_{max} of 1.15±0.11 mM and 0.06 nmol/mg FW/min, respectively, indicating that MeSUT1 belonged to high affinity/low capacity class.

3. MeSUT1 was possibly sucrose/H^+-symporter and its transport of sucrose depended on energy and proton gradients as it was inhibited by PCMBS, NEM, vanadate, CCCP and antimycin A.

4. MeSUT1 showed broad selectivity toward various sugars including sucrose, glucose, fructose, maltose and mannose suggesting that it might has different sucrose binding sites compared with other plant SUT1.

5. MeSUT2 exhibited pH-dependent sucrose transport with highest activity observed at pH ~7. The K_m and V_{max} of MeSUT2 were 12.55±3.58 mM and 0.36±0.10 nmol/mg FW/min, respectively and can be classified as low affinity SUT.It was sensitive to antimycin A and CCCP indicating the transport was dependent on proton gradient and energy.

6. Optimum pHs of MeSUT4 and MeSUT5 were 7 and 7-8 respectively. The K_m and V_{max} of MeSUT4 were 42.25±1.54 mM and 1.58±0.13nmol/mg FW/min and MeSUT5 were 51.37±5.30 mM and 1.10±0.12nmol/mg FW/min, respectively, indicating that they were low affinity/high capacity sucrose transporters.

7. MeSUT4 activity was reduced by PCMBS, NEM, vanadate and DNP but the inhibition levels were relatively low whereas MeSUT5 was not susceptible to these inhibitors. Only CCCP and antimycin A had strong effects on MeSUT4 and MeSUT5, indicating that these two MeSUTs transported sucrose in association with proton gradient and energy.

8. From their responses to various metabolic and proton gradient inhibitors, MeSUT2, MeSUT4 and MeSUT5 were proposed to be sucrose/H^+-antiporters.

9. MeSUT2 seemed to prefer monosaccharide glucose, fructose and mannose rather than sucrose whereas MeSUT4 and MeSUT5 sucrose uptake showed broader sensitive to sucrose, glucose, fructose, maltose and mannose.

10. Sucrose binding activity of root plasma membrane was pH dependent with the highest activity observed at pH 8 with K_m and V_{max} of 19.16 ± 6.44 mM and 1.47 ± 0.54 μmol/mg protein/h, respectively, in agreement with that reported in root discs.

11. All four *MeSUTs* cRNA were synthesized and expressed in *Xenopus laevis* oocytes. Expression of MeSUT2 was not successful whereas MeSUT1 and MeSUT5 expressed in oocyte showed small induced currents were and could not be used for further analysis.

12. MeSUT4 containing oocytes showed clear inward currents and the $K_{0.5}$ for sucrose transport was 12.35 mM, supporting its low affinity characteristics.

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APPENDICES

APPENDIX 1: Recipe for bacterial and yeast cultures.

1. LB agar or broth

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L
Agar	18 g/L

Adjust the pH to 7.0 with 5 N NaOH. Autoclave and store as agar or broth at 4 ºC.

2. LB/amp agar plates

Prepare LB agar as above, autoclave, and cool to 50 ºC. Add ampicillin to 50 µg/ml. Pour plates and store at 4 ºC.

3. YPD medium

Difco peptone	20 g/L
Yeast extracts	10 g/L
Agar (for plates only)	20 g/L

Add water to 950 ml. Adjust pH to 5.8 if necessary, and autoclave. Allow medium to cool to 55 ºC and then add dextrose (glucose) to 2% (50 ml of a sterile 40% stock solution).

4. Minimal media for SUSY7/ura3 culture (SDG (glucose) and SDS (sucrose))

Yeast nitrogen base without amino acids	1.7 g/L
Ammonium sulphate	5 g/L
Agar (for plates only)	20 g/L

Add water to 940 ml. Adjust pH to 5.0 with 1 M HCl. Autoclave and allow medium to cool to 50 ºC and then add

100x Tryptophane	10 ml (20mg/L)
20x Glucose or sucrose	50 ml (20g/L)

APPENDIX2: Preparations of buffer solutions for assaying sucrose binding in plasma membrane.

1. Buffer A:Resuspension buffer (500 ml)

500mM Potassium phosphate buffer (pH 7.8)	5	ml
Sorbitol powder	27.3	g
100 mM Potassium chloride	2.5	ml
500 mM DTT	1	ml
250 mM EDTA	0.2	ml

Adjust volume with dH_2O to 500 ml.

2. Buffer B: Rinsing and assay buffer (100 ml)

1 M sorbitol	30	ml
500 mM Potassium phosphate buffer (pH 7.5 or 7.8)	10	ml
20 mM $CaCl_2$	2.5	ml

Adjust volume with dH_2O to 100 ml. For stopping solution, $HgCl_2$ was added to a final concentration of 5 mM.

3. Homogenized buffer for preparation of microsomal fraction (5 Liters)

MOPH	52.32	g
Sucrose (solid)	564.80	g
500 mM EDTA	50	ml
500 mM DTT	50	ml
ascorbic acid (powder)	4.40	g
100 mM PMSF	25	ml

Adjust pH with NaOH to pH 7.5 and then adjust volume with dH_2O to 5 Liters.

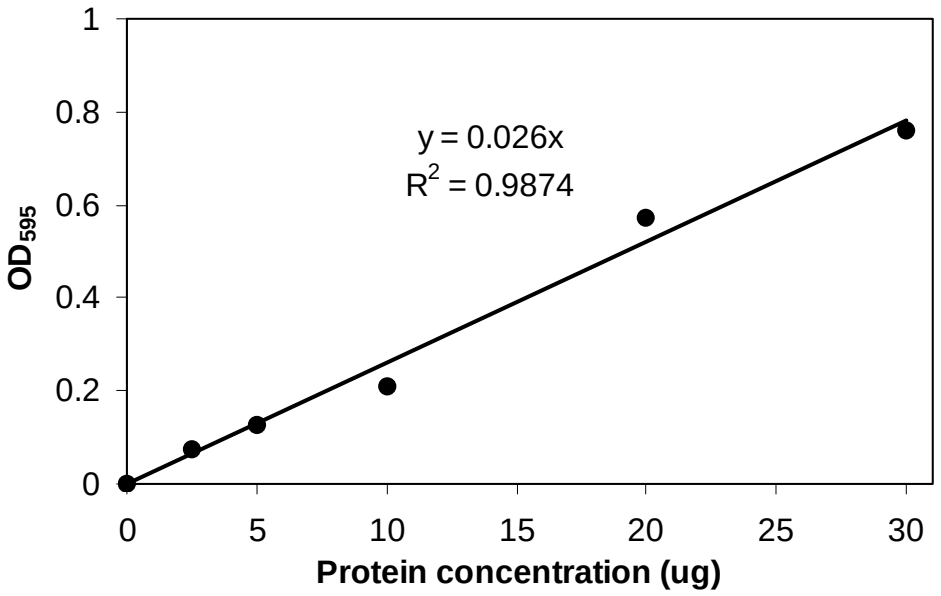
APPENDIX3: Isolation of plasma membrane by aqueous two phases. Phase misture and phase system used in plasma membrane purification.

Componets	Weight of phase mixture	
	3.0 g	**30 g**
20% (w/w) Dextran T500[a]	1.3 g	13 g
40 % (w/w) PEG 3350[a]	0.65 g	6.5 g
Sucrose (solid)	0.339 g	3.4 g
0.2 M KPO_4 Buffer, pH 7.8	0.075 ml	0.75 ml
2 M KCl	0.0075 ml	0.075 ml
0.5 M DTT in 50 mM EDTA[b]	0.008 ml	0.08 ml
Add water to a final weight of	3 g	30 ml
Sample	1 ml	10 ml
Final weight	4 ml	40 ml

[a] Preparation of polymer stock solution

[b] DTT was added fresh from a concentrated stock solution in EDTA, pH7

APPENDIX4: Standard curve of protein.



APPENDIX5: Preparation of ^{14}C-sucrose for activity assay.

Stock 1 μl in 5 ml of scintillation fluid = $5.3x10^5$ cpm.

Need $1x10^5$ cpm/rxn.; ^{14}C-sucrose = $1x10^5$ cpm/$5.3x10^5$ cpm

= 0.18 μl/rxn: 18 μl/100 rxn

Preparation of 10 mM sucrose, 2 ml (specific activity 0.18 μCi/umol)

(Specific activity of stock ^{14}C-sucrose 660mCi/mmol, 200μCi/ml)

	1000 ml contain sucrose		10 mmol
	2 ml	"	10x2/1000 = 20 μmol
0.18 μCi/μmol	1 μmol contain radioactive		0.18 μCi
	20 μmol	"	0.18x20 =3.6 μCi
200 uCi/ml	200 μCi	in	1000 μl
	3.6 μCi	in	1000x3.6/200 = 18μl **
	660 μCi	in	1 μmol
	3.6 μCi	in	3.6/660 = 0.0055 μmol
add cold sucrose	20-0.0055 = 19.99 μmol		
	2000-18 = 1982 μl **		

Concentration of cold sucrose = 19.99μmol/1982 μl = 0.01 M = 10 mM

Conversion of cpm counted from reaction to nmol sucrose.

Ex. 31 dpm were counted

$2.2x10^{12}$ dpm. = 1Ci

31 dpm. = 1Cix31dpm/$2.2x10^{12}$ dpm. = $1.41x10^{-11}$ Ci

= $1.41x10^{-5}$ μCi

stock 0.18 μCi in 1 μmol

$1.41x10^{-5}$ μCi in 1μmolx$1.41x10^{-5}$ μCi/0.18 μCi = $7.83x10^{-5}$ μmol

= $7.83x10^{-2}$ nmol**

BIOGRAPHY

Mr. Yuttana Worawut was born on September 18, 1981. He has been finished his bachelor degree (2^{nd} class honor) from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in Year 2004. He continued studying in the doctoral degree in the Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2004.