THE FUNCTIONAL CHARACTERISATION
OF
NOVEL SUCROSE TRANSPORTERS

By

Katherine Dibley
B.Env.Sc. (Hons)

Thesis submitted for the degree of Doctor of Philosophy
In
Biological Science
School of Environmental and Life Sciences
The University of Newcastle
New South Wales, Australia
June, 2012

Declarations

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

........................................
Katherine Dibley

Acknowledgement of Collaboration

I hereby certify that the data contained in Figure 4.4 and Figure 4.5 were collected by Dr. Yuchan Zhou, with the assistance of Mr. Nathan Moon, and is acknowledged as such within the text. This statement, endorsed by my supervisor, attests to the collection of this data within the course of our laboratory’s research program, and for the use of the data in this thesis with permission.

........................................
Katherine Dibley
Acknowledgements

A research higher degree is a partnership between the candidate and so many others. I have been most fortunate in my journey to have had three supervisors, each with a depth of knowledge that continues to amaze me. However, it is their eagerness to share this knowledge, and sharpen my critical thinking skills, that has made the partnership truly special. And so Prof. John Patrick, Prof. Tina Offler and Prof. Chris Grof, please accept my sincerest thanks for your intellectual stimulation, guidance, encouragement, time, energy and friendship.

I acknowledge and thank Dr. Yuchan Zhou for the cloning of the sucrose transporters that are the subject of this thesis, and for the sharing of her considerable molecular biology skills. Many thanks also to Dr. Stephen Dibley for his patient teachings in molecular biology, and for his help with trouble-shooting when things didn’t work out quite right.

I am grateful to Prof. Wolf Frommer and Prof. Carolyn Slayman for the provision of yeast strain SY1 and for the plasmid pRN90. Dr. Erwin Lamping generously provided the plasmid pSK-sec6-4-URA3. My friend and mentor, the late Dr. Michael Sheahan, provided both *Agrobacterium tumefaciens* strain LBA4404 cells and ongoing support with the transient expression of GFP-protein fusions. Dr. Xin-Ding Wang and Dr. Mark Talbot provided competent histology guidance, for which I am grateful. I also thank Mr. Kevin Stokes for the ongoing supply of healthy plants, and for his good humour.

I also wish to thank Prof. Eileen McLaughlin for confocal microscopy access and training assistance, and Prof. Marcel Maeder for access to the stopped flow fluorimeter facilities. Prof. Steve Tyerman and Dr. Christa Niemetz (University of Adelaide) generously provided training in stopped-flow fluorimetry.

It has been a delight to be part of the Plant Science Group. I have always felt so privileged to work aside such talented and friendly people. There are too many PSG members past and present to list here, but please know that every one of you have enriched my experience at the University. I particularly wish to thank Mark Talbot, Mikey Sheahan, Nigel Fisher, David Lewis and Felicity Andriunas for their friendship and support. Felicity, I am so lucky to have been able to share this journey with you, and your friendship has meant so much.
I have been blessed to have such a wonderfully supportive family, my parents Christine and Gerry, my sisters Elaine and Heather and their families, and the Dibley family. Right from the start, you have always encouraged me to do whatever I have aspired to do. I thank you so much for your patience and humour.

To my dear husband Steve, your support, patience and unwavering belief in me means more than you may ever know. Thank you with all my heart. Here’s to our next adventure together!
# Table of Contents

- Declarations ............................................................................................................. I
- Acknowledgements .................................................................................................. II
- Table of Contents ..................................................................................................... IV
- Abbreviations .......................................................................................................... VIII
- Abstract .................................................................................................................... X

## CHAPTER 1: General Introduction ................................................................. 1

1.1 Photoassimilate Movement Within Plants .................................................. 2
  1.1.1 Introduction ................................................................................................. 2
  1.1.2 Phloem Loading ......................................................................................... 3
  1.1.3 Long Distance Phloem Translocation ....................................................... 4
  1.1.4 Phloem Unloading ..................................................................................... 5
  1.1.5 Significance of Sucrose Efflux .................................................................... 7

1.2 The Developing Legume Seed as a Model System ....................................... 8

1.3 Membrane Transport of Sugars in Plants ................................................... 10
  1.3.1 Energetics .................................................................................................. 11
  1.3.2 Transported Sugar Species ........................................................................ 14
  1.3.3 Molecular Identities of Sugar Transporters ............................................. 14

1.4 Sucrose Transporter Characteristics ............................................................... 17
  1.4.1 SUT Molecular Structure ......................................................................... 17
  1.4.2 Phylogeny .................................................................................................. 19
  1.4.3 Functional Characterisation and Energetics .......................................... 22

1.5 Sucrose Transporter Function in Plants ......................................................... 23
  1.5.1 Approaches to localise SUTs in plant tissues ........................................ 23
  1.5.2 Spatial and temporal expression of SUTs ................................................. 24
  1.5.3 Sub-cellular Localisation .......................................................................... 27
  1.5.4 Regulation of SUT Expression and Activity ........................................... 27

1.6 Sucrose Efflux ................................................................................................. 28
  1.6.1 Passive Diffusion ....................................................................................... 29
  1.6.2 Transport via a Transporter Protein ........................................................... 29
    1.6.2.1 Facilitated transport via a channel or carrier protein .................... 29
    1.6.2.2 Reversal of a sucrose/H⁺ symporter .................................................. 30
    1.6.2.3 Sucrose/H⁺ antiport ......................................................................... 31

1.7 Project Objectives ........................................................................................... 33
CHAPTER 2: Functional Characterisation of Novel Sucrose Transporters Cloned from Legume Seed Coats

2.1 Introduction ...................................................................................................................... 38

2.2 Materials and Methods .................................................................................................... 41
  2.2.1 Yeast Materials ........................................................................................................ 41
    2.2.1.1 Strains ........................................................................................................... 41
    2.2.1.2 Yeast growth and preparation ......................................................................... 41
  2.2.2 Sucrose Uptake Studies .............................................................................................. 41
    2.2.2.1 Basic uptake procedure .................................................................................. 41
    2.2.2.2 Time course uptake ....................................................................................... 42
    2.2.2.3 pH dependence of novel sucrose transporters .............................................. 42
    2.2.2.4 Concentration-dependent sucrose uptake .................................................... 43
    2.2.2.5 Sucrose transport inhibitor studies ................................................................. 43
    2.2.2.6 Counter-transport and sucrose efflux ............................................................ 43
  2.2.3 TLC of Yeast Extracts .............................................................................................. 44

2.3 Results ............................................................................................................................. 45
  2.3.1 Time Course ............................................................................................................. 45
  2.3.2 pH Dependence of Sucrose Transport ................................................................... 47
  2.3.3 Concentration-Dependent Sucrose Transport ...................................................... 49
  2.3.4 Counter-Transport of Sucrose ............................................................................... 51
  2.3.5 Energisation of Transport ...................................................................................... 53
  2.3.6 Transporter Specificity ......................................................................................... 55
  2.3.7 Sucrose Efflux ...................................................................................................... 56

2.4 Discussion ....................................................................................................................... 58
  2.4.1 Sucrose/H⁺ Symporters ......................................................................................... 58
  2.4.2 Novel Sucrose Facilitators .................................................................................... 63
  2.4.3 Phylogenetic and Structural Comparisons .......................................................... 66

CHAPTER 3: Sub-cellular and Cellular Localisation of Novel Sucrose Transporters In Developing Seed Coats and Source Leaves of Pea

3.1 Introduction ...................................................................................................................... 70

3.2 Materials and Methods .................................................................................................... 76
  3.2.1 Plant Growth Conditions ....................................................................................... 76
  3.2.2 Sub-cellular Localisation of Sucrose Transporters by GFP-gene Fusion.. 76
    3.2.2.1 Construct generation ..................................................................................... 76
    3.2.2.2 Agrobacterium transformation ....................................................................... 79
    3.2.2.3 Transient expression in tobacco leaf .............................................................. 79
    3.2.2.4 Confocal microscopy ..................................................................................... 79
3.1.1 Antibody Preparation ................................................................. 80
   3.2.1.1 Antibody design ............................................................... 80
   3.2.1.2 ELISA assay ................................................................. 81
   3.2.1.3 Antibody purification ...................................................... 82
3.1.2 Western Blotting .................................................................. 82
   3.2.2.1 Plant microsomal membrane extraction .............................. 82
   3.2.2.2 Protein quantification ................................................... 83
   3.2.2.3 SDS page and immunoblotting ........................................ 83
3.1.3 Immunolocalisation .............................................................. 84
   3.2.3.1 Plant material sampling and embedding .............................. 84
   3.2.3.2 Immunocytochemistry .................................................... 84
3.2 Results ................................................................................. 86
   3.3.1 Sub-cellular Localisation of SUT/SUF Proteins ....................... 86
   3.3.2 Selection of tissue for sink and source leaf protein sampling: stage of sampling in relation to leaf development .................. 88
   3.3.3 SDS-Page and Immunoblotting of Sucrose Transporters in Pea Leaves... 91
      3.3.3.1 Anatomy of a fully expanded source leaf ......................... 92
   3.3.4 Immunolocalisation of SUTs/SUFs in Leaf Minor Veins ............ 96
   3.3.5 SDS-Page and Immunoblotting of Sucrose Transporters in Developing Seed Coats ......................................................... 100
      3.3.6.1 Seed coat anatomy ...................................................... 102
   3.3.6 Model of Sucrose Movement within Developing Seed Coats ....... 102
      3.3.6.1 Seed coat anatomy ...................................................... 105
   3.3.7 Immunolocalisation of SUTs/SUFs in Developing Seed Coats ...... 108
3.4 Discussion ............................................................................. 113
   3.4.1 Sub-cellular Localisation of SUT/SUFs ................................. 113
   3.4.2Sucrose transporters in sink leaves ......................................... 115
   3.4.3 Sucrose Efflux in Source Leaves ........................................... 116
   3.4.4 Function of Seed Coat SUT/SUFs ........................................ 122
      3.4.4.1 Post phloem pathway and release to the seed apoplastic space.... 124
   3.4.5 Sucrose Delivery across Seed Coat Development .................... 124

CHAPTER 4: Development of A System to Allow the Functional Characterisation of Sucrose Efflux Mediated by Novel Transporters .......... 128

4.1 Introduction .......................................................................... 129
4.2 Materials and Methods .......................................................... 133
   4.2.1 Yeast Strains and Plasmids ................................................ 133
   4.2.2 Yeast Culture ................................................................ 134
   4.2.3 Disruption Fragment Generation ....................................... 134
4.2.4 Yeast Transformation Methods .................................................. 135
4.2.5 Sequencing .................................................................................. 136
4.2.6 Invertase Activity Assay ................................................................. 136
4.2.7 Vesicle Isolation ............................................................................ 137
4.2.8 Proton Pumping Assay ................................................................. 138
4.2.9 Stopped-Flow Fluorimetry ............................................................... 138

4.3 Results & Discussion ....................................................................... 140
4.3.1 Phase I: sec6-4 yeast from Frommer Lab ....................................... 142
  4.3.1.1 SY1-F yeast yields functional vesicles .................................... 142
  4.3.1.2 Acid invertase is present in sec6-4 secretory vesicles ............. 143
4.3.2 Phase II: sec6-4 yeast From the Slayman Lab ............................... 151
  4.3.2.1 The kanamycin gene inserted correctly into an invertase locus ... 152
  4.3.2.2 Transformants have a reduced capacity to metabolise sucrose .... 157
4.3.3 Phase III- Engineering of sec6-4 Mutation into SUSY7 Yeast .......... 160
  4.3.3.1 The sec6-4 mutation was successfully transformed into SUSY7 .. 161
  4.3.3.2 s6s7 yeast yield functional vesicles ....................................... 163
  4.3.3.3 s6s7 vesicles shrink and swell subject to osmotic changes ...... 163
4.3.4 Vector Requirements for Sucrose Transporter Characterisation in s6s7 Yeast .......................................................... 166
  4.3.4.1 Vector requirements for s6s7 .................................................. 166
  4.3.4.2 Heat-shock element sequencing ............................................ 166
  4.3.4.3 Approach for generating a suitable sucrose transporter expression plasmid ................................................................. 167

CHAPTER 5: General Discussion ............................................................ 169

5.1 Summary of Principal Findings ...................................................... 170

5.2 Implications of Findings ................................................................. 172
  5.2.1 Sucrose Efflux Across Seed Development- A Model ................... 172
  5.2.2 Facilitated Sucrose Efflux: Same Functionality, Different Locations? 176
  5.2.3 Sucrose Transporters in Pea and Bean – A Snapshot ................... 178
  5.2.4 Sucrose Efflux Beyond SUFs ...................................................... 179

5.3 Proposed Future Directions ............................................................ 181
  5.3.1 Characterisation of SUF Proteins at the Intracellular Face ............ 181
  5.3.2 Reverse Genetics and SUF-mediated Contributions to Sucrose Transport .......................................................... 182
  5.3.3 Possible Strategies for Cloning a Sucrose- proton Antiporter ......... 182

5.4 Conclusion ...................................................................................... 184

References ......................................................................................... 185
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine-5'-triphosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CC</td>
<td>companion cell</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenol-hydrazone</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamininetetraacetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescene isothiocyanate</td>
</tr>
<tr>
<td>H+</td>
<td>proton</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N'-2-hydroxyethylpiperazine-N' -2-ethanesulphonic acid</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menton constant</td>
</tr>
<tr>
<td>LEU</td>
<td>leucine</td>
</tr>
<tr>
<td>MATE</td>
<td>multidrug and toxicity effluxer</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MES</td>
<td>2-[-N-morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>osmol</td>
<td>osmolarity</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCMBPS</td>
<td>p-chloro-mercuribenzen sulfonic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>- log (proton concentration)</td>
</tr>
<tr>
<td>pmf</td>
<td>proton motive force</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SBP</td>
<td>sucrose binding protein</td>
</tr>
<tr>
<td>SD</td>
<td>synthetic dropout media</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>sieve element</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SET</td>
<td>sugar efflux transporter</td>
</tr>
<tr>
<td>SUC</td>
<td>sucrose transporter</td>
</tr>
<tr>
<td>SUF</td>
<td>sucrose facilitator</td>
</tr>
</tbody>
</table>
SUT  sucrose transporter
SuSy  sucrose synthase
TBS  tris-buffered saline
TBST  tris-buffered saline + Tween 20
TE  tris-EDTA
TMT  tonoplast monosaccharide transporter
Tris  tris[hydroxymethyl]aminomethane
URA  uracil
$V_{\text{max}}$  maximal velocity
WRC  relative water content
WT  wild type

**Units:**

°C  degree Celsius
d  day
Da  Dalton
dpm  disintegrations per minute
g  gram
g  relative centrifuge force
h  hour
L  litre
M  molar
m  meter
min  minute
mol  mole
rpm  revolutions per minute
s  second
V  volts
v/v  volume to volume
w/v  weight to volume

**Prefixes:**

G  giga  $10^9$
M  mega  $10^6$
k  kilo  $10^3$
c  centi  $10^{-2}$
m  milli  $10^{-3}$
µ  micro  $10^{-6}$
n  nano  $10^{-9}$
Abstract

Sucrose is the predominant form in which photosynthetically-fixed carbon is transported over long distances in many plant species. Sucrose moves in the plant from regions of net photosynthetic fixation of carbon or storage (source organs) to sinks, where active growth and/or storage product accumulation occurs. The phloem serves as the long-distance transport conduit. One or more symplasmic discontinuities may occur along the pathway from source to sink, invoking plasma membrane transport steps. For instance, phloem unloading of nutrients includes an apoplastic step in a number of physiologically important sinks such as developing seeds and fleshy fruits. The uptake of sucrose into plant cells has been well described, and is mediated by sucrose/proton symporter proteins (SUTs). In contrast, little is known about the molecular identity of membrane transporters contributing to sucrose efflux in apoplastic phloem loading and unloading.

The aim of this study was to identify and characterise novel sucrose transporters involved in sucrose efflux. Seed coats of pea and bean were selected as source material, as they are functionally committed to sucrose efflux. Cloning by homology to known SUT sequences, five genes were isolated from legume seed coats - three from pea (including the previously described *PsSUT1*) and two from French bean. Complementation of the yeast strain, SUSY7, demonstrated that each gene encodes a functional sucrose transporter.

When functionally expressed in yeast, three of the five transporters studied exhibited pH- and energy-independent sucrose transport that was shown to be bi-directional. These transport properties, together with counter transport, are consistent with a sucrose facilitator (SUF) function. In addition, and unlike H⁺-coupled SUTs, their transport function was insensitive to diethylpyrocarbonate and did not bind maltose. Kinetically the SUFs functioned as low-affinity, high-capacity sucrose transporters. The physiological significance of these novel SUFs in mediating release of sucrose from coats to the seed apoplasm in developing pea and bean seeds is discussed.

Cellular and subcellular localisation studies were also carried out to determine whether SUFs are present at putative sites of sucrose efflux. The cellular and subcellular localisation of *PsSUT1*, *PsSUF1* and *PsSUF4*, cloned from pea, was carried out to determine their potential contribution to phloem loading and unloading of sucrose *in planta*. Transient expression of GFP-tagged protein showed that all three transporters
were plasma membrane localised. Thus, the likely function of the two facilitators is to mediate sucrose movement into or out of cells down a sucrose concentration gradient. In contrast, the sucrose symporter PsSUT1 would be expected to mediate sucrose retrieval from the source leaf or seed apoplasms.

Immunolocalisation studies in seed coat tissues revealed that PsSUF1 and PsSUF4 are present in the inner layers of parenchyma, the putative site of sucrose release in developing seed coats. In contrast, PsSUT1 was restricted to the seed coat vascular bundles, suggesting a role in the retrieval of leaked sucrose along the delivery pathway. PsSUF4 was also present within the vascular bundles, and its function is less apparent, given the polarity of the sucrose gradient and the transporter functions as a facilitator.

In pea, phloem loading of leaf minor veins follows an apoplastic pathway (Wimmers and Turgeon, 1991). As such, two transport events - efflux from the mesophyll symplasm and subsequent uptake by collection phloem - occur in series. Thus, source leaf minor veins were investigated as another region of symplasmic discontinuity and hence a site of sucrose efflux. SUFs were not located in putative efflux cells (bundle sheath or phloem parenchyma cells), but were present, along with PsSUT1, on the plasma membranes of sieve elements. In addition, another sucrose transporter, of unknown identity, was established to be located on plasma membranes of companion cells using a generic SUT1 antibody.

To further understand the mechanisms of sucrose efflux, the need to access and study the cytoplasmic face of the SUFs was recognised. A system was developed to enable this by utilising the sec6-4 mutation in yeast, which results in the production of inside-out plasma membrane vesicles. When the transporter gene of interest is transformed into the yeast mutant and transporter protein is incorporated into inside-out vesicles, the cytoplasmic face of the membrane protein is exposed for study. Rapid, real time evaluation of sucrose transporter activity of the membrane vesicles can be monitored using stopped-flow fluorimetry. The technology achieves this outcome by measuring changes in light scattering as membrane vesicles osmotically shrink or swell in response to sucrose transport into or from the vesicles respectively. To make this system suitable for studies of sucrose transport, the endogenous yeast invertase and maltose transporters, which also transport sucrose, needed to be removed from the yeast genome. Initial attempts to remove the invertase gene were carried out in yeast harbouring the sec6-4 mutation, relying on homologous recombination-mediated gene disruption. However, multiple attempts at disruption indicated that more than one
invertase gene was present in *sec6-4* yeast. A different strategy was adopted which involved incorporating the vesicle accumulation mutation into a suitable (invertase and maltose transporter free) yeast strain. The resultant yeast strain developed, s6s7, successfully accumulated inside-out membrane vesicles, and when combined with the appropriate expression plasmid, offers a new system to functionally characterise sucrose transporters from their cytoplasmic face.

Overall, the work presented in this thesis has increased our knowledge of the mechanisms of sucrose efflux in plants. We have demonstrated that the SUT gene family includes novel sucrose facilitators (SUFs) in addition to the sucrose/proton symporters previously reported. These plasma membrane SUFs are localised (but not restricted) to regions supporting high sucrose fluxes, including seed coats and the minor veins of source leaves. In developing seed coats, the contribution of SUFs (relative to other, as yet unidentified, energised transporters) to sucrose efflux may vary across seed development. The engineering of the s6s7 strain of yeast for sucrose transporter characterisation provides the opportunity to investigate the kinetics of the cytoplasmic face of sucrose transporters, including SUFs, SUTs and non-SUT family transporters. This will allow sucrose efflux to be better understood *in planta.*