THE FUNCTIONAL CHARACTERISATION OF NOVEL SUCROSE TRANSPORTERS

By

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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.

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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.

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Katherine Dibley

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Abbreviations

ATP  adenosine-5'-triphosphate
ATPase  adenosine-5'-triphosphatase
bp  base pairs
BSA  bovine serum albumin
CC  companion cell
CCCP  carbonyl cyanide m-chlorophenol-hydrazone
cDNA  complementary DNA
C-terminal  carboxy-terminal
DEPC  diethyl pyrocarbonate
DMSO  dimethylsulphoxide
DNA  deoxyribonucleic acid
DNP  2,4-dinitrophenol
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
FITC  fluorescene isothiocyanate
H+  proton
HCl  hydrochloric acid
HEPES  N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
kb  kilobases
Km  Michaelis-Menton constant
LEU  leucine
MATE  multidrug and toxicity effluxer
MCS  multiple cloning site
MES  2-[N-morpholino]ethanesulphonic acid
osmol  osmolarity
PAGE  polyacrylamide gel electrophoresis
PCMBs  p-chloro-mercuribenzene sulfonic acid
PCR  polymerase chain reaction
pH  - log (proton concentration)
pmf  proton motive force
RNA  ribonucleic acid
SBP  sucrose binding protein
SD  synthetic dropout media
SDS  sodium dodecyl sulphate
SE  sieve element
SE  standard error of mean
SET  sugar efflux transporter
SUC  sucrose transporter
SUF  sucrose facilitator
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<sub>max</sub> maximal velocity
WRC       relative water content
WT        wild type

**Units:**
°C        degree Celsius
d        day
Da       Dalton
dpm      disintigrations 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 \textit{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 apoplast 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 \textit{PsSUT1}, \textit{PsSUF1} and \textit{PsSUF4}, cloned from pea, was carried out to determine their potential contribution to phloem loading and unloading of sucrose \textit{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 apoplasts.

Immunolocalisation studies in seed coat tissues revealed that PsSUFL and PsSUFL4 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. PsSUFL4 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.
CHAPTER 1

General Introduction
1.1 Photoassimilate Movement Within Plants

1.1.1 Introduction

Over 90% of plant biomass is derived from photosynthetically-fixed carbon. The majority of carbon fixation occurs in leaves, which are able to acquire photosynthates in excess to their needs for growth and metabolism. Actively growing heterotrophic tissues do not have the capacity to fix enough carbon to meet their requirements. To overcome this, the products of photosynthesis are translocated from photosynthetic source tissues to heterotrophic sink tissues, where they may be used for growth, storage and as a source of energy. The formation and maintenance of the plant body relies on this translocation. Sucrose is the primary form of sugar transported between source and sink regions.

In photosynthetic tissues of green plants, atmospheric CO$_2$ is fixed into carbohydrates in chloroplasts of leaf mesophyll cells. The resulting triose phosphates are exported to the cytoplasm, where synthesis of sucrose occurs (Kühn et al., 1999). From here, sucrose can be sequestered in the vacuole and stored as hexoses (after hydrolysis by vacuolar invertase) or as fructans, which are synthesised from sucrose units by vacuolar fructosyltransferases (Vijn and Smeekens, 1999). Alternatively, sucrose may be released from photosynthetic cells for long-distance transport via the phloem.

Sucrose is a disaccharide formed from two glycoside moieties (α-glucose and fructose) linked by a 1,2 α-glycosidic bond. This linkage may be hydrolysed unidirectionally by the enzyme invertase (of which there are several isomers including extracellular, cytoplasmic and vacuolar forms), or by the intracellular enzyme sucrose synthase. Hydrolysis of sucrose by invertase yields a glucose plus a fructose molecule (which are collectively referred to as hexoses), while sucrose synthase reversibly catalyses the conversion of sucrose and UDP to UDP-glucose and fructose (Cardini et al., 1955). Although some plant species may also transport sugars as sucrose-derived oligosaccharides (such as raffinose) or polyols (for example mannitol or sorbitol), sucrose is a significant component of transported sugar in all plant species (Lemoine, 2000). This is because sucrose is an efficient and safe way to transport carbon, as it is non-reducing, thus can be circulated without risking oxidative damage to cellular components. Sucrose is also relatively resistant to metabolism, and has low viscosity.
(Arnold, 1968) even at concentrations of 0.2-1.6 M, which are the reported concentrations encountered in phloem sap (Kühn et al., 1999). Transporting carbon as sucrose rather than glucose may also enable a level of control over translocation and ultimate utilisation, by restricting metabolism to sink regions through the presence or absence of sucrose-cleaving enzymes (Arnold, 1968).

### 1.1.2 Phloem Loading

The initial step of phloem translocation is the loading of solutes from source (mesophyll) cells into sieve element-companion cell (SE-CC) complexes. The SEs and CCs of a single SE-CC complex arise from the same mother cell, and are symplasmically connected with many, often branched, plasmodesmata (van Bel, 1993).

For clarity, the term phloem loading is adopted to describe this entire pathway of translocate movement from leaf mesophyll cells to the SE-CC complex, to distinguish this from the phenomenon of sieve element loading, which refers to movement into the SE-CC complex (van Bel, 1993). In source leaves, the major site of SE loading appears to be minor veins (van Bel, 1993). The leaf minor vein phloem, with its role of net export of photosynthates from source leaves, is referred to as collection phloem (van Bel, 2003).

There are two cellular pathways along which phloem loading may occur. One route is symplasmic (Step 1, Fig. 1.1), where solutes move from mesophyll cells to the SE-CC complex via a series of cell-to-cell plasmodesmal interconnections. Alternatively, phloem loading is described as apoplastic if a symplasmic discontinuity exists along the pathway. The location of the symplasmic discontinuity is probably at the minor vein boundary, at the phloem parenchyma/SE-CC complex interface (van Bel, 1993). The resulting apoplastic route involves two membrane transport steps arranged in series (Patrick, 1997). The first step is the release of solutes into the apoplasm (Step 2, Fig. 1.1), with a subsequent step of uptake into the SE-CC complex (Step 3, Fig.1.1). In plants with an apoplastic transport route, the companion cells or phloem parenchyma cells may be highly modified. These modified cells, termed transfer cells, have cell wall invaginations leading to a greatly increased plasma membrane surface area, which in turn enables high levels of solute flux (van Bel, 1993).

In instances where symplasmic loading of the phloem occur, a mechanism for preventing the backflow of translocates along the loading pathway (from the collection
phloem back towards the mesophyll cells) may be required. One possible mechanism is polymer trapping, where sucrose (and sometimes galactinol) molecules that have diffused along the phloem loading symplasmic pathway from mesophyll cells, are used to synthesise raffinose, stachyose or verbascose in the companion cells (Turgeon, 1996). These oligosaccharides are too large to pass through plasmodesmata en route back to the mesophyll, and accumulate to high concentrations in the intermediary (companion) cells. Plasmodesmata of sufficient diameter then allow passage of oligosaccharides from companion cells to sieve elements completing the loading pathway (Turgeon, 1996). Thus, the symplasmic pathway relies on both plasmodesmatal frequency and the diameter or size exclusion limit of the plasmodesmata (Oparka and Santa Cruz, 2000). Not surprisingly, plants with a symplasmic path of phloem loading transport as much as 80% of their sugars as oligosaccharides. It has been proposed that plant species translocating sucrose rely on apoplastic loading exclusively, despite the presence of plasmodesmata, while those translocating oligosaccharides are also able to utilise symplasmic loading (Turgeon and Medville, 2004).

Phloem sap loaded into the collection phloem moves through the sieve tubes of the plant’s transport phloem to the delivery phloem (van Bel, 2003), which is proximal to sink tissues.

1.1.3 Long Distance Phloem Translocation

Translocation of photoassimilates (mainly as sucrose), amino N compounds, mineral ions, signalling molecules and water occurs over long distances in the plant through what is termed the transport phloem. Solute movement through the phloem to sink tissues occurs by bulk flow, with sink regions having low transpiration rates (Patrick et al., 2001). The Münch pressure-flow hypothesis offers a widely-accepted model of long distance phloem movement (Thorpe and Minchin, 1996), where sap movement is driven by pressure differences between the loading and unloading regions. At sites of phloem loading, the osmolarity of sap is greater, driving movement of water into the phloem from adjoining xylem and so increasing the hydrostatic pressure within the sieve tubes in these regions. At sites of phloem unloading, the decrease in solutes (such as when they are unloaded via membrane transporters) results in the net movement of water from the phloem back to the xylem (Patrick et al., 2001; water movement is shown by blue arrows, Fig. 1.1). Thus, in these regions, the relative hydrostatic pressure is reduced.
1.1.4 Phloem Unloading

The phenomenon of phloem unloading, where solutes leave sieve tubes by apoplastic or symplasmic routes, can occur throughout the entire phloem network (Patrick, 1997). Using a similar conceptual approach to that applied by van Bel (1993) for defining the phloem loading pathway, the phloem unloading pathway can be considered as the route taken by solutes moving out of sieve elements to sites of utilisation. Thus, in a symplasmic unloading pathway, no apoplastic discontinuities exist between sieve elements and sink cells (Step 4, Fig. 1.1) whereas for an apoplastic unloading pathway such a discontinuity (and so membrane transport) is present (Steps 5 and 6, Fig. 1.1).

In actively developing tissues such as fruits and root tips, it appears that symplasmic unloading of phloem usually occurs (Oparka and Santa Cruz, 2000). However, in some sinks, particularly in the case of developing seeds, apoplastic unloading occurs (Patrick, 1997). The developing seed is a peculiar sink for two reasons: firstly, at the seed coat-embryo interface, cellular connections are absent between the two genetically-distinct generations, necessitating apoplastic transport of solutes. A variety of other biotrophic relationships are known where apoplastic transport of solutes occurs, such as between root nematode-induced syncytia and adjacent tissues (Bockenhoff et al., 1996). Secondly, and unlike these other sites of solute release, developing seeds of many species (including grain legumes) provide a system where the abutting tissues can be readily separated, allowing experimental access to efflux tissues, without confounding effects of subsequent uptake mechanisms. The characteristics and considerations in the use of developing legume seeds as an experimental model for the study of apoplastic transport events is discussed in Section 1.2.
Figure 1.1. The movement of sucrose from source to sink regions in a model plant.

1- Symplasmic loading of phloem via plasmodesmata; 2- efflux to the apoplastic of source tissue; 3- apoplastic loading of phloem; 4- symplasmic unloading of phloem via plasmodesmata; 5- apoplastic unloading of phloem; 6- membrane transport of sucrose into sink tissues.

Black arrows indicate the pathway of sucrose movement. Blue arrows indicate water movement within plant tissues, which drives bulk sap flow by the generation of hydrostatic pressure gradients. Black circles indicate sites of membrane transport events via efflux (Steps 2 and 5) and uptake (Steps 3 and 6).
1.1.5 Significance of Sucrose Efflux

Although it is generally agreed that sucrose efflux from cells is transporter-mediated, no candidate transporters had been unequivocally demonstrated at the outset of this study. Since this time however, the SWEET family of sucrose facilitators have been isolated and characterised by Chen et al. (2010; 2012; examined further in section 1.3.1).

The study reported in this thesis aims to identify transporters mediating sucrose efflux, and to elucidate their mechanism of transport action and energisation.

An understanding of sucrose efflux, and the transporters mediating this, is central to our overall understanding of plant productivity and carbon allocation, as sucrose (and other sugar) efflux properties may exert a considerable influence over the sucrose flux from source to sink and hence these whole plant properties. Translocation of phloem sap relies on the osmotic gradient of solutes (such as sucrose) along the translocation pathway(s). As a consequence, sinks with high rates of sucrose efflux from the delivery phloem will be favoured over weaker sinks for phloem delivery of solutes, thus influencing carbon allocation between plant organs (Patrick, 1997).

Efflux of sucrose from plant cells is significant for whole plant productivity in that rates of efflux have an effect on photosynthesis. The accumulation of sucrose (as well as its cleavage products glucose and fructose) in mesophyll cells is known to downregulate photosynthesis (possibly through the suppression of photosynthesis genes). This suppression, a phenomenon referred to as sink-regulated inhibition of photosynthesis (Jang and Sheen, 1997), is a form of sugar signalling, which impacts upon carbon allocation within the plant (for a review of sugar signalling in plants, see Rolland et al., 2006). Thus, sucrose efflux in sink regions, as well as from phloem parenchyma-mesophyll cell continuities (van Bel, 1993) in apoplastic loaders, has the potential to influence plant productivity (and see Wardlaw, 1990).

A greater understanding of sucrose efflux mechanisms would provide the opportunity to exert some influence over biomass allocation. Sinks can be of great agronomic importance. For instance, approximately 70% of human food is sourced directly from seeds, with cereals and grain legumes accounting for the bulk of these (Bewley and Black, 1994). The step of sucrose efflux from the maternal (seed coat) tissues into the apoplasm is paramount for subsequent uptake and biomass gain by the
developing embryo and so influences seed yield. Additionally, other terminal sinks where sugars are accumulated to high concentrations (such as in tomato fruit- Ruan and Patrick, 1995) typically involve an apoplastic route of phloem unloading, hence also relying on this sucrose efflux step (Patrick, 1997). At these sites, efflux occurs via membrane transporters, as the demand for sucrose in these sink regions far exceeds that available via passive, unfacilitated diffusion across the plasma membrane. On a practical front, a greater understanding of the transporters governing efflux of sucrose from maternal (and other) tissues can provide a molecular target for plant breeding and/or transgenic approaches to improvements in crop yield.

1.2 The Developing Legume Seed as a Model System

In the developing legume seed, nutrients are delivered to the filial tissues of the seed by the surrounding maternal tissues comprising the seed coat. Here, due to symplasmic continuity, the phloem-unloading pathway can be considered to include ground tissues of the seed coat, at which efflux into the apoplasms occurs (Patrick, 1997). As the maternal and filial tissues are symplasmically isolated, two membrane transport events occur in series (Patrick et al., 2003). This system provides an excellent opportunity to study these two transport events in isolation. The seed coat has only minimal requirements for sucrose storage and metabolism, and possesses cells specialised for the release of nutrients to the developing embryo. In contrast, after an initial phase of cell expansion and differentiation, the filial tissues are committed to nutrient storage (Patrick and Offler, 2001). Legume seeds are mostly non-endospermic; with the cotyledons functioning as storage organs (Bewley and Black, 1994). The uptake of sucrose by developing cotyledons has been extensively studied (the mechanisms involved are discussed in Section 1.1.3). In contrast, little is known about the molecular mechanism of sucrose efflux from seed coat cells (Step 5, Fig. 1.1). Further, study of efflux transport at this interface may also provide information on the little studied and less accessible efflux step (Step 2, Fig. 1.1) in the apoplastic phloem loading pathway, assuming a similar mechanism is in place.

Assimilates are delivered to the developing legume seed coat via phloem contained in the funicle (Hardham, 1976). In the seed coats of pea, broad bean, and *Medicago*, the
phloem then divides to form a single chalazal vein, which has two lateral branches (Hardham, 1976; Offler et al., 1989; Wang and Grusak, 2005), while for soybean and French bean, the phloem is repeatedly branched to form an extensive network of veins throughout the seed coat (Thorne, 1981; Offler and Patrick, 1984). The seed coat vasculature is embedded within ground tissues, consisting of parenchyma cells. The innermost layers of parenchyma proximal to the cotyledons are the putative site of efflux (Patrick et al., 1995). This is supported by the trans-differentiation of these cells in some species to form transfer cells (Offler et al., 1989; Tegeder et al., 1999). Transfer cells are characterised by cell wall invaginations (Gunning and Pate, 1974) that result in a greatly increased plasma membrane area to support high levels of solute transport (Thompson et al., 2001).

The extent of symplasmic connection in developing seed coats of both broad and French bean has been studied using the movement of phloem-imported carboxyfluorescein (CF- a membrane-impermeant fluorochrome) (Patrick et al., 1995). In broad bean, it appears that the symplasmic continuity with the chalazal vein phloem extends throughout the ground tissues to the innermost layers of the seed coat cells. Studies using the fluorescent probe 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) in developing pea seed coats reveals a similar symplasmic extent (van Dongen et al., 2003). In contrast, the symplasm is less extensive in French bean, with CF movement occurring only to the ground parenchyma cells (Patrick et al., 1995). It is worth noting that studies substituting tracer dyes for assimilates (such as sucrose) in elucidating symplasmic movement rely on several assumptions, being that the dye has a similar geometry to the assimilate in question, and that plamodesmatal conductances to molecules of differing geometry are equivalent. Indeed, it appears that CF is able to move via plasmodesmata, at least within the plasmodesmata connecting ground tissue layers in broad bean and pea (Patrick et al., 1995; van Dongen et al., 2003). However, the inability of dyes to move/permeate through tissues does not exclude the transport of assimilates through plasmodesmata, where these are present. Likewise, the movement of CF to other ground tissue cell layers distant from the inner layers (such as those underlying the outer seed coat), does not necessarily implicate these cells in the cellular pathway of phloem unloading.

Further attempts have been made at identifying the cells responsible for efflux in seed coats, using the localisation of ATPases. This approach is based on the assumption that efflux is an energy-dependent process (this is discussed further in Section 1.6).
Cerium chloride deposition in broad bean identified the wall ingrowth regions of thin-walled parenchyma transfer cells as areas of high ATPase activity, hence possible sites of energy-dependent solute efflux (Wang et al., 1995). For French bean seed coats, cerium chloride was deposited within the ground parenchyma region (Wang et al., 1995). Additionally, other approaches taken by (Wang et al., 1995) in identifying putative transporter location (based on staining of protein thiols) and identifying ATPase activity (using a pH-dependent fluorochrome), as well as observations of the effect of removing inner cell layers on sucrose efflux (Patrick et al., 1995) corroborated these findings. Their conclusions of possible efflux sites concur with studies of post-phloem symplasmic extent by Patrick et al. (1995) and van Dongen et al. (2003). In the case of both French and broad bean, the cells identified as being both within the symplasm and proximal to the developing embryo were identified as being sites of efflux. However, the suggestion that unloading occurs in different layers of pea parenchyma by van Dongen et al. (2003) expands the cellular role of unloading from the thin-walled parenchyma transfer cells to other cell layers that are more distant from the maternal/filial interface.

The unequivocal identification of cells responsible for assimilate efflux would be possible by immunolocalisation of transporter protein(s) responsible for release from the post-phloem symplasm and into the seed coat-seed apoplasm.

1.3 Membrane Transport of Sugars in Plants

Diffusion rates of sugars across the lipid bilayer of membranes is too slow to meet biological demand. Membranes have a low permeability to large, uncharged molecules such as sucrose. The rate of sugar movement across the plasma membrane by simple diffusion is governed by the membrane permeability to the subject sugar species, and the driving force across the membrane, which is the trans-membrane concentration gradient (Zhang et al., 2007). This relationship obeys Fick’s Law, where:

\[
\text{Flux of sugar across membrane} = \text{permeability coefficient} \times \text{transmembrane conc. gradient}
\]

The permeability co-efficient of membranes for sucrose is estimated at \(10^{-10} \text{ m s}^{-1}\) (Cram, 1984), giving a predicted sucrose flux of \(4 \times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}\) with a typical
transmembrane sucrose difference of 40 mM (as seen between seed coat symplasm and apoplasm in broad bean-Patrick, 1994). The actual flux of sucrose observed across this membrane is $7 \times 10^{-8}$ mol m$^{-2}$ s$^{-1}$ (Offler and Patrick, 1993) and so simple diffusion only accounts for 5% of the observed sucrose movement. So, to support the observed flux, the movement of sucrose out of these cells must be facilitated by transport proteins. These transporters may act passively or be energy-coupled (see below). The mechanism of both types and their contribution to sugar transport in plants is explored.

1.3.1 Energetics

Passive transporters can either function as channels or carriers. Channels are membrane proteins that form continuous, aqueous pathways between two compartments, along which solutes can move (Bush, 1993b). Channels may be used to traffic ions (e.g., K$^+$ channels) or uncharged polar molecules (such as H$_2$O movement across membranes via aquaporins). In contrast, carriers bind the transported solute and undergo a change in 3D structure during transport. With a carrier, the number of binding sites limits the number of molecules transported in a single event, and so carriers conform to enzyme kinetics. There can be some confusion with the term carrier, as it has been applied to passive, as well as secondary active transporters (Bush, 1993b), which are described later in this section.

Evidence does exist for passive sucrose transport in plants. In sugar beet leaf discs, two transport mechanisms for sucrose uptake - one saturable and the other non-saturable- have been identified (Maynard and Lucas, 1982a). The saturable system was shown to be energy-dependent, while the non-saturable component may be at least partly attributable to passive transport. A similar biphasic arrangement has been observed in other plant tissue systems, such as in soybean cotyledon protoplasts, with the non-saturable component again being an energy-independent mechanism (Lin et al., 1984 and see also Delrot et al., 2001).

On a molecular level, several candidates for mediating energy-independent sucrose transport have now been identified. The first of these is the sucrose binding protein (SBP- Hitz et al., 1986). Although SBP is associated with the extracellular side of the plasma membrane (Overvoorde and Grimes, 1994) it is structurally more similar to storage proteins than membrane transporters (Contim et al., 2003). Despite this, it was reported that GmSBP, a 62kDa protein isolated from soybean cotyledons (Grimes et al., 1992) mediated sucrose uptake when expressed in SUSY7 yeast (Overvoorde et al., 11
However, subsequent investigation of a broad bean SBP did not find any evidence of sucrose transport by yeast cells expressing this protein (Heim et al., 2001). Nevertheless, SBP appears to be membrane-localised, with VrSBP1 from mung bean being localised to the tonoplast (Wang et al., 2009). Additionally, studies of the in planta location of VfSBP in developing broad bean seeds does show the presence of SBP in sucrose release cells of seed coats (Harrington et al., 1997b). Similarly, SBP was also localised to cells involved in sucrose uptake, including sink leaf mesophyll and developing cotyledon transfer cells (Grimes et al., 1992). Furthermore, SBP antibodies inhibited sucrose uptake by transfer cells of *V. faba* (Fieuw et al., 1992), while SBP-antisense tobacco plants had impaired sucrose translocation (Pedra et al., 2000; Delú-Filho et al., 2000). An alternative role to directly mediating transport proposed for SBP is a regulatory one in carbon partitioning, possibly by affecting activity of other enzymes (including sucrose synthase, ADP-glucose pyrophosphorylase and invertases) involved in carbohydrate metabolism, rather than directly mediating sucrose transport (Pedra et al., 2000; Delú-Filho et al., 2000; Waclawovsky et al., 2006).

A second, more recently described group of sucrose transporters are the SWEET proteins (Chen et al., 2010). This family represents a new class of sugar transporter conserved across many organisms, including plants, humans and *Caenorhabditis elegans*. In *Arabidopsis*, two SWEETs, *AtSWEET11* and *AtSWEET12* were highly expressed in source leaves (Chen et al., 2012) and were implicated in phloem loading. Both of these transporters are energy-independent sucrose uniporters, with transport unaffected by pH gradients (Chen et al., 2012).

Active transporters are carriers that rely on energy to transport molecules against a chemical (non-electrolytes) or electrochemical (electrolytes) gradient. Primary active transport proteins utilise energy directly (usually generated by ATP hydrolysis) to transport a molecule across a membrane against an electrochemical gradient. Two well-known groups of primary active transporters are the ATPases and the ABC (ATP-binding cassette) transporters. ATPases are numerous in plants, and are known to function as proton or calcium pumps. ABC transporters, which in prokaryotes carry out peptide, sugar and lipid transport, have only recently been identified in plants, with more than 50 ABC transporter genes identified in *Arabidopsis* (Martinoia et al., 2002). The functions of these are largely unknown, but some may be involved in stomatal movement (Klein et al., 2003) and detoxification of herbicides (Kreuz et al., 1996).
Secondary active transport proteins utilise the electrochemical gradient of electrolytes (for example H$^+$ or Na$^+$), harnessing co-transport to move another charged or uncharged molecule against a concentration gradient. The electrochemical gradient harnessed is created by primary active transport, (for example, a proton gradient generated by an H$^+$-ATPase) and so the secondary active transport is energy-dependent. Co-transport can occur as either symport or antiport. In symport, both the charged or uncharged substrate and the co-transported ion move across the membrane in the same direction. In antiport, an uncharged molecule is moved in exchange for an ion, thus the electrochemical gradient of the ion can be exploited even if it is in the reverse direction.

Across a membrane, the electrochemical gradient is composed of both the electrical potential difference and the chemical potential difference, and is described by the Nernst equation:

Electrochemical potential = chemical potential + electrical potential

\[ \Psi = RT\ln C + zFE \]

Where:  
- \( R = \) Universal gas constant (8.314 J mol$^{-1}$ K$^{-1}$)  
- \( z = \) Ion valency  
- \( F = \) Faraday constant (9.649 x 10$^4$J mol$^{-1}$ V$^{-1}$)  
- \( T = \) Absolute temperature (°K)  
- \( \Psi = \) Membrane potential difference (V)  
- \( C = \) Concentration (mol m$^{-3}$)

The Nernst equation can be used to predict the net outcome of the electrical and chemical gradients present in a system on the movement of a molecule (such as an uncharged sugar molecule) across a membrane by co-transport with an electrolyte. At electrochemical equilibrium across the membrane when there is no net movement of the substrate:

\[ RT\ln C_o + zF E_o = RT\ln C_i + zF E_i \]

However, in the case of symport, the chemical potential of the uncharged molecule plus the chemical and electrical potential of the electrolyte influence the transport of the uncharged molecule:

\[ \text{Electrolyte } + \text{ uncharged molecule} = \text{Electrolyte } + \text{ uncharged molecule} \]
\[
\text{RTlnC}_{o1} + z\text{FE}_{o1} + \text{RTlnC}_{o2} = \text{RTlnC}_{i1} + z\text{FE}_{i1} + \text{RTlnC}_{i2}
\]

\[0 = \text{outside cell}; \quad i = \text{inside cell} \quad 1; = \text{electrolyte}; \quad 2 = \text{non-electrolyte}\]

Now, in the case of \(\text{H}^+\)-coupled symport, pH can be substituted into the equation as the electrolyte concentration gradient, where \(\text{pH} = -\log[\text{H}^+]\). For a univalent cation and at \(25^\circ\text{C}\), \(\frac{\text{RT}}{F} = 25.6\ \text{mV}\), while \(\ln = 2.30\ \log_{10}\) and \(z = 1\) for \(\text{H}^+\), and so this equation can be simplified:

\[
\Delta\Psi = 59(\log_{10} C_{o2} - \log_{10} C_{i2} - \Delta\text{pH})
\]

(Equation 1.1)

Where: \(\Delta\text{pH} = \text{pH difference across the membrane}\)

### 1.3.2 Transported Sugar Species

Although sucrose is the major transported sugar in most plants, other sugars can also be of significance in particular plant species or tissues. Hexose transport mechanisms play an important role in systems where extracellular invertases are present. In over 100 plant species polyols are also transported across cell membranes (Stoop et al., 1996). Polyols have been shown to increase under abiotic stress, such as water or cold stress, possibly for osmoprotection (Noiraud et al., 2000), and the amount of carbohydrate transported in the form of polyols can be significant (Stoop et al., 1996). In addition, many woody plant species (with high plasmodesmal frequencies at sites of symplasmic phloem loading) transport raffinose series sugars (Gamalei, 1985). It is perhaps not surprising that plant membrane transporters have been identified on a molecular level for several of these sugar species.

### 1.3.3 Molecular Identities of Sugar Transporters

The first sugar transporter identified in plants on a molecular level was an \(\text{H}^+/\text{hexose}\) symporter. Sauer and Tanner (1989) isolated and characterised the \(\text{H}^+/\text{hexose}\) symporter HUP1 from the unicellular alga *Chorella kessleri* by differentially screening cDNA libraries after inducing cells to express the glucose transporter, which is achieved by incubating cells in high exogenous levels of glucose. Following this, a higher plant \(\text{H}^+/\text{hexose}\) symporter was cloned (Sauer et al., 1990), by screening an *Arabidopsis* cDNA library with a HUP1 probe. \(\text{H}^+/\text{hexose}\) symporters, which are part of the monosaccharide transporter (MST) superfamily, are now the most well characterised group of plant sugar transporters, including on a structural level. They form an
extensive gene family in higher plants: in *Arabidopsis* 66 putative MSTs have been identified (for a review, see Lalonde *et al.*, 2004).

Sucrose transporters (SUTs), also designated as SUCs or Scr (sucrose carriers), are transport proteins that have been shown to mediate plasma membrane sucrose transport in plants. For clarity, sucrose transporters will be referred to collectively as SUTs. Cloning the first SUT was achieved by the development of the SUSY7 yeast strain, which lacks both invertase and the yeast maltose transporter (which is also capable of transporting sucrose), and is transformed to express an intracellular plant sucrose synthase. These characteristics render SUSY7 unable to grow on sucrose, as it can neither transport it nor cleave it into hexoses for uptake. However, when complemented with cDNA encoding a sucrose transporter, the transformed SUSY7 can then take up sucrose and utilise it via hydrolysis by sucrose synthase, thus can grow on sucrose as a sole carbon source. So, transforming a spinach cDNA library into this strain allowed the identification of the first functional sucrose transporter SoSUT1 (Riesmeier *et al.*, 1992). The SUT-expressing SUSY7 yeast was then used to functionally characterise the cloned transporter, which was identified as a sucrose/H\(^+\) symporter (Riesmeier *et al.*, 1992). Functional characterisation of SUTs will be further examined in Section 1.4.3.

In addition to SUTs, other sugar transporters have been identified in plants. Several polyols have been shown to have their movement across membranes mediated by transport proteins. A mannitol transporter from celery has been isolated and characterised as a H\(^+\)/mannitol symporter (AgMaT1; Noiraud *et al.*, 2001). This was achieved by producing a yeast strain constitutively expressing mannitol dehydrogenase, which enables the yeast strain to grow on mannitol as a sole carbon source. Likewise, two sorbitol transporters (SOTs) were isolated from sour cherry (Gao *et al.*, 2003) being H\(^+\)/sorbitol symporters also. To isolate these genes, homology cloning was carried out from cDNA of appropriate tissues (celery petiole for AgMaT1; several stages of fruit for SOTs) using a degenerate primer set based on sequence homology of a range of previously identified sugar transporters (including those for glucose, arabinose, galactose and myo-inositol; Noiraud *et al.*, 2001). Complementary DNA libraries were screened with the PCR fragment to obtain a full-length sequence, which was then used to transform the appropriate yeast strain. Subsequently, two *Plantago* SOTs (PmPLT1 and PmPLT2) were identified (by screening a cDNA library with AgMAT1-derived radiolabelled probe) and also characterised as symporters (Ramsperger-Gleixner *et al.*, 2004). In addition to these sorbitol and mannitol transporters, a H\(^+\)/polyol transporter
that has the ability to transport a wide range of sugars was isolated from Arabidopsis, after in silico prediction of gene function based on homology to PmPLT genes. This gene was demonstrated to transport both linear and cyclic polyols (including sorbitol, mannitol, glycerol and myo-inositol), hexoses and pentoses, but not sucrose (Klepek et al., 2005; Reinders et al., 2005). This is an interesting scenario, given that Arabidopsis primarily translocates sugars in the form of sucrose, with no polyols present in the phloem (Klepek et al., 2005). It was proposed that this transporter plays a role in retrieving sugars produced by the degradation of carbohydrates during floral abscission (Reinders et al., 2005).

Many more plasma membrane sugar transporters remain to be discovered in plants. Using a bioinformatics approach to the fully sequenced Arabidopsis genome, Ward (2001) identified some 4589 putative proteins with two or more predicted membrane-spanning domains, which is an indicator of membrane transport function. There is a distinct possibility that some of these may belong to as yet unidentified new groups of transporters. Over 2500 of the putative transporters identified in Arabidopsis had no homology to proteins of known function from any organisms (Ward, 2001).

Knowledge of the transporters responsible for movement of sugars across intracellular membranes, particularly the tonoplast, has accelerated in recent years. The localisation of sucrose/H⁺ symporters from barley (HvSUT2) and Arabidopsis (AtSUT4) to the tonoplast of mesophyll cells was demonstrated using both proteomic and GFP-fusion approaches (Endler et al., 2006). Sucrose movement had previously been shown to occur across tonoplast vesicles both passively (in barley leaf vacuoles - Kaiser and Heber, 1984) and via sucrose/H⁺ antiport (in red beet root storage tissues - Briskin et al., 1985). Similarly, glucose has been shown to move across the tonoplast by passive (celery tonoplast vesicles - Daie and Wilusz, 1987) and active mechanisms (3-O-methylglucose/H⁺ antiport in sugarcane tonoplast vesicles - Thom and Komor, 1984). Despite this knowledge, only one possible candidate protein has been identified on a molecular level. Chiou and Bush (1996) used degenerate primers to isolate a putative sugar transporter cDNA from sugar beet leaves. The presence of the protein in the tonoplast membrane fraction was confirmed using immunolocalisation. However, they have not been able to demonstrate sugar transport mediated by the protein, and so its putative function is unconfirmed to date. In a subsequent study, the cDNA sequence was found to be very similar to that of the sour cherry sorbitol transporters PcSOT1 and
PcSOT2, for which plasma membrane sorbitol transport was demonstrated in transformed yeast (Gao et al., 2003).

SUTs are likely candidates for mediating sucrose transport to a greater extent than currently identified. In several plant species, more than one SUT family member exists; in Arabidopsis nine SUT homologs have been identified, and at least five differentially expressed SUTs exist in rice (Aoki et al., 2003). Additionally, SUTs have been shown to be expressed in a range of plant tissues, both source and sink. Furthermore, there may be interactions between co-localised SUTs, with both homo- and hetero-oligomerisation of proteins possible (Reinders et al., 2002a).

For these reasons the characteristics of SUTs will be further investigated, and the possibility of their involvement in sucrose efflux evaluated.

1.4 Sucrose Transporter Characteristics

1.4.1 SUT Molecular Structure

The hypothesised SUT protein membrane arrangement is envisioned to consist of 12 transmembrane domains (TMDs), with a large central cytoplasmic loop, particularly for SUT2 transporters. In terms of sequence similarity within the protein, it is a 6 + 6 TMD arrangement, suggesting that a duplication and fusion of sequence occurred some time in the distant past (Lalonde et al., 2004).

The molecular structure of SUTs places them in the major facilitator superfamily (MFS). The MFS is a diverse group of proteins that include plant hexose/H⁺ symporters, animal glucose transporters (including human GLUT1), bacterial organophosphate:Pi antiporters, bacterial sugar efflux:H⁺ antiporters and plant chloroplast triosephosphate translocators (Pao et al., 1998; Saier, 2000). SUTs are members of the glycoside-pentoside-hexuronide:cation (GPH) symporter family (Saier, 2000), one of more than 40 families within the MFS (Chang et al., 2004), which are designated based on nucleotide sequence similarity. The MFS has a motif of G-X-X-D/E-R/K-X-G-[X]-R/K-R/K in the cytoplasmic loop following trans-membrane domains 2 and 8, a feature which is generally conserved across members (Pazdernik et al., 2000). Members of the GPH family generally take up sugars in symport with H⁺ or Na⁺ (Chang et al., 2004). However, mutants of a Streptococcus thermophilus lactose permease (a GPH family lactose/H⁺ symporter) have been observed to act as energy-independent uniporters.
Poolman et al., 1995). No transporters homologous to SUTs have been identified in animal systems to date (Rentsch et al., 1998). However a SUT homolog has been identified in the yeast, Schizosaccharomyces pombe, that exhibits a 40% similarity with the potato transporter, StSUT1 (Reinders and Ward, 2001).

Little is known about the 3D structure as well as the sucrose or proton binding sites of SUTs, since efforts to date have failed to obtain a crystalline structure of a sucrose transporter. As a consequence, very little is known about the transport mechanism at the protein molecular level and only a few studies have been carried out on SUT binding site characteristics. Bush (1993a) observed that sucrose uptake by sugar beet plasma membrane vesicles was inhibited by the histidine-modifier diethyl pyrocarbonate (DEPC), which is membrane-impermeant. However, when DEPC was added to vesicles pre-incubated in sucrose, DEPC has less of an inhibition effect, suggesting that the sucrose protected the transporter-binding site from DEPC. Based on these observations, Bush (1993a) concluded that a DEPC-sensitive histidine residue was located within the binding site of the sucrose transporter on the extracellular side of the plasma membrane. In a subsequent study, Lu and Bush (1998) analysed the amino acid sequences of six SUT proteins, and noted that His-65 was the only histidine residue conserved across all sequences. Further, topological modeling of putative protein structures showed that His-65 was located on the extracellular face of the transporter. Lu and Bush (1998) investigated the significance of His-65 in transporter kinetics using site-directed mutagenesis. When the basic His-65 of AtSUC1 was substituted with a lysine or arginine residue (both basic amino acids) and expressed in yeast, an enhanced maximal sucrose transport rate resulted, while replacement with a cysteine or aspartic acid residue (polar and acidic respectively) resulted in a loss of function. They also demonstrated that His-65 was the DEPC-sensitive histidine residue within AtSUC1, as sucrose uptake by yeast expressing modified SUC2 (with amino acids other than histidine at position 65) was not sensitive to DEPC. All SUTs reported to date have a conserved histidine residue corresponding to His-65 (Lalonde et al., 2004).

The substrate specificity of SUTs has been investigated to gain a functional understanding of the binding site characteristics. It appears that the hydroxyl groups of the glucosyl moiety of sucrose were involved in substrate recognition by sucrose transport proteins, while the fructosyl moiety did not appear to be directly involved (Hitz et al., 1986). This finding was corroborated with transport studies of a range of carbohydrates into Xenopus oocytes expressing AtSUC2. Using voltage patch clamping,
it was demonstrated that SUC2 was able to transport several carbohydrates, including the disaccharides sucrose, maltose and turanose (a sucrose isomer with a 1,3 α-linkage), which all contain glucosyl moieties (Chandran et al., 2003).

Sugar competition studies have been reported for a range of SUTs. Successful competition of a sugar with sucrose indicates that the particular transporter is able to bind the sugar in question, although not necessarily transport it across the membrane. Of a range of sugars tested, only maltose (a glucose dimer) has been found to compete with sucrose for all SUTs tested. Glucose has been shown to stimulate uptake in all SUTs tested; this is most likely due to ATP activation (Lemoine, 2000). In addition, (Ludwig et al., 2000) made the somewhat surprising discovery that a SUT, AtSUC5, was also capable of transporting biotin (vitamin H), which is structurally dissimilar to sucrose.

1.4.2 Phylogeny

SUTs have been classified based on sequence homology with the resulting phylogenetic tree showing three distinct clades (Lalonde et al., 2004 and see Fig. 1.2). Clade I contains SUT1/SUC2 transporters, which are generally high affinity, low capacity (HALC) sucrose transporters. These have Michaelis-Menten constant ($K_m$) values in the order of 0.5 to 2 mM when heterologously expressed in yeast (Lalonde et al., 2004). To date, all transporters in Clade I are from dicots, with none appearing in monocots. There is a tendency to have multiple copies of Clade I SUTs in a particular plant species - for example, in Arabidopsis, seven of the nine SUT homologs identified fall into this clade (Lalonde et al., 2004). It appears that Clade I SUTs either appeared in dicots after divergence of monocots and dicots, or were lost early in monocot evolution (Reinders et al., 2006).
Figure 1.2. Phylogenetic tree of known sucrose transporter (SUT) sequences showing 3 distinct clades. Numbers in red circles indicates average amino acid length of the central loop. Ab, Asarina barclaina; Am, Alonsoa meridionalis; Ap, Apium graveolens; At, Arabidopsis thaliana; Bo, Brassica oleracea; Bv, Beta vulgaris; Cs, Citrus sinensis; Dc, Daucus carota; Ee, Euphorbia esula; Gm, Glycine max; Hv, Hordeum vulgaris; Le, Lycopersicon esculentum; Lj, Lotus japonicus; Nt, Nicotiana tabacum; Os, Oryza sativa; Ps, Pismum sativum; Rc, Ricinus communis; So, Spinacea oleracea; St, Solanum tuberosum; Ta, Triticum aestivum; Vf, Vicia faba; Vv, Vitis vinifera; Zm, Zea mays.

Reproduced from (Lalonde et al., 2004).
Clade II contains what are often referred to as SUT4 transporters. These have a low affinity and high capacity (LAHC) for sucrose transport. Typical apparent $K_m$ values for this clade are approximately 5-15 mM. Clade III SUTs have been identified in both monocots and dicots, and it appears that only a single homolog is usually present in a given plant species (Lalonde et al., 2004). However, two distinct SUT4 clade members in soybean have been revealed by genome sequencing (Payyavula et al., 2011). Further functional studies are needed to determine whether both of these encode functional proteins.

The remaining clade, Clade III, contains SUT2/SUC3 proteins, which are represented in both monocots and dicots. In dicots, it appears that only a single Clade III protein is present in a single species. In contrast, several homologs may appear in a single monocot species - for example, four of the five SUT homologs identified in rice belong to this clade (Aoki et al., 2003). In a recent review, Kühn and Grof (2010) further subdivide this clade into three clades, two of which are monocot specific. Proteins of Clade III generally have an extended central cytoplasmic loop in comparison to those of Clades I and II. It has been proposed that this central loop serves a particular purpose, as its sequence is highly conserved across monocots and dicots (Lalonde et al., 2004).

The in planta function of SUT2/SUC3 proteins has been the subject of much conjecture and debate. One speculation is that the large central loop may be for sucrose sensing, as in yeast, cytoplasmic hexose sensing appears to be carried out by transporters with large cytoplasmic domains, similar to that of SUT2 (Lalonde et al., 1999). However, conflicting evidence for sucrose sensing in plants by SUT2 exists. In one study, no sucrose transport function was detected for AtSUT2 (=AtSUC3) and LeSUT2 (Barker et al., 2000), leading to the suggestion that they served instead as sucrose sensors. This reasoning was based on the presence of the large central cytoplasmic loop, as well as the co-localisation of LeSUT2 with the functional sucrose transporters LeSUT1 and LeSUT4 in tomato petioles. In a subsequent study however, LAHC transport by AtSUC3 was demonstrated (Meyer et al., 2000), a finding subsequently corroborated by Schulze et al. (2000). There has also been confusion over the in planta localisation of SUT2-LeSUT2. This SUT appears to be associated with sieve elements of the transport phloem, with a similar situation observed for AtSUT2 promoter-GUS fusion plants (Barker et al., 2000). In contrast, AtSUC3 has been localised in cells adjacent to the vascular bundles, as well as in the sub-epidermal carpel cell layer (Meyer et al., 2000) and sieve elements (Meyer et al., 2004). The SUT2
transporter PmSUC3 from *Plantago* is also localised to sieve elements (Barth et al., 2003). Additionally, the localisation of AtSUC3 to regions of symplasmic discontinuity and source organ unloading led to speculation that this transporter may in fact function as a sucrose effluxer (Meyer et al., 2000). However, the authors were not able to reconcile the symport mechanism observed by AtSUC3 in yeast with the physiological conditions for unloading in these regions, suggesting that either a pH-dependent switch or interaction between SUTs may occur to allow efflux. Conflicting reports also exist for studies of SUT2 co-localisations with other SUTs. Neither AtSUC3 (Meyer et al., 2004) nor PmSUC3 (Barth et al., 2003) localise with AtSUC2 or PmSUC2 respectively, unlike LeSUT2 which is reported to co-localise with both LeSUT1 and LeSUT4 (Barker et al., 2000). However, as at least nine SUT homologs exist in *Arabidopsis* (Lalonde et al., 2004), the possibility of co-localisation of AtSUC3 with one or more of the other seven putative SUTs has not been excluded. Overall however, the conflicting reports on SUT2’s transport ability and localisation make it difficult to speculate at this stage on a global function for SUT2 in planta.

### 1.4.3 Functional Characterisation and Energetics

Functional characterisation of SUT kinetics and transport mechanisms have been carried out in heterologous expression systems. For biochemical characterisation, the yeast *Saccharomyces cerevisiae*, is commonly used. Oocytes from the amphibian *Xenopus laevis* are used in biophysical studies, such as in membrane potential investigations (for example, see Boorer et al., 1996). Because of their large size, *Xenopus* oocytes provide a suitable system to enable such manipulations as microinjection of solutions and electrode voltage patch clamping.

As SUT symporters rely on a proton gradient, their activity is affected by extracellular pH. Gahrtz et al. (1994) found that when tested over a pH range of 4.5 - 7, the rate of sucrose uptake by PmSUC2 (SUT1 clade) decreased with increasing pH: at pH 7.0 the uptake rate was only 11% of that at pH 4.5. This pH optima is typical of that reported for SUTs to date. Gahrtz et al. (1996) discuss transporters in terms of being “acidic” or “neutral” based on their pH dependence. AtSUC1 and PmSUC1 are referred to as “neutral”, as there is little change in transport activity from pH4 to 6. Both of these SUTs have been characterised as energy-coupled symporters. Gahrtz et al. (1996) propose that “acidic” transporters (such as AtSUC2 and PmSUC2) are phloem loaders, with PmSUC2 localised to the companion cells throughout the plant, including those of
the major and minor veins, roots and petioles (as demonstrated by Stadler et al., 1995).
Lu and Bush (1998) examined the effect of mutations at His-65 on pH dependence of
AtSUC1, to investigate whether proton binding was affected. As similar optima (of pH
6) were found, they concluded that His-65 is not directly involved in pH sensitivity
through proton binding.

To determine the transport mechanism of heterologously expressed SUT proteins in
yeast, inhibitors can be used (see Appendix 1 for a summary of commonly used
inhibitors). These inhibitors usually target a particular aspect of transport. For example,
to investigate whether transport relies on a proton gradient, the protonophores carbonyl
cyanide m-chlorophenyl hydrazone (CCCP), carbonyl cyanide-r-trifluoromethoxy-
phenyl hydrazone (FCCP) or 2,4-dinitrophenol (2,4-DNP) can be used. Protonophores
insert into cell membranes, making them leaky to protons and so dissipate pre-existing
proton gradients. Any transporter relying on the presence of a proton gradient (for
example a SUT sucrose/ H⁺ symporter) will have a decrease in function. To confirm
that transport is ATP-dependent, antimycin or arsenate can be used to inhibit cellular
ATP generation (Manning et al., 2001). Agents that modify SUT amino acid residues
have been used to gain functional understanding of the protein’s extracellular face. The
membrane-impermeant sulfhydryl modifier pCMBS binds with sulfhydryl groups on
cysteine residues, and so can affect any transport protein with these residues exposed.
Similarly, diethylpyrocarbonate (DEPC) is another membrane impermeant inhibitor that
acts by affecting exposed histidine residues (Lemoine, 2000). Thus by exposing SUTs
to a series of inhibitors, an understanding of the energetics and exposed residues of
transporters can be gleaned.

1.5 Sucrose Transporter Function in Plants

1.5.1 Approaches to localise SUTs in plant tissues

The spatial and temporal expression of transcript and protein levels can provide
cues to the in planta function of SUTs. Several approaches to detection exist. The
organ or tissue-specific quantification of mRNA transcript can be carried out using
semi-quantitative and real-time PCR methods, as well as in situ transcript visualisation
in fixed histological sections. These approaches, while indicating tissues in which
transcript exist, do not necessarily confirm the presence of SUT proteins. To enable this,
fluorescent anti-SUT antibodies or SUT promoter-GFP fusion approaches are required. The resolution provided by these approaches enables protein localisation on both cellular and sub-cellular scales. In addition, protein or RNA extracts can be prepared from whole plant organs, tissues, cell populations or sub-cellular components, then separated and probed with labelled antibodies or oligonucleotides to detect the presence of a particular protein or transcript. In addition, the use of SUT under- and over-expressing plants can be a useful tool in deducing the in planta role of SUTs.

1.5.2 Spatial and temporal expression of SUTs

Terminal sinks, particularly developing seeds, appear to rely heavily on SUTs for acquiring sucrose. The importance of OsSUT1 for sucrose import into developing rice grains was demonstrated using antisense rice plants, with knockout plants demonstrating smaller grains with impaired germination, but with no impact on plant photosynthetic rates (Scofield et al., 2002).

The temporal expression of SUTs in developing seeds has also been well studied, with the evidence indicating a role for SUTs (particularly those belonging to Clade I) in seed sucrose nutrition. PmSUC1 (Clade I) mRNA is abundant in non-vascular tissues of the Plantago flower during early seed development, with protein localised to young ovules (Gahrtz et al., 1996). Similarly, a peak in expression of AtSUC5 (Clade I) from Arabidopsis occurring between 4 and 9 days after flowering also suggests a role in early seed development (Baud et al., 2005). AtSUC5 mutants showed a marked but transient reduction in filial tissue fatty acid concentration, and a small delay in embryo development (Baud et al., 2005). In the soybean seed, protein levels of GmSUT1 increased rapidly across development, with this reflected in seed sucrose levels (Aldape et al., 2003). In developing broad bean cotyledons, SUT transporters (with homology to LeSUT1) were immunolocalised to the epidermal transfer cells abutting seed coat tissues (Harrington et al., 1997a), with temporal expression coinciding with the cotyledon expansion phase (Harrington et al., 1997b). In developing pea cotyledons, PsSUT1 protein was localised to storage parenchyma in addition to epidermal transfer cells, with higher transport activity occurring in the latter (Tegeder et al., 1999; Rosche et al., 2002), while in differing French bean cultivars, differences in final cotyledon biomass was reflected in SUT activity at the epidermal layer (Tegeder et al., 2000).
Thus, it appears that SUT1 proteins are central to seed nutrition and biomass gain during development.

The localisation of some SUTs suggests a specialised role in providing energy to support other biotrophic functions, such as nutrition for pollen tube growth. For example, NtSUT1 from tobacco and AtSUC1 from *Arabidopsis* (both Clade I) appear to play a role in supplying energy to developing pollen tubes (Lemoine *et al.*, 1999; Stadler *et al.*, 1999). Anti-LeSUT2 (Clade III) tomato plants were affected in fruit and seed development, leading to the conclusion that this transporter played an important role in pollen tube growth (Hackel *et al.*, 2006). Similarly, the rice transporter OsSUT2 (also a Clade III transporter) appears to be expressed in a pollen-specific manner (Takeda *et al.*, 2001).

The Clade II member LjSUT4 from *Lotus japonicus* is most likely involved in carbohydrate provision to developing root nodules, showing expression in vascular bundles and inner root cortical cells (Flemetakis *et al.*, 2003). Additionally, the LAHC StSUT4 localises to the SEs in the minor veins of source leaves, as does AtSUT4 from *Arabidopsis* and LeSUT4 from tomato, indicating a possible role for SUT4 (Clade III) transporters in phloem loading (Weise *et al.*, 2000). However, in the case of AtSUT4 from *Arabidopsis*, recent studies by Endler *et al.* (2006) suggest this transporter localises at the tonoplast when expressed in mesophyll cells.

As SUTs from the three different clades have different biochemical and perhaps expression profiles, having a suite of SUTs present in a single species allows different sucrose transport requirements to be met. In potato, the function and location of StSUT1 (Clade I), StSUT4 (Clade I) and StSUT2 (Clade III) have been studied in detail. StSUT1 localises to the SEs of young potato tubers (a sink organ), suggesting a role in either the direct unloading of sucrose from SEs and/or retrieving sucrose leaked to the apoplasm along the phloem translocation pathway (Kühn *et al.*, 2003). Further investigation of antisense potato SUT1 plants by comparing both constitutive and companion-cell specific antisense expression systems on leaf anatomy and plant productivity found that all SUT1-suppressed plants had increased levels of starch accumulation in leaf epidermal and mesophyll cells and sucrose accumulation in mature leaves. Thus, SUT1 appears to play a role in sucrose export from the leaves. For plants with companion-cell specific antisense expression, a marked decrease in tuber yield was also observed (Schulz *et al.*, 1998).
SUT expression is subject to temporal changes, both throughout development and also diurnally. Kühn et al. (1997) found that the expression of LeSUT1 and StSUT1 were diurnally regulated. LeSUT1, which is associated with SEs in source leaf minor veins (Barker et al., 2000) had peak mRNA levels in source leaves after 4 hours exposure to light, while in potato, StSUT1 protein levels decrease after 15 hours of darkness. This shows that a regulatory system is in place to optimise SUT protein levels and that SUT proteins have a relatively high turnover rate (Kühn et al., 1997). Likewise, Shakya and Sturm (1998) found that the carrot DcSUT1 responsible for phloem loading in source leaves was diurnally regulated, with the highest levels of SUT mRNA in the middle of the day. This corresponded with peak photosynthetic levels, which would be when higher levels of phloem loading are required. In contrast, DcSUT2, which showed high levels of expression in sink organs, was not diurnally regulated (Shakya and Sturm, 1998).

In leaves, SUT expression often changes over a longer developmental period, changing as leaves mature. For example, PmSUC2 expression triples during the transition of leaves from sink to source (Gahrtz et al., 1996). In developing fruits, SUT expression profiles change with development, being linked with the sugar species available for transport. In developing grape berries, mRNA levels for VvSUT1 increased at the onset of ripening and were sustained through until maturity (Ageorges et al., 2000). Although hexoses are the main form of sugar imported by developing grape berries, the presence of VvSUT1 may indicate incomplete cleavage of sucrose by apoplastic invertases, or an efflux mechanism for sucrose (Ageorges et al., 2000).

A question that has arisen for SE-located SUTs is one of regulation, given that mature SEs are enucleate. Kühn et al. (1996) observed that SUT mRNA in potato was located mainly in SEs, particularly near plasmodesmata. However, active SEs lack nuclei, so must rely on importing mRNA to maintain SUT protein levels. It was shown that potato plants with CC-specific SUT-antisense expression had decreased mRNA levels (Kühn et al., 1996), while the SUT proteins are localised to the SEs (Kühn et al., 1997). The possibility of trafficking of proteins was excluded, as GFP-SUT1 fusion proteins remained restricted to the CC (Lalonde et al., 2003). Taken together, these data confirm the trafficking of CC-derived mRNAs to SEs via plasmodesmata, indicating CC control over SE-located SUTs (Kühn et al., 1997). However, there are conflicting reports localising this same protein to the CCs of Arabidopsis (Stadler and Sauer, 1996), as well as PmSUC2 (=SUT1) in companion cells of Plantago (Stadler et al., 1995).
Many SUTs appear to have a direct role in sucrose loading of phloem. In antisense potato SUT1 plants, mature leaves contained up to 20 times more soluble carbohydrates and 5 times more starch than in wild-type leaves, with bleaching and curling of leaves an evident antisense phenotype. The excess carbohydrates correlated to decreased SUT1 mRNA levels, indicating a role of this transporter in apoplasmic phloem loading (Riesmeier et al., 1994). The tobacco transporter NtSUT1 transcript is expressed highly in mature leaves. Antisense SUT1 plants (under a constitutive promoter) had decreased transcript levels in mature leaves and a chlorotic phenotype. These mature leaves had higher sucrose content, which, together with NtSUT1 protein localisation to SE plasma membranes (Kühn et al., 1997) indicates a role in phloem loading.

1.5.3 Sub-cellular Localisation

Knowledge of the sub-cellular localisation of SUTs is fundamental in developing an understanding of SUT function. Using GFP-fusion proteins, SUTs have been shown to localise to either the plasma membrane or tonoplast. Transporters belonging to the SUT1 clade appear to be plasma membrane localised, along with some SUT4 clade members including LeSUT4 from tomato and StSUT4 from potato (Chincinska et al., 2008). In contrast, non-solanaceous SUT4 proteins from barley, Arabidopsis (Endler et al., 2006) lotus (Reinders et al., 2008) and poplar (Payyavula et al., 2011) are reported as being tonoplast localised. Further, the tonoplast SUT4 transporter in Arabidopsis, AtSUC4, mediates sucrose release from the vacuole in symport with protons \textit{in vivo} (Schultz et al., 2011).

1.5.4 Regulation of SUT Expression and Activity

In recent years, much experimental evidence relating to the regulation of SUT expression and activity has been obtained. Not surprisingly, factors that influence the production of photoassimilates such as diurnal patterns, have been shown to impact upon SUTs. For example, it has been shown that transcript levels of OsSUT1 (a companion-cell specific SUT from rice) increase in response to light and endogenous sugar levels (Matsukura et al., 2000). Additionally, expression may also be influenced by prevailing abiotic factors. Similar diurnal expression patterns are reported for
ZmSUT1 (a SUT2 clade member), with increases in transcript level correlating with high levels of sucrose and glucose. ZmSUT1 is also highly expressed after greening occurs in blades of young and mature leaves. A basipetal gradient of expression developed along expanding leaves, decreasing from tip to base, reflecting leaf development from a sink to source organ (Aoki et al., 1999). A similar pattern of expression has also been observed for AtSUC2 promoter-GFP fusion proteins in tobacco leaves as they approach maturity (Wright et al., 2003). It appears that this expression is closely linked with the production of photoassimilates: feeding leaf blades with sucrose led to an increase in ZmSUT1 transcript levels, suggesting a positive regulation by sucrose levels (Aoki et al., 1999).

In contrast to these reports, SUT1 expression in broad bean cotyledons cultured at high sucrose levels (150 mM) was reduced (Weber et al., 1997a), while the activity of BvSUT1 in leaves fed with high levels of sucrose was also found to decrease (Chiou and Bush, 1998). Thus, it appears that sucrose levels act as a regulator of SUT expression and activity, however the direction of regulation is possibly dependent on a number of factors, including the plant tissues in question and endogenous levels of sucrose and possibly other sugars. The underlying mechanism of this sugar-mediated regulation has not yet been identified (and is beyond the scope of this work). However, it is tempting to speculate that endogenous sugar levels (particularly that of sucrose) are most likely to assert influence over the expression of sucrose efflux genes as well.

SUT expression also appears to be regulated by other environmental cues, particularly those that require an osmotic response to optimise plant performance or survival. For example, under salt stress, which is a condition that favours mannitol transport (as an osmotic defence) levels of AgSUT1 mRNA in celery decreased, particularly in roots (Noiraud et al., 2000). Shiozaki et al. (2005) also report salt stress-induced SUT expression in rice.

1.6 Sucrose Efflux

The efflux of sucrose from cells is a physiological necessity where sucrose transport occurs across a symplasmic discontinuity, such as in phloem loading in mature leaves (for plants with an apoplastic pathway) or from maternal seed coat tissues to the developing filial tissues. Two different modes of action for this efflux of sucrose appear possible: the passive diffusion of sucrose through the plasma membrane and the
movement of sucrose via a transporter protein. The requirements and physiological evidence for each of these are considered in turn.

1.6.1 Passive Diffusion

The rate of passive diffusion of sucrose across a lipid bilayer is directly proportional to the permeability of the membrane to sucrose and the concentration difference (Δ[C]) across the membrane, where:

\[
\text{Flux} = \text{permeability coefficient} \times \Delta[C]
\]

Assuming a plant cell plasma membrane permeability of \(10^{-10} \text{ m s}^{-1}\) (Cram, 1984) to sucrose, and a trans-membrane difference of 10 mM, the rate of flux would be \(10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}\). For French bean in similar experimental conditions, a rate of flux approximately 10 times this predicted value was observed (Offler and Patrick, 1984; Patrick, 2006) indicating that transport must be carrier-mediated.

1.6.2 Transport via a Transporter Protein

Several options exist for the molecular action of sucrose efflux from the plasma membrane. These possible options are:

- passive diffusion across the plasma membrane
- facilitated diffusion through either a non-selective channel or carrier protein, according to concentration gradient
- facilitated diffusion by the reversal of a sucrose/\(H^+\) symporter
- active transport by a sucrose/\(H^+\) antiporter

The thermodynamic considerations and experimental evidence for each of these possibilities will be examined in turn.

1.6.2.1 Facilitated transport via a channel or carrier protein

Experimental evidence for the possible mechanism of efflux has been gathered from tissue studies, using seed coats from several different grain legume species. de Jong et al. (1996) and van Dongen et al. (2001) reported evidence for the presence of a non-selective, energy-independent pore (or channel) in pea seed coats, arguing that a sucrose gradient between the seed coat release cells and apoplasm of only 2 mM is sufficient to deliver the required sucrose to the developing embryo. Studies of inhibitor effects on efflux from detached seed coats of both broad bean (Fieuw and Patrick, 1993) and French bean (Walker et al., 1995) also found that 50% of sucrose efflux was energy-
independent, while evidence for facilitated, energy-independent uptake of sucrose was found for both species at high sucrose concentrations (Ritchie et al., 2003).

Can a SUT-like protein become uncoupled from the $H^+$ gradient and act as an energy-independent carrier? The localisation of putative sucrose/ $H^+$ symporters in tissues committed to efflux - for example PsSUT1 in pea seed coats (Tegeder et al., 1999) suggests this possibility. In several instances, SUTs that have been characterised as symporters have had transcript located to sites of efflux, for example, AtSUC3 (Meyer et al., 2004).

SUTs also appear to play a role at sites of sucrose efflux in monocots. In developing wheat grains, SUT proteins were immunolocalised to both maternal (nucellar projection transfer cells) and filial tissues, that is, sites of both sucrose efflux and uptake (Bagnall et al., 2000). It was concluded that these SUT-type proteins are candidates for mediating sucrose efflux, with further studies characterising the efflux as occurring via facilitated diffusion (Wang and Fisher, 1995).

This raises the possibility that these SUTs may become energy-uncoupled (perhaps due to sequence variations or under certain conditions), acting as a passive carrier instead. If this is the case, it is possible that sucrose efflux (via facilitated transporters) may be mediated by SUT- like proteins. A scenario similar to this is known in the lactose transporters of bacteria, where a single amino acid change results in proton-uncoupling (Pao et al., 1998).

### 1.6.2.2 Reversal of a sucrose/ $H^+$ symporter

Another possibility to account for the localisation of putative symporters to regions of efflux is that the transporter may have undergone a reversal in the direction of sucrose and proton transport, allowing these SUTs to efflux sucrose. However, the proton gradient driving sucrose uptake is inward-directed, and so from an energetics perspective, the reversal of symport requires a sucrose gradient that is steep enough to overcome the proton electrochemical gradient. The intracellular sucrose concentration required for a given extracellular concentration to enable reversal can be predicted by rearranging the Nernst Equation given in Equation 1.1:

$$\log_{10} C_i = \log_{10} C_o - \frac{(\Psi + 59\Delta p\text{H})}{59}$$

(Equation 1.2)

Where:

- $\Psi$ = membrane potential difference (V)
- $C_o$ = Extracellular concentration (mol m$^{-3}$)
- $C_i$ = Intracellular concentration (mol m$^{-3}$)
From Equation 1.2, it can be seen that a smaller $\Delta$pH and less negative membrane potential reduce the sucrose gradient required to enable symporter reversal.

Carpaneto et al. (2005) demonstrated reversal of the symporter ZmSUT1 heterologously expressed in *Xenopus* oocytes, where sucrose gradients are reversed (with a high intracellular concentration relative to that in the apoplasm) and membrane potentials are collapsed. However, it is questionable whether such a reversal could occur in planta under the physiological conditions present. In the seed coat of French bean, for example, a membrane potential of ca. -40 mV and pH gradient of one unit exist (Walker et al., 1995). Under these conditions, and with a seed apoplasm sucrose concentration of 70 mM (Patrick, 1994) to reverse a seed coat symporter to one capable of efflux:

$$\log_{10} C_i = \log_{10} C_o - (\Psi + 59\Delta pH)/59$$

$$= \log_{10} 70 - (-40 + -59)/59$$

$$= 3.53$$

So, $C_i = 3388$ mM

Under these conditions, a theoretical intracellular concentration of over 3 M is required to reverse the sucrose flux from one of uptake to that of efflux. This required seed coat symplasmic sucrose concentration greatly exceeds the measured values of 80 mM for French bean (Patrick, 1994). So, although SUTs are expressed in seed coat efflux tissues, it appears that symport reversal is unlikely to account for sucrose release from these cells.

### 1.6.2.3 Sucrose/ $H^+$ antiport

From an energetics perspective, a sucrose/ $H^+$ antiporter is a more feasible mode of energy-dependent sucrose efflux due to the inward-directed pmf present. Indeed, experimental evidence exists to support the existence of such transporters. The development of the attached empty seed coat technique, where the embryo is surgically removed and replaced with solution (Patrick, 1983; Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983), has facilitated the study of assimilate release by maternal tissues. The use of this technique, in conjunction with various inhibitors (such as protonophores, sulfhydryl modifiers and ATPase inhibitors), has progressed understanding of efflux energetics in vivo. Initial experiments using the empty seed coat technique showed that sucrose release by soybean seed coats was transporter mediated, as treatment with the sulfhydryl modifier (and so transporter protein inhibitor) and
mercury derivative p-chloromercuriphenylsulfonic acid (pCMBS) abolished sucrose efflux; this was partially restored by subsequent dithiothreitol (dtt) addition (Thorne and Rainbird, 1983). In broad bean and French bean seed coats, evidence of both active and passive sucrose efflux exists (Fieuw and Patrick, 1993; Walker et al., 1995). For French bean, it was observed that the ATPase stimulator fusicoccin stimulated sucrose efflux. Additionally, it was demonstrated that increasing the transmembrane pH difference also stimulated sucrose efflux. However, pCMBS diminished seed coat sucrose efflux by 50%, and made any subsequent addition of fusicoccin ineffective. Taken together, these data indicate that two mechanisms of sucrose efflux exist: one that is energy-coupled and reliant on an inward-directed proton gradient (a putative sucrose/ H⁺ antiporter), and one independent of energy. Similarly, treatment of broad bean seed coats with the protonophore CCCP reduced efflux by 50% (Fieuw and Patrick, 1993). In contrast to these reports, sucrose release from seed coats of developing peas is reported to be largely energy independent, through a non-selective pore (de Jong et al., 1996) with some evidence of SUT symporter activity being the only energy-coupling reported for this tissue (de Jong and Bortslap, 2000).

The first plant sucrose/H⁺ antiporters have been recently identified. Originally described as tonoplast monosaccharide transporters, AtTMT1 and AtTMT2 were also shown to transport sucrose in antiport with protons when studied using patch clamping (Schulz et al., 2011). As a tonoplast-localised transporter, the authors proposed its role in the loading of sucrose into the vacuole. However, no plasma membrane localised counterpart responsible for sucrose efflux from the cell has yet been described.

A family of H⁺/sugar antiporters has also been identified in bacteria. The sugar efflux transporter (SET) family (Liu et al., 1999) has members identified in both gram-positive and gram-negative bacteria. SetA and SetB from E. coli were characterised as encoding a transporter capable of effluxing glucose and the disaccharide lactose (but not sucrose) in antiport with protons. No SET homologs have been identified thus far in plants. Several other classes of antiporters have been identified in plants, with many involved in cation exchange (see Fox and Guerinot, 1998 for a review) and transport of secondary metabolites and transition metals by multidrug and toxicity effluxers (MATEs- reviewed by Omote et al., 2006).

To date, the majority of sucrose transporters cloned from plants are sucrose/H⁺ symporters. It is perhaps not surprising that few antiporters have been identified, given the reliance on the SUSY7 complementation system. Where cloning by
complementation is used, the ability to uptake sucrose is selected for, so any cDNA encoding a functional antiporter would not be detected, due to a presumably outward-directed mechanism for sucrose transport. Cloning novel SUTs by homology could potentially isolate effluxers, however if SUSY7 growth on sucrose is used as an indicator of functional clones, antiporters would once again be excluded. To isolate and characterise effluxers, a cloning strategy that relies on the ability to efflux, rather than uptake, sucrose is required, together with a technique to access the intracellular face of the putative antiporter.

The presence of nine putative SUTs in *Arabidopsis*—of which all but two of them appear to be functional (Sauer et al., 2004)—raises the possibility that the SUT genes include more than simply a single HALC and LAHC plasma membrane sucrose transporter for a particular plant species. The identification of several SUT4 clade members that localise to the tonoplast (AtSUT4 from *Arabidopsis* and HvSUT2 from barley—Endler et al., 2006; LjSUT4 from lotus—Reinders et al., 2008) confirms that SUTs function is not restricted to the plasma membrane. Furthermore, numerous reports locate SUTs, along with H\(^+/\)ATPases (suggesting energy-coupled transport) at sites of sucrose efflux, in such places as seed coat release cells of broad bean (Harrington et al., 1997a) and pea (Tegeder et al., 1999). Is it possible that SUTs are also able to function as sucrose antiporters? Given these findings, the further examination of this group of transporters as possible sucrose effluxers is warranted.

## 1.7 Project Objectives

A survey of the literature reporting current understanding of sucrose translocation throughout the plant clearly identifies a deficit in the area of sucrose efflux mechanisms. In particular, no transporters responsible for sucrose efflux from the developing seed coat have been identified at a molecular level. The identification of such transporters, and their mode of function, will in turn allow us to integrate and deepen our knowledge of carbon partitioning on a whole plant scale, as well as provide possible target genes for selective breeding programs to optimise plant yields.

The seed coat of developing legume seeds is functionally committed to sucrose efflux to the developing embryo. As such, it may be enriched in transcripts for genes mediating sucrose- efflux. Given the extensive evidence presented in the previous sections, we hypothesised that sucrose efflux transporters may be SUT-like proteins.
We have therefore taken a homology cloning approach to identify several full-length putative sucrose transporter genes from coats of developing pea and bean seeds, with the intention of examining their function.

The investigations described in this thesis aim to:

1. Functionally characterise these novel sucrose transporters from their outer (extracellular) face, by expression in yeast
2. Investigate the role of these novel sucrose transporters in solute unloading, through:
   a. the sub-cellular localisation of transporter protein, to determine whether it is localised to the plasma membrane, tonoplast, or another membrane.
   b. cellular localisation of transporter protein in developing seed coats and leaf tissues at sites of sucrose efflux.
3. Develop a novel system to functionally characterise novel sucrose transporters from their inner face (cytoplasmic), through engineering an inside-out vesicle accumulating yeast strain.

The novel sucrose transporters cloned from seed coats were expressed and functionally characterised in SUSY7 yeast. This was carried out by studying uptake of $^{14}$C-labelled sucrose. In this work, a particular emphasis was placed on understanding the energetics of transport, which was achieved by investigating the effects of inhibitors on sucrose uptake. Concentration-dependent sucrose uptake kinetics and transporter substrate specificity was also studied, as well as the ability of the transporters to mediate sucrose efflux from pre-loaded yeast cells. These studies are described in Chapter 2.

To determine whether the transporters are responsible for plasma membrane or tonoplast transport, the sub-cellular localisation of transporter protein was investigated. This was carried out using transient expression of GFP-tagged transporters in a tobacco leaf. The cellular localisation of transporters in developing pea seed coats and source leaves, was carried out to determine their potential contribution to phloem loading and unloading of sucrose in planta (Chapter 3).

The need to access the cytoplasmic face of putative sucrose effluxers for kinetic and energisation studies was recognised. To enable this, a system capable of producing inside-out membrane vesicles was adapted to make it suitable for sucrose transporter study (Chapter 4).
The information gleaned on transporter characteristics and localisation is used as a basis to develop a speculative model for the role of novel sucrose transporters facilitating efflux to the apoplasms of legume seed coats (Chapter 5). Our current state of knowledge of sucrose efflux is reviewed and gaps identified. The use of appropriate tools, such as the inside-out membrane vesicle accumulation system developed for sucrose transporters, to address these gaps offers the opportunity to further understand this most fundamental component of plant biology.
Chapter 2

Functional Characterisation of Novel Sucrose Transporters Cloned from Legume Seed Coats
2.1 Introduction

Sucrose is the major form of carbohydrate released from seed coats to the developing embryo during legume seed development (Patrick and McDonald, 1980; Rochat and Boutin, 1991). Seed coat tissues function to efflux sucrose to the apoplasm for subsequent uptake by the developing embryo (Patrick and Offler, 2001). In planta rates of sucrose release from grain legume seed coats greatly exceed those attributable to simple diffusion across efflux site membranes (Cram, 1984; Offler and Patrick, 1984). The release of sucrose and other assimilates from the seed coats of several grain legumes (broad bean - Wolswinkel et al., 1992; Fieuw and Patrick, 1993, pea- de Jong and Wolswinkel, 1995; de Jong et al., 1996, French bean- Walker et al., 1995) has been shown to be sensitive to known inhibitors of membrane transporters such as pCMBS. As such, these seed coat tissues may be a source of putative sucrose effluxers, and a molecular investigation of the cDNA transcripts of these tissues provides the possibility of identifying the transporters responsible for the observed sucrose efflux.

Studies of whole tissues of legume seed coats have revealed that sucrose efflux may occur via several transport mechanisms, including energy-independent, non-selective pores in pea (de Jong et al., 1996; van Dongen et al., 2001). In French and broad bean seed coats, it appears that half of sucrose efflux occurs in antiport with protons in an energy-dependent manner with the remaining flux mediated by some form of facilitated diffusion (Fieuw and Patrick, 1993; Walker et al., 1995). Although usually associated with sites of sucrose influx, including developing cotyledons (for example see Tegeder et al., 1999; Aldape et al., 2003) sucrose transporter (SUT) genes, which encode sucrose/H\(^+\) symporters, are also expressed in seed coat tissues (Weber et al., 1997a; Tegeder et al., 1999). It has been demonstrated that under conditions of depolarized membrane potentials and high intracellular sucrose levels, SUT reversal can be induced, and so SUTs have been suggested as possible candidate transporters responsible for sucrose efflux (Carpaneto et al., 2005). SUTs were the only sucrose transporters unequivocally identified in plants at the commencement of this study (prior to the identification of SWEET sucrose transporters- Chen et al., 2010; 2012). The possibility that effluxers may share homology with SUTs was recognised, were a variation or loss in energisation may result from only minor sequence variation. Such a case exists in the lactose transporters of bacteria, where it is known that a single amino acid change
results in a shift from transport via proton-coupled symport to an energy-independent carrier (Pao et al., 1998).

Consequently, five genes homologous to SUTs were cloned by Dr. Yuchan Zhou prior to this study (Zhou et al., 2007). Of these, two novel sucrose transporters, along with the previously described PsSUT1 (Tegeder et al., 1999) were isolated from pea seed coats, with the remaining two novel transporters being isolated from French bean seed coats. Phylogenetic analysis (Fig. 2.1) showed that the isolated transporters clustered closely with other sucrose transporters. Adopting the SUT classification system of Lalonde et al. (2004), one of the isolates from pea fell within Clade II, clustering most closely with LjSUT4 from lotus (Flemetakis et al., 2003), the only full-length legume SUT4 previously cloned. In addition, other partial legume SUT sequences available in public databases, including two from peanut, a second sequence from lotus and one each from Medicago truncatula and soybean fell within this same cluster. The remaining two pea isolates clustered in Clade I, along with other dicot SUT1s. PsSUT1 clustered most closely with legume SUTs GmSUT1 (Aldape et al., 2003) and VfSUT1 (Weber et al., 1997a), from soybean and broad bean respectively. The two novel sucrose transporters cloned from French bean also clustered in Clade I, forming a subclade with the two novel pea isolates (Fig. 2.1). The naming of these novel genes was based on both phylogenetic analysis and studies of function carried out in this study. Transporters shown to be sucrose/H\(^{+}\) symporters (based on evidence presented later) were designated as SUTs, as previously characterised SUTs were found to have this mode of energy-coupling (Lalonde et al., 2004). Those transporters shown to mediate energy-independent sucrose transport were assigned the descriptor sucrose facilitator (SUF). Thus, the two transporters from pea were named PsSUF1 and PsSUF4, with the two from French bean named PvSUT1 and PsSUF1. Comparisons between the phylogeny, structure and function of SUTs and SUFs are discussed in Section 2.4.3.

These novel sucrose transporters cloned from seed coats were functionally characterised in SUSY7 yeast, with a particular emphasis on understanding the energetics of transport, concentration-dependent sucrose uptake kinetics and transporter substrate specificity. As predicted, several novel transporters were characterised as sucrose effluxers (the first such transporters reported), being energy-independent carriers. In addition, two transporters were identified as sucrose/H\(^{+}\) symporters. The
possible physiological roles of both SUFs and SUTs, in light of what is known of
sucrose efflux from whole tissue studies, are discussed.

Figure 2.1. Phylogenetic analysis of known sucrose transporters. Clades I, II and III
are shown, adopting the system devised by Lalonde et al., 2004.
2.2 Materials and Methods

2.2.1 Yeast Materials

2.2.1.1 Strains

Yeast of the mutant SUSY7/ura3 strain (Riesmeier et al., 1992; Barker et al., 2000) were previously complemented with SUT cDNAs obtained from seed coats of pea and French bean. The sucrose utilisation capacity of each clone of complemented yeast had been demonstrated by culture on SD media (ura-) supplemented with 2% sucrose as the sole carbon source (Zhou et al., 2007).

2.2.1.2 Yeast growth and preparation

Each transformed clone of yeast was grown in liquid SD media containing 2% glucose and all necessary amino acids except uracil, as SUSY7/ura3 has a uracil auxotrophy, which the transporter-encoding pDR196 plasmid complements (Barth et al., 2003). Cultures were grown on a rotary shaker at 250 rpm at 30 °C, to an OD_{623} of 0.8-0.85 units, which approximates to 3 \times 10^7 yeast cells per mL. Yeast cells were harvested from early log phase liquid cultures by centrifugation at 3000 rpm for 10 min, then washed with (30 mL) 25 mM 2-(N-morpholino)ethanesulfonic acid/ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (MES/HEPES) buffer of appropriate pH, and re-centrifuged at 3000 rpm for 10 min. The harvested yeast cells were resuspended in 25 mM MES/HEPES buffer of the appropriate pH at the rate of 36 \mu L of buffer per mL of liquid culture harvested, giving a concentration of 8.3 \times 10^8 cells per mL (an OD_{600} of approximately 25 units/mL). All uptake experiments were performed in a shaking water bath (140 rpm) at 25 °C.

2.2.2 Sucrose Uptake Studies

2.2.2.1 Basic uptake procedure

The following basic uptake procedure was used for all uptakes, except where noted. All reactions were performed in a shaking water bath (140 rpm) at 25 °C. For each uptake reaction, 360 \mu L of yeast suspension was used. At 1 min prior to the addition of sucrose, 40 \mu L of 200 mM glucose (or 2 M ethanol for PvSUF1, as glucose was found to be a competitive inhibitor for sucrose with the transporter) in 25 mM MES/HEPES, buffered at the appropriate pH, was added to energise the yeast. Their final reaction
concentrations were 10 mM glucose or 100 mM ethanol (see Sauer and Stolz, 1994). 400 µL of sucrose uptake solution (unlabelled sucrose in 25 mM MES/HEPES, supplemented with uniformly labelled $^{14}$C]-sucrose (Amersham; 18.5 kBq/mL), was then added to the reaction mixture. Sucrose uptake was terminated after 4 min by the addition of 4 mL of ice-cold milliQ H$_2$O. Yeast cells were collected immediately by vacuum filtration onto glass microfibre filters (1.2 µm, Whatman) using an Amicon VFM-111 vacuum filtration manifold connected to a vacuum pump (DynaVac VGH series). The internal vacuum pressure in the manifold was in the range of 5-10 kPa. Filters were washed with an additional 4 mL cold milliQ H$_2$O during filtration, to remove any remaining extracellular label. Filters were then collected into individual 5 mL scintillation vial inserts, supplemented with 500 µL cold milliQ H$_2$O and left for 30 min. 2.5mL of Ecolite™ (+) liquid scintillation cocktail (MP Biomedical) was added and incubated at room temperature overnight before radioassay in a Packard Tri-Carb Liquid Scintillation Counter with quench correction. Aliquots of $^{14}$C]-sucrose uptake solutions were also included to measure specific activity in DPM (disintegrations per minute) values to enable calculation of $^{14}$C]-sucrose uptake. Transport rates for yeast transformed with the empty vector (pDR196) were also measured, and the rates subtracted from all presented data, with the exception of the time-course studies.

2.2.2.2 Time course uptake

To demonstrate transporter-mediated rather than passive, non-facilitated diffusion of sucrose, time course uptake of $^{14}$C]-labelled sucrose was carried out using 0.2 mM sucrose in 25 mM Na-Pi buffer at pH 5.5. Yeast cells were energised by the addition of 10 mM glucose (or 100 mM ethanol for PvSUF1 and in sugar competition studies) 1 min prior to sucrose solution addition. Uptakes were performed over 10 min, with 800 µL aliquots withdrawn and washed every 2 min. Yeast washing, harvesting and radio-assay were performed as described in Section 2.2.2.1.

2.2.2.3 pH dependence of novel sucrose transporters

Sucrose uptakes at pH 4.0, 4.5, 5, 6, 6.5, 7 and 8 were performed as per the procedure in Section 2.2.2.1. A sucrose uptake solution with a final concentration of 1 mM sucrose, which approximates the K$_m$ of previously described transporters of the SUT1 clade (Lalonde et al., 2004), was used. The 1 mM sucrose solutions were prepared in 25 mM HEPES/MES (of appropriate pH) and supplemented with $^{14}$C]-
sucrose (8.5 kBq/ mL). Sucrose uptake was terminated after 4 min and assayed as described in Section 2.2.2.1.

2.2.2.4 Concentration-dependent sucrose uptake

Uptakes were carried out using $^{14}\text{C}$-sucrose labelled solutions for concentrations ranging from 250 µM to 250 mM sucrose. Solutions were osmotically adjusted (to 250 mOsm kg$^{-1}$) with sorbitol to compensate for the osmotic effects of high sucrose concentrations, using the standard uptake procedure described in Section 2.2.2.1.

2.2.2.5 Sucrose transport inhibitor studies

To dissect the energetics of sucrose transport, the effects of several metabolic inhibitors on transport were studied. The mitochondrial electron transport chain inhibitor antimycin A was used (at a final concentration of 10 µM) to inhibit cellular ATP production (Riesmeier et al., 1992), and so assess whether transport is energy-dependent. To differentiate between primary active transport (such as via an ATP-dependent transporter such as those in the ABC transporter family) and secondary active transport via utilisation of an energy-generated proton gradient, the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to dissipate the trans-plasma membrane proton gradient (see Gaskova et al., 1998). The slowly-permeating sulfhydryl reagent DEPC was also used (added to a final concentration of 1.5 mM) to interfere with any exposed histidine residues, which have been previously shown to be involved with substrate binding in SUTs (Lu and Bush, 1998). For these studies, the inhibitor of interest was added to energised yeast 30 sec prior to the addition of 1 mM sucrose in MES/HEPES buffer (pH 4.5). Sucrose uptake was terminated after 4 min and assayed as described in Section 2.2.2.1.

2.2.2.6 Counter-transport and sucrose efflux

Yeast cells were prepared as described in Section 2.2.2.1 then pre-loaded with unlabelled sucrose by incubating cells in 50 mM sucrose in Na-Pi (buffered at pH 5.5) for 30 min. An iso-osmotic control was also incubated in buffered 50 mM sorbitol for 30 min. Cells were then washed with ice-cold water, harvested by centrifugation and resuspended in 50 mM sorbitol. Time course uptakes, as described in Section 2.2.2.2, were then carried out.

Sucrose efflux measurements were made by first preloading yeast cells with $^{14}\text{C}$-sucrose in a 0.2 mM sucrose solution (buffered at pH 4.5 with 25 mM HEPES–MES) at
25°C for 10 min. Apoplastic [14C]-sucrose was removed by washing cells with 4 mL ice-cold water. Cells were then washed over a 10-min period with 20 mL of fresh 25 mM HEPES–MES buffer (pH 4.5) on microfibre filters. Cells were then radioassayed to determine the amount of 14C label remaining.

2.2.3 TLC of Yeast Extracts

To determine whether any intracellular hydrolysis of sucrose label occurs within a 10-min timeframe, yeast cells were pre-loaded with [14C]-sucrose for 10 min, from a 0.2 mM sucrose solution buffered at pH 4.5 with 25 mM MES/HEPES, followed by 10 min of incubation with sucrose-free buffer. Yeast cells were collected via centrifugation at 3000 rpm for 10 min. Yeast cell extracts were prepared from preloaded cells before and following the 10-min incubation by hot water extraction at 85 °C for 40 min, followed by snap-freezing in liquid nitrogen and freeze drying. Samples were resuspended in 70% ethanol prior to loading onto TLC plates.

The procedure followed for TLC is as described by Ruan (1995). 20 x 20 cm glass plates pre-coated with 0.5 cm silica gel (60-F254, Merck) were immersed in 100 mM sodium bisulfite as an impregnant, then oven-dried at 100 °C for 30 min. Following cooling of the plates, samples were co-chromatographed with 10 mM glucose and sucrose standards in 70% ethanol (spiked with the appropriate [14C]-labelled sugar) for 110 min in an ascending solvent of ethyl acetate: acetic acid: methanol: H2O at a ratio of 12:3:3:2 (Ghebregzabher et al., 1976; Fried and Sherma, 1999). Sugars were visualised by spraying plates with 1-naphthol-sulfuric acid and heating to 100 °C for 5 min to develop colour (Fried and Sherma, 1999). The plate was divided horizontally into 1.2 cm bands and the silica gel scraped off the plate and transferred to a scintillation vial. 0.5 mL of H2O and 4.5 mL of Ecolite™ (+) liquid scintillation cocktail was added prior to radioassay.
2.3 Results

2.3.1 Time Course

Time course studies of $[^{14}\text{C}]-\text{sucrose}$ uptake by SUT/SUF-transformed SUSY7/ura3 were undertaken to observe transport of sucrose across the plasma membrane and into yeast cells. For these studies, a 0.2 mM sucrose solution was selected, as this is below the apparent $K_m$ values previously reported for SUTs in the literature (0.5 to 2 mM for SUT1 clade; for a review see Lalonde et al., 2004). A sub-$K_m$ concentration of sucrose prevents saturation of transporters and excessive rates of non-facilitated diffusion across the plasma membrane, making transporter-mediated uptake a major route of sucrose entry into the yeast cells. The sucrose uptake solution was buffered at pH 5.5 by a zwitterionic buffer (25 mM MES/HEPES). This pH value was selected as a trade-off between the previously reported pH optima for characterised SUTs (see for example Weise et al., 2000; Aldape et al., 2003) and the estimated seed coat apoplasmic pH of 6.5 for French bean (Walker et al., 1995).

The time course uptakes confirmed that all five genes mediated sucrose transport, with uptake rates exceeding that of yeast transformed with an empty pDR196 vector alone (Fig. 2.2). Transport in each case was linear over the first four minutes. The initial rates of sucrose transport over this time varied between transporters (Table 2.1). In particular, PvSUT1 exhibited a high initial rate of sucrose uptake, being five to ten times greater than that of the SUFs (Fig. 2.2B; Table 2.1). PsSUT1 and PvSUT1 exhibited linear rates of sucrose uptake over the 10 min trial period. In contrast, uptake by PsSUF1, PsSUF4 and PvSUF1 began to plateau after approximately 6 min of uptake. In the case of PsSUF4, significant ($p<0.05$) sucrose efflux occurred between 6 and 10 min (Fig. 2.2A). It was determined that subsequent uptake studies would be undertaken over a four minute period, due to the linear nature of uptake, which would be indicative of influx across the yeast plasma membrane.
Figure 2.2. Time-course of $[^{14}C]$-sucrose uptake by SUT/SUF-transformed SUSY7/ura3 yeast, for SUT/SUFs cloned from A. pea and B. French bean. Uptake was from a 0.2 mM sucrose solution, buffered at pH 5.5. Values are means ± SEs of at least 3 replicates per treatment. All experiments were energised with 10 mM glucose (or 100 mM ethanol for PvSUFI) added 1 min prior to commencement of sucrose uptake.
Table 2.1. Initial rates of sucrose uptake by SUT/SUF-transformed SUSY7/ura3 yeast, for SUT/SUFs cloned from pea and French bean. Rates were calculated using the first 4 min of uptake from a 0.2 mM sucrose solution, pH 5.5. Values are means ± SEs of at least 3 replicates per treatment.

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<th>Pea</th>
<th>French Bean</th>
<th>Vector</th>
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<td></td>
<td>PsSUT1</td>
<td>PsSUF1</td>
<td>PsSUF4</td>
</tr>
<tr>
<td>Initial Uptake Rate (nmol sucrose 10^-8 cells min^-1)</td>
<td>0.21 ± 0.01</td>
<td>0.08 ± 0.004</td>
<td>0.12 ± 0.10</td>
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2.3.2 pH Dependence of Sucrose Transport

The sucrose uptake activity of the transporters was measured over a pH range of 4.0 to 8.0. Sucrose uptake activity for PsSUT1 was shown to be pH-dependent, with an optimal pH of 4.0. Activity then steadily declined until pH 6.0, where uptake was only 30% of values at pH 4.0. This suggests that an inward-directed proton gradient may be required to drive sucrose transport for PsSUT1. In contrast, PsSUF1 and PsSUF4 were shown to be pH independent, with no change in uptake over the pH range of 4.0 to 8.0 (Fig. 2.3A).

PvSUT1 showed an optimal pH of 5.0 for sucrose uptake, which steadily decreased until pH 8.0, while PvSUF1 uptake was pH-independent. At pH 5.0, uptake by PvSUT1 was 5 times that of PvSUF1 and 2 times that of PsSUT1; at pH 8.0 they exhibit similar uptake rates (Fig. 2.3A, B).
Figure 2.3. pH-dependent uptake of \([^{14}C]\)sucrose by SUT/SUF-transformed SUSY7/ura3 yeast, for SUT/SUFs cloned from A. pea and B. French bean. Uptake was from a 1 mM sucrose solution, buffered at the appropriate pH. Values are means ± SEs of at least 6 replicates per treatment. All experiments were energised with 10 mM glucose added 1 min prior to commencement, with the exception of PvSUF1, which was energised with 100 mM ethanol.
2.3.3 Concentration-Dependent Sucrose Transport

Uptake studies performed over a range of sucrose concentrations revealed that all sucrose transporters, except for PvSUF1, showed saturation kinetics (Fig. 2.4). PvSUF1 had low levels of uptake, and is almost linear with increasing sucrose concentrations (Fig. 2.4B). Interestingly, PsSUF1 and PsSUF4 show similar curves initially, however PsSUF1 saturates much earlier (Fig. 2.4A). Eadie-Hofstee transformations were carried out and estimates of the apparent $K_m$ and $V_{max}$ values for each transporter for sucrose were made (Table 2.2). The apparent $K_m$ estimate of 1.5 ± 0.2 mM for PsSUT1 is similar to those previously reported for SUT1s (Lalonde et al., 2004). For PsSUF1, PvSUF1 and PvSUT1, apparent $K_m$ values ranged from 8.5 to 99.8 mM sucrose, which are 5- to 20-fold higher than typical SUT1 values for sucrose. Similarly, the apparent $K_m$ value of PsSUF4 is far in excess of previously reported $K_m$s for Clade II (SUT4) transporters. The calculated $V_{max}$ values vary greatly between transporters. As $V_{max}$ is a function of the number of transport proteins present, it is likely to be very sensitive to variations in heterologous expression of the different transporters in yeast, and so may be of little relevance to the study of transporter properties.
A. Uptake (nmol 10⁻⁸ cells min⁻¹) vs. Sucrose (mM)

B. Uptake (nmol 10⁻⁸ cells min⁻¹) vs. Sucrose (mM)

C. Uptake (nmol 10⁻⁸ cells min⁻¹) vs. Sucrose (mM)
Table 2.2. Estimates of apparent $K_m$ and $V_{max}$ values for sucrose uptake by SUTs/SUFs cloned from pea and French bean.

<table>
<thead>
<tr>
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<th>Pea</th>
<th>French bean</th>
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<tr>
<td></td>
<td>PsSUT1</td>
<td>PsSUF1</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>1.5 ± 0.2</td>
<td>99.8 ± 10.0</td>
</tr>
<tr>
<td>$V_{max}$ (nmol 10^8 cells min^{-1})</td>
<td>4.8 ± 0.4</td>
<td>79.2 ± 3.7</td>
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2.3.4 Counter-Transport of Sucrose

To determine whether sucrose uptake by SUFs was via channels or carriers, counter-transport studies were undertaken. A defining feature described for carriers (and not channels) is the phenomenon known as counter-transport. This is the ability of a transporter to transiently accumulate a substrate against a concentration gradient, with efflux temporarily driving uptake (Stein, 1986). To test if SUFs enable counter transport, yeast cells were pre-loaded with a high concentration of unlabelled sucrose, and then transferred to a lower concentration of $^{14}$C-sucrose. The movement of radiolabel can then be monitored to assess whether counter transport is occurring. After an initial burst in $^{14}$C-sucrose accumulation during the first 2 min, PsSUF1, PsSUF4 and PvSUF1 did not continue to uptake $^{14}$C-sucrose (Fig. 2.5). This initial burst in $^{14}$C-sucrose accumulation was driven by the efflux of unlabelled sucrose, and continues until the trans-membrane sucrose concentration gradient is abolished. At this point, net movement of sucrose ceases, indicating that the SUFs are functioning as facilitators. In contrast, PsSUT1 and PvSUT1 exhibited a linear uptake of $^{14}$C-sucrose over the 10 min. Taken together with the time-course data (dashed lines, Fig. 2.5), this suggests the ability to of the SUTs to uptake sucrose independent of the trans-membrane concentration gradient.
Figure 2.5. Counter-transport study showing time course of $^{14}$[C]-sucrose uptake by SUT/SUF-transformed SUSY7/ura3 yeast preloaded with unlabelled sucrose, for SUT/SUFs cloned from A. pea and B. French bean. Yeast cells were preloaded with unlabelled sucrose by incubation in a 50 mM solution, pH 5.5, for 30 min prior to counter-transport experiment using a 0.2 mM sucrose solution, pH 5.5. Solid lines - time course of preloaded cells; dashed lines - time course of unloaded cells.

### 2.3.5 Energisation of Transport

For an energy-coupled transporter, accumulation of a solute independent of a concentration gradient should be possible, given that an energetically favourable
condition exists. For sucrose uptake in symport with protons, this would involve an inward-directed pmf, driven by a lower external pH relative to the intracellular conditions, and a negative membrane potential. To test whether such a symport mechanism was in place for the SUTs and SUFs investigated, measurements of sucrose uptake from a low (0.2 mM) sucrose, acidic (pH 5.5) solution were made after 10 min (or 6 min for PsSUF4) (Fig. 2.2). A low sucrose concentration was selected to ensure that transporters were not saturated, and to emphasise accumulation of sucrose by energy-dependent mechanisms by minimising non-facilitated passive diffusion (across the plasma membrane) by having a minimal inward-directed sucrose gradient. These data were then used to compute internal sucrose concentrations (Cᵢ) after this incubation, assuming that 10⁸ yeast cells have a cellular volume of 5 µL (Cirillo, 1989). The ratio of intracellular to extracellular sucrose (i.e. Cᵢ:Cₒ) was then calculated to determine whether accumulation against a concentration gradient had occurred. Both PsSUT1 and PvSUT1 had Cᵢ:Cₒ ratios over one, indicating active accumulation. In contrast, PsSUF1, PsSUF4 and PvSUF1 had values at or near one, which indicated equilibrium with the outside concentration and so a passive movement of sucrose according to the sucrose concentration gradient alone across the plasma membrane (Table 2.3).

Table 2.3. Estimated Cᵢ:Cₒ ratio for SUT/SUF-transformed SUSY7/ura3 yeast. Cᵢ values were calculated after 10 min of sucrose uptake (6 min for PsSUF4) from a 0.2 mM sucrose solution buffered at pH 5.5.

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<tr>
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<th>Pea</th>
<th>French bean</th>
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<tbody>
<tr>
<td></td>
<td>PsSUT1</td>
<td>PsSUF1</td>
</tr>
<tr>
<td>Estimated Cᵢ:Cₒ Ratio</td>
<td>1.81</td>
<td>1.03</td>
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To further confirm that sucrose transport is energy-coupled, and investigate the underlying mechanisms, the effects of the metabolic inhibitors antimycin A and CCCP on uptake rates were investigated. Antimycin A is an inhibitor of mitochondrial electron transport, while CCCP acts as a protonophore, collapsing the pH gradient across the plasma membrane. PsSUT1 and PvSUT1 were sensitive to both antimycin A and CCCP, with significantly reduced uptake rates in the presence of either inhibitor. This indicates that sucrose transport by these proteins is energy-coupled, and dependent on
the generation of a trans-membrane proton gradient. In contrast, sucrose uptake by PsSUF1, PsSUF4 and PvSUF1 was not affected by antimycin A or CCCP (Fig. 2.6), and so transport of sucrose was mediated in an energy-independent manner.

Figure 2.6. Inhibition of 14[C]-sucrose transport in the presence of 10 µM Antimycin A and 50 µM CCCP by SUT/SUF-transformed SUSY7/ura3 yeast. Uptake was from a 1 mM sucrose solution, buffered at pH 4.5. Values are means ± SEs of at least 6 replicates per treatment. All experiments were energised with 10 mM glucose added 1 min prior to commencement, with the exception of PvSUF1, which was energised with 100 mM ethanol. Control transport rates (nmol 10⁻⁸ cells min⁻¹) were: PsSUT1- 1.046 ± 0.060; PsSUF1- 0.882 ± 0.054, PsSUF4- 0.938 ± 0.029; PvSUT1- 3.212 ± 0.320; PvSUF1- 1.038 ± 0.069. *- significance at P < 0.05.

The histidine-residue modifier diethyl pyrocarbonate (DEPC) was used to probe the outer face of the sucrose transporters, to see whether the exposure of histidine residues varied between transporters. Sequence analysis has shown that all sucrose transporters (including the novel transporters reported here) possess a conserved histidine residue (Lu and Bush, 1998). DEPC treatment significantly inhibited sucrose transport by both PsSUT1 and PvSUT1, while PsSUF1, PsSUF4 and PvSUF1 were not affected (Fig. 2.7).
Figure 2.7. Percent changes in sucrose uptake rates in the presence of 1.5 mM DEPC. Uptake was from a 1 mM sucrose solution, buffered at pH 4.5. Values are means ± SEs of at least 6 replicates per treatment. All experiments were energised with 10 mM glucose added 1 min prior to commencement, with the exception of PvSUF1, which was energised with 100 mM ethanol. *- significance at P < 0.05.

2.3.6 Transporter Specificity

To test the specificity of SUTs/SUFs for sucrose, competition studies were carried out using various sugars that are either known to be transported, or are structurally similar to sucrose. The hexoses glucose and fructose were included as they are the monosaccharide units of sucrose, and contribute to seed nutrition in the early stages of seed development (for a review, see Weber et al., 1997b). Maltose, a glucose dimer, has been previously shown to compete with sucrose for SUTs. Raffinose sugars, which are sucrose-derived oligosaccharides, are of transport significance for several plant families and are known to accumulate in seeds, particularly those of grain legumes (Kuo et al., 1988; Turgeon, 1996). The trisaccharide raffinose was included in the specificity studies, to investigate whether the transporters are able to bind this sugar. In addition, the polyol mannitol was included, as it (along with sorbitol and dulcitol) is also transported by several different plant families (Turgeon, 1996).

A ten-fold excess of the various competing sugar species was added to uptake assays, to see whether any competed with sucrose for binding sites of the sucrose transporters.
Maltose, which consists of two glucose units, significantly reduced sucrose uptake by PsSUT1 and PvSUT1, while glucose and fructose stimulated their sucrose transport rates (Fig. 2.8). This latter result is probably due to an energisation effect on the yeasts, which increases ATPase activity and so proton-coupled sucrose transport. In contrast, glucose, fructose and palatinose (a sucrose analog) significantly reduced sucrose transport by PvSUF1. Raffinose, maltose and mannitol did not compete with sucrose for any of the facilitators.

![Figure 2.8. Percent changes in sucrose uptake rates in the presence of 10 mM competing sugars.](image)

**Figure 2.8. Percent changes in sucrose uptake rates in the presence of 10 mM competing sugars.** Uptakes measured were from a 1 mM $^{[14]C}$-sucrose solution, pH 4.5. Values are means ± SEs of at least 6 replicates per treatment. All experiments were energised with 100 mM ethanol added 1 min prior to commencement of sucrose uptake. *- significance at P < 0.05.

### 2.3.7 Sucrose Efflux

The yeast strain SUSY7/ura3 is engineered to express the potato sucrose synthase gene (Riesmeier *et al.*, 1992), and so is able to metabolise sucrose. To confirm that sucrose loaded into SUT/SUF-transformed SUSY7/ura3 yeast remained intact after loading for 10 min (and so is available for efflux), thin-layer chromatography was carried out on SUT/SUF-transformed yeast preloaded with sucrose. Analysis was carried out on sucrose-loaded yeast prior to, and after, a 10 min post-loading incubation, to estimate the extent of sucrose metabolism during this time. TLC revealed that after a
10-min incubation, $^{14}$[C]-sucrose accounted for 98% of $^{14}$[C]-label present in the yeast cell extract, and for 99% of the $^{14}$[C]-sucrose originally loaded into cells (data not shown). Thus, any significant loss of $^{14}$[C]-label from $^{14}$[C]-sucrose-loaded yeast cells during a 10-min wash would be attributable to sucrose efflux only.

PsSUF1, PsSUF4 and PvSUF1 effluxed significant amounts of sucrose, with 50 to 60% of preloaded sucrose effluxed in 10 min (Fig. 2.9). In contrast, PsSUT1 and PvSUT1 efflux over the same period was minimal, and comparable to the control (untransformed) efflux.

![Figure 2.9](image-url)

**Figure 2.9.** Percentage of pre-loaded $^{14}$[C]-sucrose effluxed during a 10-min incubation of SUT/SUF-transformed SUSY7/ura3 yeast preloaded with sucrose. Yeast cells were preloaded with $^{14}$[C]-sucrose from a 0.2 mM solution, pH 4.5 for 10 min, and then washed for 10 min in sucrose-free buffer at pH 4.5. Data presented are means ± SEs for at least 6 replicates per treatment. *- significance at P < 0.05.
2.4 Discussion

We have demonstrated that all five SUT/SUFs, when heterologously expressed in SUSY7 yeast, mediate sucrose uptake (Fig. 2.2). It appears that these transporters fall into two distinct groups, based on energisation and specificity characteristics, which will be examined in turn.

2.4.1 Sucrose/H+ Symporters

PsSUT1 and PvSUT1 both appear to be sucrose/H+ symporters similar to those previously reported in the literature (Riesmeier et al., 1992; Weise et al., 2000). Identifying one or more of our cloned transporters as symporters was a likely scenario, even from ‘effluxer’ tissue such as developing seed coats, as the homology-based cloning approach used known symporter (SUT) sequences. The ability of PsSUT1 and PvSUT1 to accumulate intracellular sucrose above the outside concentration within 10 min – with an 8-fold accumulation in the case of PvSUT1 (Table 2.3) – indicates the expenditure of energy for uptake. Diminishing cellular ATP levels (by addition of the mitochondrial inhibitor antimycin A) reduced the capacity for sucrose uptake (Fig. 2.6), a phenomenon also observed for other SUTs heterologously expressed in yeast (e.g. SoSUT1 - Riesmeier et al., 1992; AtSUT2 - Schulze et al., 2000; AtSUT4 - Weise et al., 2000). The energisation of transporters can be primary active (directly reliant on ATP action such as with ABC transporters- Rea, 2007) or secondary (via coupling with an energy-dependent gradient of ions, such as protons- see Bush, 1993). Additional evidence for energy-coupling of sucrose transport was provided by sucrose pre-loading studies, where SUT-transformed yeast did not efflux sucrose when faced with an outward-directed sucrose gradient (Fig. 2.9). Also, SUTs did not show counter-transport (Fig. 2.5), as the experimental conditions of a large inward-directed pmf relative to the outward-directed sucrose gradient may have simply acted to strongly favour influx over efflux.

To identify the mode of energy coupling, further inhibitor studies were carried out using the protonophore CCCP and, as previously reported, SUTs were shown to be CCCP sensitive and so proton-coupled (see for example Riesmeier et al., 1992). Evidence for CCCP action exists for yeast using the dye diS-C3-3 (3,3'-dipropylthiacyarbocyanine iodide). DiS-C3-3 is a fluorescent dye that moves across membranes according to membrane potential, with a colour shift occurring when dye
molecules enter the cytoplasm (de la Peña et al., 1982; Plasek et al., 1994; Kinclova-Zimmermannova et al., 2006). Addition of CCCP (at a concentration of 5 µM) shows the dissipation of membrane potential, which occurs by abolishing ∆pH in yeast cells via proton movement (de la Peña et al., 1982; Gaskova et al., 1998). As sucrose uptake by PvSUT1 and PsSUT1 are appreciably inhibited by CCCP (by 60% and 30% respectively- Fig. 2.6) it can be concluded that the transport of sucrose by these proteins relies on proton coupling. In addition, it appears that the inhibition of sucrose transport by antimycin A was effected through the H⁺-ATPase, with diminished ATP availability impairing proton pumping.

Furthermore, several additional lines of evidence for sucrose/H⁺ symport exist. Firstly, sucrose transport rates increased in the presence of high proton concentrations (lower pH conditions). This has been reported extensively for other SUTs in both yeast (e.g. Riesmeier et al., 1992; Sauer and Stolz, 1994; Weise et al., 2000) and Xenopus (Boorer et al., 1996; Zhou et al., 1997; Zhou and Miller, 2000; Sivitz et al., 2005) heterologous expression systems. As putative sucrose/H⁺ symporters, SUTs rely on an inward-directed pmf for the co-transport of sucrose into the cell. The two components of the pmf, being pH gradient (∆pH) and membrane potential (∆ψ), determine the magnitude and direction of the pmf, which can be calculated at standard temperature by:

\[ \text{pmf (mV)} = \Delta \psi - 59 \Delta \text{pH} \]  

(Equation 2.1)

An inward-directed pmf is required to enable co-transport of sucrose and protons into the yeast cell. The time-course uptake data for PsSUT1 and PvSUT1 (Fig. 2.2) and estimates of membrane potentials of yeast cells (Table 2.4) can be used to show that the pmf present in these conditions can fully account for the sucrose uptake observed. The membrane potential of yeast cells in an external pH of 4.5 has been measured at -45 mV, and the trans-membrane ΔpH at a bathing media pH of 5.5 is 1.0 unit (de la Peña et al., 1982; Table 2.4), giving a pmf of -110mV. The Nernst equation at standard temperature (Equation 2.2) can then be used to estimate the intracellular sucrose concentration (Cᵢ) that can be achieved at electrochemical equilibrium under our experimental conditions, assuming a sucrose:proton stoichiometry of 1:1 as previously reported for SUTs (Boorer et al., 1996).

\[ \log_{10} C_i = \log_{10} C_o - \frac{[\Delta \psi - 59 \Delta \text{pH}]}{59} \]  

(Equation 2.2)
So, for an external sucrose concentration of 0.2 mM:

\[
\log_{10} C_i = -0.70 - \frac{[-45 - 59]}{59} = -0.70 + 1.76 = 1.06 \therefore C_i = 11 \text{ mM}
\]

So, under our experimental conditions, when the internal sucrose concentration approaches 11 mM, electrochemical equilibrium is reached, thus the inward-directed pmf is balanced by the outward-directed sucrose gradient. Note that after 10 min equilibration, intracellular concentrations of 0.36 and 1.66 mM were observed for PsSUT1 and PvSUT1 yeast cells respectively (Table 2.3). Thus, the pmf generated is an order of magnitude above that required to account for the observed sucrose uptake.

### Table 2.4. Published values for membrane potential and ∆pH across the plasma membrane for yeast cells at varying extracellular pH (pH<sub>o</sub>.

<table>
<thead>
<tr>
<th>pH&lt;sub&gt;o&lt;/sub&gt;</th>
<th>Membrane potential (mV)</th>
<th>∆pH (units)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>-45</td>
<td>1.8</td>
<td>de la Peña et al., 1982</td>
</tr>
<tr>
<td>5.5</td>
<td>n/d</td>
<td>1.0</td>
<td>“</td>
</tr>
<tr>
<td>7.0</td>
<td>-90</td>
<td>0.1</td>
<td>“</td>
</tr>
</tbody>
</table>

The assertion that PsSUT1 is a sucrose/H<sup>+</sup> symporter concurs with the co-localisation of PsSUT1 with the H<sup>+</sup>-ATPase in developing cotyledons, particularly in epidermal transfer cells (Tegeder et al., 1999). Additionally, the function of this transporter is highly reliant on ATPase activity in whole tissues. For instance, when cotyledons were treated with the ATPase inhibitor EB, sucrose uptake was reduced by 75% (Tegeder et al., 1999).

The pH dependence pattern reported for the majority of SUTs characterised to date shows a peak of transport activity at pH 4 to 5, followed by a rapid decline over increasing pH. This reflects the decrease in pmf as pH increases, and is similar to the pH dependence pattern seen for PsSUT1 (Fig. 2.3). There are some exceptions to this trend, including AtSUC9 (Sivitz et al., 2007) and AtSUC1, which are both insensitive to the diminishing pmf between pH 5.0 and 6.5 (Sauer and Stolz, 1994).

Despite the central role of pmf in SUT function, the relationship between pH and sucrose uptake is not necessarily proportional. In addition to its contribution to pmf generation, pH may exert additional influences on the transport rate by affecting the
surface geometry and possibly oligomerisation of the transporter protein. For example, in an overly acidic environment, pH may adversely affect the active site of a SUT, (which could inhibit binding of substrate, or the transport of bound substrate) resulting in reduced transport despite the strong inward-directed pmf. Lu and Bush (1998) carried out site-directed mutagenesis studies on the conserved histidine residue at the sucrose binding site of AtSUC1, and showed that replacing this with a lysine or arginine residue increased both pH sensitivity and sucrose transport rates at low pH conditions. This demonstrated that the pH-dependence of sucrose transporters is a function of the allosteric properties of the transporter face in addition to the pmf.

The different pH dependency profiles of PsSUT1 and PvSUT1 show that pH may affect these transporters differently. For PsSUT1, activity generally decreases with a decrease in proton gradient. In contrast, PvSUT1 activity increases significantly from pH 4 to 5, (despite the decrease in proton gradient), suggesting that some conformational change may occur on the extracellular face of the transporter over this range (Fig. 2.3). At pH 6, PvSUT1 is still at near-maximal rates of transport, whereas PsSUT1 has the same lower rates as those seen up to pH 8. This again suggests an allosteric effect of pH on PsSUT1 in this range. Interestingly, the rate of uptake is lower than that seen for the energy-independent SUFs, which reinforces this notion. PvSUT1 does not appear to have this allosteric pH sensitivity, with uptake rates decreasing linearly from pH 6 to 8 along with pmf. At pH 8.0, PvSUT1 still has an uptake rate above that of the SUFs. If a membrane potential of -90 mV is assumed for an external pH of over 7.0 (Table 2.4), Equation 2-1 predicts that an outward-directed pH gradient of 1.5 units (and so an extracellular pH of at least 8.6) would be required to abolish the pmf (and so sucrose transport). So, despite the proton gradient reversal at pH 8, pmf may still contribute to uptake as the intracellular environment has an overall negative charge relative to the outside. It is a possibility that above pH 8.5 (where protons cease to aid sucrose uptake into the cell) the SUTs may become uncoupled to proton transport and operate as facilitators, relying solely on trans-membrane sucrose concentration gradient. The data suggest that this scenario is a possibility for PsSUT1 above pH 6.0. Patch-clamping studies of this protein in Xenopus oocytes over this range could be used to confirm or reject this possibility.

The apparent $K_m$ value for PsSUT1 of $1.5 \pm 0.2$ mM (Table 2.2) is similar to others reported at pH 4.5, including the closely-related GmSUT1 from soybean, which has a $K_m$ of $1.4$ mM (Aldape et al., 2003). The apparent $K_m$ value for PvSUT1 ($8.5 \pm 0.7$...
mM) is somewhat higher than the range usually reported for members of Clade I (being 0.2 to 2 mM; Lalonde et al., 2004). However, it should be noted that these uptakes were performed at pH 4.5 rather than the optima for this transporter of pH 5.5. Furthermore, VfSUT1 from broad bean has a reported apparent $K_m$ value of 5.6 mM at pH 4.0 (Weber et al., 1997a).

The hexoses glucose and fructose stimulated sucrose transport by PsSUT1 and PvSUT1 (Fig. 2.8), most likely by energising the yeast system and so increasing the trans-membrane $\Delta$pH generated by the $\text{H}^+$-ATPase. Similar effects of hexoses on SUT transport activities have been reported by others (for example Riesmeier et al., 1992; Gahrtz et al., 1994). It is a possibility that these hexoses also competitively bind with sucrose, with the effect being masked by the stimulating effects of the hexoses. Indeed, it has been demonstrated that a glucose moiety is involved in substrate recognition and transport by SUTs (Hitz et al., 1986). Whether or not SUTs are able to bind glucose, electrophysiological investigation of the Clade I SUTs AtSUC2 and AtSUC9 in *Xenopus* oocytes shows that glucose is not transported by these proteins (Chandran et al., 2003).

Physiological investigations of sucrose transport in developing legume seed coats support the finding of sucrose/$\text{H}^+$ symporters from these tissues. For developing French bean seed coats, an energy-dependent, pCMBS-sensitive sucrose uptake mechanism was described for sucrose concentrations of less than 10 mM (Ritchie et al., 2003). These characteristics can be accounted for by PvSUT1, which is both energy-dependent and DEPC-sensitive. Similarly for pea, a symport component of sucrose transport has been identified in pea seed coat plasma membrane vesicles (de Jong and Borstlap, 2000), which may be mediated by PsSUT1.

Several possible explanations have been suggested to explain the presence of SUTs, which mediate sucrose uptake, in seed coat tissues that are carrying out an efflux role. One proposal is that although SUTs are expressed in the cells, the protein is sequestered in membrane vesicles rather than functionally incorporated into the plasma membrane (de Jong and Borstlap, 2000). Another is that the SUTs act in reversal mode to efflux sucrose. For instance, ZmSUT1 was shown to be reversible under conditions where the driving force from the outward-directed sucrose concentration exceeded the inward-directed $\text{pmf}$ (Carpaneto et al., 2005; for further discussion, refer to Section 1.5.3.2). It has also been proposed that these SUTs may function to retrieve sucrose leaked to the apoplasm along the delivery pathway, to ensure delivery to sites of efflux (Patrick,
1997). Immunolocalisation of SUTs in seed coats should provide additional insight into their physiological function in these tissues; a question explored in Chapter 3.

### 2.4.2 Novel Sucrose Facilitators

SUFs exhibited initial rates of sucrose transport (Table 2.1) similar to those previously reported for SUT1 and SUT4 proteins (Table 2.5). However, beyond these initial similarities, SUFs exhibited markedly different transport properties from SUTs, with SUFs showing characteristics of facilitated diffusion rather than the usual sucrose/proton symport typical of SUTs. There are multiple lines of evidence in this study indicating that these novel transporters function as energy-independent facilitators rather than sucrose/H⁺ symporters.

**Table 2.5. Sucrose transport rates reported for a 0.2 mM sucrose solution buffered at the specified pH for a) SUT1 and b) SUT4 transporters heterologously expressed in SUSY7/ura3 yeast.**

<table>
<thead>
<tr>
<th>SUT</th>
<th>Initial Transport Rates (nmol sucrose/10⁸ cells/min)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Clade I (SUT1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SoSUT1</td>
<td>0.1</td>
<td>5.5</td>
<td>Fig. 5 Riesmeier <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>DcSUT2</td>
<td>0.33</td>
<td>5.5</td>
<td>Fig. 4 Shakya and Sturm, 1998</td>
</tr>
<tr>
<td>VvSUT1</td>
<td>0.7 (0.25mM sucrose)</td>
<td>5.0</td>
<td>Fig 3. Ageorges <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>b) Clade II (SUT4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DcSUT1</td>
<td>0.66</td>
<td>5.5</td>
<td>Fig 4. Shakya and Sturm, 1998</td>
</tr>
</tbody>
</table>

Firstly, sucrose transport by SUFs is not affected by inhibition of cellular ATP production, as indicated by antimycin A insensitivity (Fig. 2.6). In addition, SUF activity is not affected by the protonophore CCCP, which confirms transport independent of a proton-coupling mechanism. This concurs with the observation that these transporters are able to mediate uptake in the absence of an inward-directed proton gradient (Fig. 2.3). Uptake rates are similar across the pH profile, from pH 4, where the inward directed pmf would be greatest (due to an excess of extracellular protons), through to pH 8 where pmf is greatly diminished (Fig. 2.3). It does not appear that the pH-dependence of the non-saturable sucrose uptake mechanism has been previously studied. However, as a proton gradient is not utilised by this mechanism, it is reasonable
to assume that the scope of pH influence on sucrose uptake should be limited to allosteric effects. The pH-independence however suggests that any allosteric effects over a pH range of 4 to 8 on these SUFs are minimal.

The second line of evidence that exists for indicating facilitated diffusion by SUFs is that the direction of sucrose transport across the plasma membrane is governed by the direction of the trans-membrane sucrose concentration gradient. Evidence for this is provided by the inward-directed sucrose movement observed initially in the time-course studies (Fig. 2.2). The rates of uptake then begin to plateau within the 10-min period, as the trans-membrane concentration difference approaches zero. This is reflected in the calculated C_i:C_o values (Table 2.3) which show an equilibration of sucrose between the cytoplasmic and intracellular compartments. Similarly, the observed significant sucrose efflux from SUF-expressing cells preloaded with sucrose (Fig. 2.7) reflects the outward direction of the trans-membrane sucrose concentration gradient. This energy-independent nature of sucrose transport, mediated by the facilitators PsSUF1, PsSUF4 and PvSUF1, is a novel finding, being the first report of the identity of a sucrose facilitator within the SUT family.

The phenomenon of counter-transport was observed in our study for SUFs (Fig. 2.5), confirming that they act as carriers rather than channels (Stein, 1986). This shows that these transporters are not the non-selective pores described in previous studies. For instance in seed coats of pea, it has been proposed that movement of a range of solutes, including sucrose, glucose and amino acids occurs through non-selective pores (de Jong et al., 1996; van Dongen et al., 2001). The substrate specificity of PsSUF1 and PsSUF4, together with their carrier rather than channel mode of translocation, excludes them from accounting for this role. Rather, our study shows the existence of additional and sucrose-specific carriers in developing pea seed coats, possibly the pCMBS and EB-insensitive transporters observed in broad bean seed coats by Fieuw and Patrick (1993).

Another distinctive property of SUFs is their unusually high apparent $K_m$ values for sucrose transport (Table 2.2) in comparison to SUTs, with apparent $K_m$ values ranging from 30-100 mM. However, these are reflected in apparent $K_m$ values estimated for native membranes of pea (e.g. de Jong et al., 1996) and French bean (Ritchie et al., 2003).

Of the SUFs tested, only PvSUF1 appears able to bind hexoses (Fig. 2.8). This pattern of PvSUF1 specificity for hexoses as well as sucrose is in good agreement with data previously obtained from native membranes of French bean seed coats. Here
Ritchie et al. (2003) reported that a ten-fold excess of hexoses inhibited sucrose uptake by seed coat protoplasts by at least 60%. In addition, PvSUF1 may account for the energy-independent component of sucrose efflux—some 50% of total sucrose efflux—observed for French bean seed coats (Walker et al., 1995). Expression studies of PvSUF1 in seed coats throughout their development may provide some interesting insights into the physiological function of this transporter. Of particular interest would be to observe whether the expression of this transporter varies between the pre-storage and storage phases of seed development, and whether it correlates with the high activity of cell wall invertases (resulting in an apoplastic pool of hexoses) observed early in seed development (Weber et al., 1995; Déjardin et al., 1997). While this period in development would provide a strong sink for the efflux of sucrose via energy-independent carriers such as PvSUF1, the ability to bind hexoses on the extracellular face of the transporter may inhibit sucrose efflux. It is tempting to speculate that PvSUF1 may provide feedback to efflux cells through either an increase in cellular hexose levels (if hexoses are able to not only bind but also be transported) or through increasing cytoplasmic sucrose pools due to the inhibition of efflux.

The inability of any of the SUFs or SUTs to transport raffinose is perhaps not surprising. Although raffinose sugars often accumulate in legume seeds, it appears likely that de novo synthesis within the seed accounts for this rather than by import, with raffinose synthase expressed in developing legume seeds (Castillo et al., 1990). Similarly, no binding with mannitol was detected for any transporter. In recent years, a family of polyol transporters with low homology to SUTs have been cloned (e.g. see Noiraud et al., 2001; Gao et al., 2003; Klepek et al., 2005). Interestingly, these polyol transporters are generally able to bind glucose and fructose (which are the monosaccharides moieties of sucrose), but not sucrose.

When tested for other SUTs (for example SoSUT1- Riesmeier et al., 1992 and StSUT1- Riesmeier et al., 1993), the sucrose analogue palatinose did not compete with sucrose (Fig. 2.8). However, in this study, PvSUF1 was able to bind palatinose, although the other two SUFs were not affected. This, along with the binding of hexoses to PvSUF1, suggests that the extracellular face of this transporter differs from the other two SUFs, which are similar to one another in terms of sugar binding. AtTMT1/2, a pair of vacuolar sugar transporters recently described by Schulz et al. (2011), are able to transport both glucose and sucrose in antiport with protons. Thus, the ability to bind and transport disaccharides does not appear to preclude the transport of monosaccharides.
2.4.3 Phylogenetic and Structural Comparisons

It does not appear that the phylogenetic relationships of the SUTs and SUFs in this study (Fig. 2.1) are an absolute reflection of transporter characteristics. PsSUF1, PvSUF1 and PvSUT1 create a sub-clade within Clade I, despite having apparent $K_m$ values ranging from 8.5 to 99.8 mM and both energy-dependent and –independent transport mechanisms. Instead, the dispersal of energy-independent SUFs through more than one clade suggests that the gain or loss of a proton-coupling mechanism may have occurred at multiple points during sucrose transporter evolution.

Topology modelling of SUFs (Zhou et al., 2007) shows that these transporters have the typical structure of SUT family proteins: 12 trans-membrane domains, with a highly conserved histidine residue (His-65) present in the extracellular Loop I/II (Lu and Bush, 1998; Fig. 2.10). The amino acid sequences of the three novel SUFs show that this is also conserved for these proteins (Zhou et al., 2007). Using DEPC-inhibition studies, Lu and Bush (1998) previously concluded that His-65 (with which DEPC interacts) is located within the sucrose-binding site of the SUT protein extracellular face. However, in our study DEPC failed to inhibit sucrose transport by SUFs, despite the presence of His-65 (Fig. 2.10). In contrast, DEPC affected sucrose transport by PsSUT1 and PvSUT1. It certainly appears that the extracellular face of the transporter differs between the SUT and SUF groups tested. Less certain now however is the role of His-65 in sucrose binding to the active site, particularly for SUFs. One possibility is that the sucrose binding site is located elsewhere for SUFs, due to either a conformational change due to amino acid variations between the SUT and SUF groups (particularly in the extracellular loops) or the availability of other sites due to the lack of proton binding. A major difference in the extracellular face between the two groups is that a proton binding site may not be required for SUFs, as indicated by transport independent of pmf.

It has previously been asserted that Clade I (SUT 1) members able to transport a range of natural α- and β-glucosides, have a lesser specificity for sucrose than other clades (Chandran et al., 2003; Sivitz et al., 2007). Here, we have shown that the ability of SUFs to bind competing sugars differs from that seen for the SUTs investigated in this study, with SUFs unable to bind maltose. In contrast, the specificity profile of PsSUT1 and PvSUT1 is more akin to that usually seen for SUTs.
As energy-independent sucrose transporters, SUFs share functional similarities to other transporters outside of the SUT family of proteins. The sucrose binding protein (SBP- Ripp et al., 1988) GmSBP from soybean was functionally characterised in yeast as a sucrose facilitator that was non-saturable at sucrose concentrations (up to 30 mM) tested (Overvoorde et al., 1996). Thus, it appears that SBP has a high $K_m$ for sucrose, similar to the SUFs described here. Additionally, GmSBP activity was pH-independent and relatively insensitive to protonophores and sulfhydryl modifying agents.

A recently described family of sucrose transport proteins- termed SWEETs (Chen et al., 2010; 2012) also appear functionally similar to SUF proteins. The apparent $K_m$ value for sucrose uptake for AtSWEET12 is about 70 mM (again similar to apparent $K_m$ values for SUFs), and SWEETs are also pH-insensitive. In terms of sugar species specificity SWEETs and SUFs are similar, in that they do not bind maltose. In this respect the binding site of SUFs may be more similar to SWEETs than SUTs, which are able to both bind and transport maltose (Fig. 2.8; Chandran et al., 2003; Sivitz et al., 2007).

![Figure 2.10. Generalised structure of SUT proteins, showing 12 trans-membrane domains. Adapted from Lemoine, 2000.](image)

**Conclusion**

The functional characterisation of sucrose transporters cloned from efflux-committed tissues of developing seed coats has enabled the identification of novel, energy-independent facilitators that are capable of sucrose efflux. Characterisation by
heterologous expression in yeast plasma membranes has enabled a greater understanding of the extracellular face of these proteins. However, to investigate more fully their contribution to *in planta* sucrose efflux, a detailed investigation of their cytoplasmic face is required. Membrane vesicles in an inside-out configuration would be required to further examine the efflux transport characteristics of SUFs. A strategy to achieve this outcome is described in Chapter 4.
CHAPTER 3

Sub-cellular and Cellular Localisation of Novel Sucrose Transporters In Developing Seed Coats and Source Leaves of Pea
3.1 Introduction

The functional characterisation of novel sucrose transporter proteins was reported in Chapter 2. These were the facilitators PsSUF1 and PsSUF4 and symporter PsSUT1 from pea, and one of each transport mechanism, PvSUF1 and PvSUT1, cloned from bean seed coats. By studying protein expression on an anatomical scale, and combining this with knowledge of transport characteristics (Chapter 2), it becomes possible to make inferences about the in planta role of these transporters, particularly as the different modes of energisation for sucrose transport observed raises questions about their possible physiological function. In this context, it was decided to carry out localisation studies of these transporters both in their tissues of origin (developing seed coats) as well as in other sites in the plant heavily committed to nutrient fluxes, particularly the minor veins of source leaves. Furthermore, GFP-fusion studies have shown that sucrose transporters can localise in vivo to different cellular membranes - the plasma membrane (Sivitz et al., 2007; Chincinska et al., 2008) or tonoplast (Weschke et al., 2000; Endler et al., 2006; Reinders et al., 2008; Payyavula et al., 2011). Different sub-cellular localisation of these proteins would also reflect differences in physiological function. Thus, this chapter investigates protein localisation at the cellular and sub-cellular levels, to determine where these transporters are located within developing seed coats, being a plant organ committed to efflux, as well as the minor veins of source leaves.

The three transporters cloned from pea (PsSUT1, PsSUF1 and PsSUF4) were selected for study over those cloned from bean for two reasons. Firstly, it was reasoned that the suite of three transporters cloned from pea may represent a more complete set of transporters than the two from bean. Secondly, the vasculature of the pea seed coat is structurally very similar to that of the closely related and well-studied broad bean seed coat (Zhang et al., 2007), providing a model that is transferrable to our subject.

The sub-cellular localisation of sucrose transporters, in particular those of the SUT4 clade, varies between plant species. Members of the SUT1 clade investigated to date, as well as several SUT4 clade members (LeSUT4, StSUT4) localise to the plasma membrane of cells when GFP fusion constructs are expressed in a heterologous plant expression system (Chincinska et al., 2008). Similarly, immunogold localisation of an apple SUT4 (named MdSUT1) indicates a plasma membrane localisation (Peng et al., 2011). Other SUT4s, including HvSUT2 (Weschke et al., 2000), AtSUT4 (Endler et al., 2006),
PtaSUT4 (Payyavula et al., 2011) and the legume LjSUT4 (the closest known relative to PsSUF4 - Reinders et al., 2008) are tonoplast-localised. Although PsSUT1, PsSUF1 and PsSUT4 all mediated sucrose transport when heterologously expressed in yeast (Chapter 2), this cannot be taken as definitive evidence of plasma membrane localisation in planta, as mis-targeting may occur. For instance, although AtSUT4 was found to mediate sucrose uptake in yeast (Weise et al., 2000), it was later identified as a vacuolar transporter (Endler et al., 2006). Hence, there is the need to carry out subcellular localisation studies in a plant system.

For the facilitators PsSUF1 and PsSUF4, a plasma membrane localisation would enable these transporters to mediate sucrose movement into or out of the cell according to the prevailing sucrose concentration gradient. In contrast, the symporter PsSUT1 could act as a sucrose influxer, retrieving sucrose from the apoplasm.

In the case of tonoplast localisation, the facilitators would act as equilibrators between the two cellular compartments (cytoplasm and vacuole). Kaiser and Heber (1984) reported energy independent, carrier mediated sucrose movement across the tonoplast of barley leaf protoplasts, with a $K_m$ of 20 mM. It is conceivable that our facilitators could carry out a similar function in the source leaves of pea, enabling sucrose to be transported into the vacuole for storage during the day. Such a system could work in concert with a vacuolar acid invertase, which would cleave sucrose into hexoses, thus maintaining an inward-directed sucrose concentration gradient (Martinoia et al., 2000). For a symporter such as PsSUT1, localisation to the tonoplast would imply a role in pumping sucrose out of the vacuole and into the cytoplasm, as the prevailing pH and electrical gradients would drive movement in this direction.

The source leaf is of significance to the question of membrane transport of sucrose as it is the site of sucrose synthesis within the plant. The route of sucrose movement is from the mesophyll cells, to the collection phloem in the minor veins. Here, sucrose is loaded into sieve element-companion cell (SE-CC) complexes for export from the leaf. In sucrose translocating species such as pea, which have a paucity of plasmodesmatal connections between surrounding cells and minor vein SE-CC complexes (Wimmers and Turgeon, 1991), it is proposed that apoplastic loading of phloem occurs. Thus, two plasma membrane transport events occur in series - an efflux and subsequent uptake event. It has been shown that minor vein loading in pea leaves is pCMBS-sensitive, thus is apoplastic and probably transporter-mediated (Turgeon, 1984).
The source leaves of beet, an apoplastic loader, have been used extensively in gaining an understanding of the mechanism of collection phloem sucrose loading. In source leaf discs, an experimental system which behaves similarly to whole leaves (Giaquinta, 1977), three distinct mechanisms of phloem sucrose loading have been described: simple diffusion, a saturable H⁺-coupled high affinity (Kₘ 0.4 – 1.5 mM) mechanism and a non-saturable and probably energy-independent mechanism. The latter two mechanisms are attributable to transporter proteins, possibly members of the sucrose transporter family. The saturable mechanism is sensitive to pH changes, protonophores and sulfhydryl modifiers such as pCMBS; the non-saturable mechanism less so (Maynard and Lucas, 1982a; 1982b). These characteristics reflect those observed for the SUTs and SUFs respectively, as studied in Chapter 2.

There have been several reports that implicate sucrose transport family members in at least one of these transporter-mediated steps. For instance, Gottwald et al. (2000) reported that Arabidopsis plants homozygous for a disruptive insert in SUC2 (SUT1 clade member) had impaired sucrose export ability in source leaves, and accumulated excessive amounts of stored starch. Other studies have shown that, in source leaves of apoplastic loaders, including plantain, Arabidopsis and solanaceous plants, SUT1 clade members localise to sieve elements (Kühn et al., 1997; Lalonde et al., 2003) or companion cells (Stadler et al., 1995; Stadler and Sauer, 1996; Schmitt et al., 2008).

Less is known of the mechanisms responsible for the release of sucrose into source leaf apoplasm prior to the loading step. This is largely due to the experimental difficulty in isolating and studying the cells mediating sucrose release to the apoplasm. However, by conducting tracer studies in whole leaves, it has been determined that the mesophyll cells are probably symplasmically continuous with cells of the vascular bundle (Madore et al., 1986). Intuitively, it makes sense that the site of sucrose release would be in close proximity to the site of uptake into the SE-CC complexes, as sucrose release at the mesophyll cell membrane presents several challenges. In particular, the movement of sucrose through the mesophyll cell walls would be in the opposite direction to the transpiration stream (Giaquinta, 1983). In addition, the presence of free sucrose in the mesophyll cell walls, which are exposed to intercellular air spaces, would provide an easy means for infection by airborne fungal and bacterial pathogens (Geiger, 1975). Instead, limited experimental evidence suggests that the site of sucrose efflux in apoplastic loaders is within the vascular bundle (Madore et al., 1986), although it has not been established conclusively which cell(s) are responsible for release. The sucrose
concentration in the mesophyll in broad bean is high relative to the surrounding apoplasm (20 - 200 mM vs 1.5 mM; Ntsika and Delrot, 1986; Lohaus et al., 2001). Thus, it appears that sucrose would move down a concentration gradient, and so facilitated transport may be a possible mechanism for sucrose efflux. It is possible that the SUFs may be mediating sucrose release in the leaf. Indeed, expression analysis of RNA transcript of these transporters across plant organs showed that the facilitator *PvSUF1* is expressed highly (relative to other organs) in source leaves (Zhou et al., 2007).

It is tempting to speculate that the SUT and SUFs we have cloned from pea may mediate one or possibly both of these transport events in source leaves - a facilitator for the release of sucrose to the leaf apoplasm, and the energy-coupled symporter *PsSUT1* to load and concentrate sucrose in the SE-CC complexes.

For the facilitators, they may play a role in the efflux of sucrose from cells in source leaves, possibly the bundle sheath or phloem parenchyma, for subsequent sucrose uptake into the collection phloem of the leaf minor veins. Indeed, it appears that the recently cloned sucrose facilitators *AtSWEET11* and *AtSWEET12* (Chen et al., 2012) may carry out a similar function. These proteins localise to non-SE cells (probably phloem parenchyma) in the vascular bundles, and knockout plants are defective in phloem loading (Chen et al., 2012). For the sucrose uptake event, it is likely that energy-coupling is required to concentrate sucrose into the phloem from the surrounding apoplasm. In source leaves, it is likely that *PsSUT1* may act as the major sucrose uptake transporter where sucrose is concentrated from the apoplasmic space around the vascular cells and into the SE-CC complexes.

The coat of developing legume seeds is functionally committed to providing the developing embryo (including the cotyledons) with nutrients to meet their growth and storage requirements. Sucrose is one such nutrient, and represents the major transported form of carbon released from seed coats to fuel the growth of the enclosed embryo (Patrick and McDonald, 1980).

Sucrose initially enters the coat of developing pea seeds via their funicle. The seed coat vasculature in pea and the closely related broad bean consists of a single chalazal vein with two lateral phloem strand branches (Hardham, 1976). Studies of the symplasmic extent of the unloading pathway reveal that sucrose may leave the phloem symplasmically, and move throughout the chlorenchyma and thin-walled parenchyma both radially and laterally before being effluxed from the inner layers of thin-walled
parenchyma cells (Tegeder et al., 1999; van Dongen et al., 2003). Often, these thin-walled parenchyma cells have wall ingrowths characteristic of transfer cells, to increase their plasma membrane surface areas to support high fluxes of carrier-mediated nutrient transport (Offler and Patrick, 1993).

The symporter PsSUT1 has been cloned and its involvement in sucrose uptake by the embryo studied previously (Tegeder et al., 1999). Similarly, the facilitators PsSUF1 and PsSUF4 have been previously investigated by Zhou et al. (2009), who examined their expression and regulation in pea cotyledons. As such, our study focuses on seed coats and excludes cotyledons from investigation.

In broad bean, the trans-membrane, outward-directed sucrose concentration gradient measured at these release cells is about 30 mM in seeds that are undergoing rapid biomass gains (Patrick, 1994), with released sucrose being cleaved by extracellular invertases in seeds in this pre-storage phase of development (Weber et al., 1995). These invertases act to maintain an outward-directed sucrose gradient. It is estimated that only about 3% of sucrose release is by simple diffusion across membranes (Zhang et al., 2007) thus the bulk of sucrose release is via transporter proteins. Previous studies of seed coat tissues have revealed that, from an energetics perspective, sucrose secretion has both energy-coupled and passive components. Metabolic inhibitor studies show that, for instance, in broad bean seed coats, facilitated diffusion accounts for 60% of sucrose efflux (Ritchie et al., 2003). So, approximately 40% of sucrose release is via energy-coupled transport (Walker et al. 1995).

Five SUT-homologous genes were cloned from developing legume seed coats (Zhou et al., 2007). When heterologously expressed in yeast (Chapter 2), they were characterised as being facilitators (PsSUF1, PsSUF4, PvSUF1) and sucrose/H+ symporters (PsSUT1, PvSUT1). These sucrose facilitators (SUFs) could account for the passive component of transporter-mediated sucrose efflux from seed coats. In the case of PsSUT1, it is known that symporters can function in reversal mode as sucrose effluxers if the trans-membrane sucrose concentration gradient is sufficient to overcome the inward directed pmf. However, in seed coats, assuming a trans-membrane pH difference of 1 unit and a membrane potential of -50 mV (Walker et al., 1995), the Nernst equation predicts that a trans-membrane sucrose difference of at least 80 mM is needed to drive this reversal. As this falls short of the 30 mM difference measured by Patrick (1994), it is unlikely that PsSUT1 plays a role in effluxing sucrose from developing seed coats at the release cells.
As two of the three transporters (PsSU1 and PsSU4) have been characterised as sucrose facilitators, it is hypothesized that these may be mediating sucrose efflux from the thin-walled parenchyma cell layers in developing pea seed coats. As for the symporter PsSU1, it may play a role where active uptake of sucrose against a concentration gradient is required. In pea seed coats, this would most likely be in a role of retrieving sucrose leaked prematurely from the sieve elements along the pathway of delivery to seed coat tissues, such as that proposed for broad bean by Ritchie et al (2003).
3.2 Materials and Methods

3.2.1 Plant Growth Conditions

Plants of *Pisum sativum* (cv. BC3) were raised under glasshouse conditions (partial temperature control of 20–26 °C by day and 18–24 °C by night, with supplementary lighting provided by tungsten incandescent lights to provide a 14 h photoperiod) in 9 L pots (4 plants per pot) containing a mixture of coarse sand, coconut fibre and perlite (3:1:1). Nutricote (Yates) slow-release fertilizer was applied at 70 and 140 d after sowing, while liquid fertilizer (Wuxal Liquid Foliar Nutrients) was applied fortnightly.

3.2.2 Sub-cellular Localisation of Sucrose Transporters by GFP-gene Fusion

3.2.2.1 Construct generation

The Gateway cloning protocol (Hartley *et al.*, 2000; Fig. 3.1 A) was followed to generate the in-frame sucrose transporter-GFP construct for transient expression in tobacco leaves. The BamHI and NotI restriction sites (Fig. 3.2 B) were used to enable the cloning of PsSUT1, PsSUF1 and PsSUF4 into the pENTR1A entry vector. The coding regions of the SUT/SUF genes were amplified using pDR196-PsSUT1, pDR196-PsSUF1 and pDR196-PsSUF4 plasmids as templates, with primers designed (Table 3.1) to incorporate the appropriate restriction sites (BamHI and NotI) enabling transfer into the Gateway-compatible entry vector pENTR1A, and to remove the stop codon from the end of the coding region.

Amplification was carried out using Pfu Ultra II Fusion high fidelity DNA polymerase (Stratagene), according to the manufacturer’s instructions, using a standard PCR program (Sambrook and Russell, 2001) with 32 cycles; 60°C annealing temperature and a 1 min extension time at 72°C.

PCR products were purified from agarose slices using the Wizard® SV Gel and PCR Clean-Up System (Promega A9282), and blunt-end cloned into the pJet1.2 plasmid using a CloneJET™ PCR Cloning Kit (Fermentas), according to the manufacturer’s instructions. Following heat-shock transformation into competent *E. coli* DH10β cells (Inoue *et al.*, 1990), ampicillin selection, and colony PCR confirmation of SUT/SUF
gene presence in *E. coli* transformants, plasmids were extracted from overnight *E. coli* cultures using Qiagen’s plasmid miniprep extraction kit (according to the manufacturer’s instructions). The SUT/SUF gene inserts were digested out of pJet1.2 using BamHI and NotI (Fermentas) restriction enzymes. The purified DNA fragments (of approximately 1.5 kb), consisting of the gene flanked by restriction sites BamHI and NotI, were directionally cloned into the 2.3 kb fragment of the BamHI/NotI digested plasmid pENTR1A (Fig. 3.1A) using T4 DNA Ligase (Fermentas) and a 3:1 insert to vector molar ratio. The pENTR-SUT/SUF plasmids were introduced into *E. coli* DH10β cells and selected upon kanamycin (50 µg/mL). Restriction enzyme digestion was carried out on plasmids isolated from SUT/SUF-positive colonies (as determined by colony PCR) to confirm the presence and size of inserts.

**Table 3.1.** Forward and reverse primers designed to amplify gene-coding regions with the addition of restriction sites (sequences for BamHI and NotI respectively, shown underlined) to enable transfer into the Gateway entry vector pENTR1A. The nucleotide bases in bold show the position of the start codon.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsSUT 1</td>
<td>GGGGGATCCGGATGGAG CCTCTCTCTTCCAC</td>
<td>GGGGCCGGCCGGCAATGAAAGCCTC CTCCAGTAGC</td>
</tr>
<tr>
<td>PsSUF 1</td>
<td>GGGGGATCCGGATGGATA ATCCTCCACCAATGA</td>
<td>GGGGCCGGCCGGCAATGAAATCCAC CCGCAATGGAA</td>
</tr>
<tr>
<td>PsSUF 4</td>
<td>GGGGGATCCGGATGCCGA ATCCCGACTCTTC</td>
<td>GGGGCCGGCCGCGATACTGGTTTC TAGGCTTTTGAG</td>
</tr>
</tbody>
</table>

Recombination of the in-frame SUT/SUF genes from pENTR into the destination vector pMDC83 was carried out to create pMDC-SUT/SUFs (Fig. 3.1A, final step). The recombination was carried out using Gateway® LR Clonase™ II (Invitrogen). Reactions were incubated overnight at 25 ºC and introduced into *E. coli*. Colonies harbouring the vector were selected using kanamycin (50 µg/mL). Plasmid DNA was isolated from SUT/SUF positive colonies, digested with appropriate restriction enzymes to confirm plasmid and insert size, and the SUT/SUF insert sequenced to confirm correct gene sequence.
Figure 3.1. Cloning of sucrose transporters into plasmids for the transient expression of GFP-tagged proteins.

A. The Gateway cloning protocol (Hartley et al., 2000) used to clone SUT/SUF genes in-frame with the GFP sequence.

B. Plasmid map (from Invitrogen) of the pENTR1A entry vector, showing restriction enzymes within the multiple cloning site.

C. Detail of the pENTR1A multiple cloning site. BamHI and NotI were used for the directional cloning of SUT/SUF genes.
3.2.2.2 *Agrobacterium* transformation

The confirmed pMDC83-SUF/SUT binary vectors were introduced into *A. tumefaciens* strain LBA4404 cells (Hoekema et al., 1983), which were kindly provided by Dr. Michael Sheahan from the University of Newcastle. Electro-competent *A. tumefaciens* were generated from an overnight culture in 3 mL YEP medium (1% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0), supplemented with 50 µg/mL streptomycin and 50 µg/mL rifampicin. Cells were pelleted at 1,500 g for 4 min, washed three times with 1 mL sterile ice-cold 1 mM HEPES (pH 7.0), washed once with 500 µL sterile ice-cold 10% (v/v) glycerol and resuspended in 40 µL of 10% glycerol. Twenty ng of pMDC83-SUT/SUF vector was used, with electroporation effected using a BioRad Gene Pulser according to the manufacturer’s instructions. Cells were plated on YEP plates supplemented with 50 µg/mL streptomycin, 50 µg/mL rifampicin and 50 µg/mL kanamycin to select for bacterial colonies harbouring the pMDC83-SUT/SUF vector.

3.2.2.3 Transient expression in tobacco leaf

Four mL cultures of *A. tumefaciens* harbouring plasmids with SUT/SUF inserts (in YEP medium supplemented with 50 µg/mL streptomycin, 50 µg/mL rifampicin and 50 µg/mL kanamycin) were grown for 24 h at 28 ºC in darkness, with moderate shaking (~200 rpm).

Cultures were pelleted at 5000 g for 5 min, and pellets washed twice with AIB (50 mM MES, pH 5.6, 0.5% (w/v) glucose, 2 mM sodium hydrogen phosphate, 100 µM acetosyringone), and diluted to an OD$_{600}$ of 0.8 in AIB. Agro-infiltration (Kapila et al., 1997) of near fully-expanded tobacco leaves was carried out in the morning (when stomata are more likely to be open) via leaf undersides. Leaves were harvested and viewed approximately 72 h after infiltration. Control infiltrations of tobacco with *A. tumefaciens* transformed with empty pMDC83 vector and pBIN.mgfp5-ER (Haseloff et al., 1997; a gift from Dr. Michael Sheahan) were also carried out.

3.2.2.4 Confocal microscopy

Fresh leaf discs were harvested, mounted in Milli-Q H$_2$O and viewed using a Zeiss LSM510 confocal laser scanning microscope with a 40x C-Apochromat water immersion objective (NA 1.2; Zeiss). GFP fluorescence was observed using a 488 nm argon laser and BP500-530IR filter, while for chloroplast autofluorescence a 543 nm
He-Ne laser and LP650 filter were used. Z-series images (0.3 µm optical thickness, 10 images per stack) were acquired, and subsequently processed using ZEN 2008 Light Edition software (Zeiss).

### 3.1.1 Antibody Preparation

#### 3.2.1.1 Antibody design

In previous studies of SUT localisation, polyclonal antibodies were raised against different regions of the SUT protein. Probing for SUTs in developing wheat grains, Bagnall et al. (2000) used a highly conserved sequence of amino acids from the intracellular loop between trans-membrane domains (TMDs) II and III from StSUT1. Aoki et al. (2004) and Furbank et al. (2001) used polyclonal antibodies raised against epitopes from the cytoplasmic loops between TMD II and III, the central loop and the c-terminus. Rae et al. (2005) used an epitope from the intracellular central loop of ShSUT1. Similarly, the central loop of LeSUT4 was used by Weise et al. (2000) to raise polyclonal antibodies. They found that these antisera also cross-reacted with StSUT4, which is 90% identical at the amino acid level to LeSUT4 in the central loop region. This highlights the importance of selecting unique sequences in the work described herein, to avoid cross-reaction between SUT antisera.

Epitopes, each 20 amino acids in length, were selected from the central cytoplasmic loop of PsSUT1, PsSUF1 and PsSUF4, as this is the most divergent region between these three transporters (Fig. 3.2). The epitope sequences selected were:

- **LIAEKAVVTAE**DGGSNGM**P** (residues 255 - 274) for PsSUT1,
- **IESQSQTQ**TQTQ**E**PEQQ**V**S** (residues 251 - 270) for PsSUF1 and
- **SSGEPDAE**AE**E**GESG**G**SA**E**EA** (residues 254-273) for PsSUF4.

The selected epitopes were synthesised, conjugated to diphtheria toxoid by Mimotopes Pty Ltd (Melbourne), and used to raise polyclonal antibodies in rabbits by The Institute for Medical and Veterinary Science (IMVS; Adelaide). The PEP2 antibody (Bagnall et al., 2000) that was raised against a highly conserved sequence (from the loop between the 2\textsuperscript{nd} and 3\textsuperscript{rd} TMDs of StSUT1 – residues 87-106: GYYSDNCSSRFGRRRPFI\textsubscript{A}A), was used as a positive control in the immunolocalisation studies.
Figure 3.2. Aligned amino acid sequences from PsSUT1, PsSUF1 and PsSUF4, with the region of greatest sequence divergence within the central loop outlined. Sequences twenty amino acids in length from this region were used for polyclonal antibody production.

### 3.2.1.2 ELISA assay

An ELISA assay was undertaken to confirm antibody presence in 7-week post-inoculation rabbit serum. Unconjugated peptide was diluted 1:5 in phosphate buffered saline (PBS- 145 mM NaCl, 2 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4). Ninety six-well high-binding flat bottom ELISA plates (Microlon 600, Greiner) were sensitised by adding 100 µL diluted serum per well and incubated overnight at 4 °C. Plates were washed using 200 µL per well, with PBS plus 0.05% (v/v) Tween 20 (PBST), followed by blocking with 1% (w/v) skim milk powder in PBST for 30 min at room temperature. Plates were then washed 6 times with PBST. Final dilutions of rabbit test bleed serum ranging from 1:100 to 1:102,400 in PBST were added (100 µL per well) and incubated at room temperature for 2 h. An identical dilution range of the pre-vaccination serum
was also included as a negative control. Upon completion of the incubation period, plates were washed 6 times in PBST, and 100 uL of secondary antibody (horseradish peroxidase conjugated anti- rabbit antibody diluted 1:4000 in PBST) was added to each well, and incubated at room temperature for 2 h. Following the incubation period, unbound secondary antibody was removed by washing plates 6 times in PBST.

One hundred µL of a freshly prepared substrate solution, required to generate a colour reaction, consisting of 1 mM 2,2'-Azino-bis3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (A1888, Sigma; stock solution of 50 mM), 0.03% H2O2 in 100 mM citric acid (pH 4.2) was added to each well. Plates were incubated at room temperature for 20 min prior to visual assessment of colour development.

3.2.1.3 Antibody purification

Affinity purification columns (Mimotopes Pty Ltd, Melbourne) of thiopropyl sepharose-conjugated peptide were used to separate specific antibodies from whole rabbit serum, as per the manufacturer’s directions. Briefly, columns were incubated in whole rabbit serum for 12 h at 4 ºC, washed with PBS, and bound antibody eluted by washing the column with several volumes of 100 mM glycine buffered at pH 2.5 and 11.5 respectively. Antibody eluate was adjusted to pH 7.0, before freeze-drying and re-suspension in distilled H2O to the original volume of whole rabbit serum used. Aliquots of antibody were stored at -20 ºC until required.

3.1.2 Western Blotting

3.2.2.1 Plant microsomal membrane extraction

Tissues were collected from young leaves (prior to emergence from stipules-see results and Fig. 3.4 for details). fully expanded mature leaves, as well as developing seeds at pre-storage (12 days after flowering, daf) and storage (20 daf) phases, and snap frozen in liquid nitrogen. Seed coats were surgically separated from cotyledons on ice prior to membrane isolation.

The method for extraction of total plant organ microsomal membranes is essentially that described by Ripp et al. (1988). Plant material was ground under liquid nitrogen and suspended in 250 mM sucrose, 2.5 mM DTT, 10 mM EGTA, 10 mM MgSO4, 25 mM Mes-KOH (pH 7.0), at the ratio of approximately 600 mg ground plant material per mL. This mixture was passed through three layers of cheesecloth and centrifuged twice
at 13,000 g (10,500 rpm in a Sorvall SS-34 rotor) for 10 min at 4 °C. The supernatant was then passed through filter paper before ultracentrifugation at 85,000 g (40,000 rpm in a Beckman TLA100.4 rotor) for 35 min at 4 °C. The microsomal pellet was washed by re-suspending in 10 mM DTT, 10 mM Mes-KOH (pH 6.0) and spinning again at 85,000g for 35 min at 4 °C. The pellet was then re-suspended in 300 uL of the DTT/Mes buffer supplemented with 3 μL of a protease inhibitor cocktail (P9599, Sigma). Membrane fractions were stored at -80 °C until required.

3.2.2.2 Protein quantification

Protein quantification was carried out by diluting samples and using Bradford reagent (B6916, Sigma; Bradford, 1976) in accordance with the manufacturer’s instructions. Briefly, 1 part of protein sample (or BSA standard) was mixed with 30 parts of Bradford reagent, vortexed and incubated at room temperature for 15 min to allow for colour development. A standard curve for BSA was constructed over a range of 0.25 to 1.0 mg/mL at OD595 and used to estimate sample protein concentrations.

3.2.2.3 SDS page and immunoblotting

Plant microsomal membrane samples were separated on a 10 % SDS-polyacrylamide gel using a BioRad Mini-gel apparatus, loading 30 μg protein per lane. Proteins were stacked at 100 V for 15 min, then separated at 200 V for 45 min. Separated proteins were then transferred electrophoretically to nitrocellulose membrane (0.2 μm pore size) in SDS-PAGE transfer buffer (25 mM tris, 192 mM glycine, 20% (v/v) methanol) at 30 mV at 4 ºC for 10 h, and membranes stained in 0.2% (w/v) Ponceau S in 3% (w/v) acetic acid to verify protein transfer. The nitrocellulose membrane was blocked for 2 h (with gentle agitation) in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 0.2% (w/v) Na-azide, pH 7.4) plus 0.2% Triton X-100 (TBST) containing 5% (w/v) non-fat milk powder, 1% (w/v) BSA and 5% (v/v) glycerol. Excess blocking solution was rinsed off with TBST, and membranes incubated in primary antibody (1:250 dilution in TBST + 1% BSA) overnight at 4 ºC. The membranes then underwent three 10 min washes in TBST, and were incubated for 2 h in a 1:2000 dilution of anti-rabbit IgG-alkaline phosphatase conjugate (A9919, Sigma) in TBST + 1% BSA at 25 °C. Excess secondary antibody was removed with three 10-min washes in TBST, before overlaying the membrane with 1-2 mL Western Blue (Promega). This was incubated for up to 1 h (depending on colour development) with the reaction terminated by washing the
membrane in Milli-Q H₂O. Membranes were subsequently imaged using a Bio-Rad ChemiDoc XRS with white light illumination.

3.1.3 Immunolocalisation

3.2.3.1 Plant material sampling and embedding

Very young axial through to fully expanded leaves were sampled to observe leaf venation development. The leaves were decolourised by boiling in 85% ethanol for 1 h, then cleared by incubating in 15% NaOH overnight. Staining with 1% (w/v) basic fuchsin was carried out to highlight venation.

The first fully expanded leaf was sampled for microscopy. The leaf tissue between the first order of major veins (excluding the midrib) in the lower third of the leaf was excised and tissues were fixed on ice in 3% glutaraldehyde, 4% paraformaldehyde, 10 mM sucrose and 25 mM sodium cacodylate buffer (pH 6.8) for 4 h. Three 10-min washes with 25 mM sodium cacodylate buffer were carried out, followed by three 10-min washes in Milli-Q H₂O. Fixed tissues were then dehydrated at 4 °C through an ethanol series, 10% to 100% ethanol in 10% increments, for 30-45 min per ethanol concentration, followed by an overnight incubation in 100% ethanol at 4 °C. Two 10-min rinses of tissue in 100% ethanol followed. All of the following resin infiltration steps were performed on a rotator at room temperature. Tissues were immersed in 5% LR White (ProSciTech Inc, Qld.) for 2 h, followed by 10% LR White for 2 h, 20% LR White for 2 h, 40% LR White for 2 h, 60% LR White for 2 h, 80% LR White for 2.5 h, 90% LR White for 4 h, then 100% LR White for 7 d, with the LR White replaced with fresh solution every 2-3 d. Tissue was then placed on partly polymerized LR White in gelatin capsules, which were then filled with fresh LR White, and polymerized by heating the capsules to 60 °C for 24-48 h.

3.2.3.2 Immunocytochemistry

One µm thick sections of embedded tissues were cut using a Reichert Ultracut microtome, and dried onto gelatin-coated slides. Slides were blocked in TBST + 1% BSA for 1 h, rinsed briefly with TBST, and then incubated in primary antibody (diluted in TBST + 1% BSA) overnight at 4 °C. Following optimization, the following antibody dilutions were used routinely: 1:50 for PsSUT1, 1:20 for PsSUF1 and PsSUF4, 1:100 for PEP2 and 1:20 for pre-immune serum. Following overnight incubation, slides were
washed for 4 x 10 min with TBST-1% BSA. Secondary antibody, being anti-rabbit IgG-FITC-conjugate (F0382; Sigma), was applied at a dilution of 1:100 in TBST-1% BSA, and incubated in the dark for 2 h at 25 °C.

The slides then underwent two 15 min washes in TBST-BSA, two 15-min washes in TBST and finally two 15 min washes in Milli-Q H₂O, before being mounted in 20 µL mowiol+ 0.1% phenylenediamine under a coverslip. Samples were viewed on a Zeiss Axiophot fluorescent microscope using epifluorescence with excitation wavelengths between 450-490 nm, and photomicrographs captured via an Axiocam CCD camera (Zeiss) using Axiovision 4.0 (Zeiss).
3.3 Results

As the sub-cellular localisation of the transporters will have a fundamental impact on their likely physiological function, these findings will be examined first. The leaves, as the source of sucrose within the plant will then be examined for transporter cellular localisation, with a focus on the loading pathway from the mesophyll cells to the minor veins. The developing seed coat will be examined in turn, as it provides a readily accessible system for observing tissues committed to sucrose efflux.

3.3.1 Sub-cellular Localisation of SUT/SUF Proteins

Previous studies show that SUT family proteins localise to either the plasma membrane or tonoplast, the latter being exclusively for SUT4 proteins of non-solanaceous species. The epidermal cells of tobacco transiently expressing sucrose transporter-GFP fusion protein were investigated using confocal laser scanning microscopy. Although considered to be of little photosynthetic importance, epidermal cells from the lower surface of the tobacco leaf are known to contain small chloroplasts (Dupree et al., 1991). As chloroplasts are located within the cytoplasmic compartment of the cell, the red autofluorescence of chlorophyll allows the relative location of GFP labelling to be assigned to either the plasma membrane or tonoplast. Given the location of the chloroplasts (white arrowheads, Fig. 3.3 B-D) enclosed within the GFP-labelled membranes, it appears that PsSUT1, PsSUF1 and PsSUF4 are all plasma-membrane localised. PsSUF4 localised in a punctate way, and also showed labelling of the perinuclear ring (Fig. 3.3 D). A pattern of dual targeting to both the plasma membrane and perinuclear ring (endomembrane system) has been reported for the SUT4 family transporters (e.g., Chincinska et al., 2008 for StSUT4). However, unlike other non-solanaceous SUT4 clade members, no evidence of tonoplast labelling was observed. In contrast to the punctate distribution of PsSUF4, PsSUT1 and PsSUF1 showed a more uniform labelling of the plasma membrane only (Fig. 3.3 B, C). The empty construct-transformed negative controls had no GFP labelling (Fig. 3.3 A), whereas the ER-GFP-transformed positive control had a GFP-labelled perinuclear ring (similar to that seen for PsSUF4), as well as labelling throughout the cytoplasm (Fig. 3.3 E).
Figure 3.3. Transient expression of N-terminus sucrose transporter- GFP6 protein fusions in epidermal cells of *A. tumefaciens*-infiltrated *Nicotiana tabacum* leaves. Z-stack overlays of confocal fluorescence micrographs of whole leaf tissue.

**A.** Empty construct- transformed negative control.

**B.** PsSUT1-GFP6 protein localised to plasma membrane, as indicated by the location of chloroplasts (labelled) on the inner side of the line of labelling.

**C.** A low level PsSUF1-GFP6

**D.** Plasma membrane localisation of PsSUF4-GFP6 fusion protein. Distribution is somewhat punctate, with the perinuclear ring (starred) also labelled. Note location of chloroplasts (arrow) in relation to plasma membrane label.

**E.** ER-GFP positive control- transformed cells showed GFP localisation throughout the cytoplasm and to the perinuclear ring (starred). Again, note location of chloroplasts (arrow) adjacent to cytoplasmic label.

A-E: bar = 20 µm.

**F.** Detail of plasma membrane localisation of PsSUF4-GFP6 fusion protein, showing punctate labelling. Bar = 10 µm.
Having determined the plasma membrane localisation of all three transporters, it appears that the likely physiological role for PsSUT1 involves the uptake of sucrose into cells against the prevailing gradient, while for the facilitators, a role in equilibrating sucrose between the cytoplasm and apoplasm appears likely.

With these likely physiological roles in mind, the source leaf was chosen as a candidate organ for protein profile and localisation studies. As the site of both synthesis and subsequent phloem loading of sucrose, source leaves offer a system where the two functions - equilibration or passive release and subsequent retrieval of sucrose - occur in succession in the apoplastic loading pathway present in pea leaf minor veins. To enable comparison with leaves that have not yet gained this export capacity, it was also decided to investigate sink leaves for transporter protein studies. This would then enable observations to be made on the changes in sucrose transporter protein profiles as leaves undergo the sink to source transition.

### 3.3.2 Selection of tissue for sink and source leaf protein sampling: stage of sampling in relation to leaf development

The pea leaves examined (Fig. 3.4 B-G) have 7 to 8 orders of veins (excluding the midrib) when fully expanded. In this study, the definition of minor veins will follow that of Wimmers and Turgeon (1991) as those veins having less than seven sieve elements, a total vein diameter of less than 90 µm and being the veins that delimit the areoles within leaf tissues. Thus, the first 3 vein orders in fully expanded leaves are major veins, being 90 µm or greater in diameter and extending across the leaf, rather than enclosing areoles. The acquisition of minor vein orders throughout leaf expansion was examined to form a developmental profile (Fig. 3.4).

The stage at which leaves gained export capacity was investigated by observing the development of major and minor vein networks. This was carried out to determine at which stage leaves should be sampled for Western blotting to capture protein profiles of leaves at two distinct developmental stages, being sink and source stages. It is anticipated that sink leaves have major veins present, a differentiating but not yet functional minor vein network, and are net importers of sucrose. In contrast, the source leaf minor vein network will be fully differentiated, experiencing high levels of sucrose...
Figure 3.4. Pea leaf morphology throughout development.

A. Sequence of arrangement of leaf relative to stipules (see text for details).

B. Cleared leaves of *P. sativum*, showing increasing number of vein orders sampled from the lower third of the leaflet throughout development. The approximate surface area (SA) of each leaflet is given in parentheses.

- Young leaflets enclosed within two stipules (SA: 56 mm²)
- Leaflets enclosed within one stipule (SA: 94 mm²)
- Leaflets emerging from closed stipule (SA: 140 mm²)
- Leaflets emerged and folded (SA: 212 mm²)
- Leaflets open and expanding (SA: 470 mm²)
- Fully expanded leaflets (SA: 700 mm²)

Although stages B- D are still enclosed within stipules and so are not functioning as source leaves, the vein network is extensively developed, with up to 7 vein orders present. An additional order of veins was recorded in fully expanded leaflets, which would have undergone the sink to source transition. Bar= 200 µm.
delivery from the surrounding tissues, and loading into the SE-CC complexes. At this point in development, any SUTs playing a role in sucrose export should be represented in the protein profile sampled from these leaves.

Leaves were sampled at six different stages of development, from the smallest practicable size (to enable manipulation for fixation and staining) through to the first fully expanded leaf. All sampling was carried out in the lower third of the leaf. Pea leaves of the cultivar studied are pinnately compound, having two pairs of leaflets attached to a central rachis, a basal stipule and a terminal tendril (Fig. 3.4 A). The leaflets of very young leaves are folded in half down the midrib, and fully enclosed by the folded stipule, which serves a role in protecting young tissues. As the leaf grows, the leaflets expand, the rachis elongates and the still-folded leaflets emerge from the stipule, which also eventually unfolds.

Very young leaves (of only 56 mm²), that were enclosed within growing tips, were found to have a fully developed major vein network. Minor vein development however was still progressing during this developmental stage, with 6 vein orders present at this stage (Fig 3.4B; noting that the midrib was not counted in vein ordering). At the next developmental stage, where leaves have increased to about 10% of their final size, there are still only 6 vein orders present (Fig 3.4C). So, although no additional minor vein orders are added, the vein network must still be expanding to accommodate leaf growth.

By the time leaves had expanded to 20% (Fig 3.4D) of their final size an additional vein order had appeared, with these 7 orders of veins representing the majority of the ultimate leaf venation. Although the leaf is still folded down the midrib and only just beginning to emerge from within the stipules, the majority of the venation network required for the export of photosynthates appears to have been laid down. Indeed, leaves that were two-thirds fully expanded and had unfolded to enable maximal light capture still had 7 vein orders (Fig 3.4F). It is worth noting here that functional maturity of minor veins progresses in a basipetal direction (Wright et al., 2003), and so the sink to source transition occurs first nearer the leaf tip; the tissue sampled in Fig. 3.4 was from the lower third of the leaf. Pea leaf minor vein maturity, as measured by the ability to export 14C-labelled assimilates, shows that maturity coincides with the differentiation of companion cells into transfer cells, and occurs when the leaf is 60 to 80 % fully expanded (Gunning and Pate, 1974). Thus, it is expected that in leaves sampled in Fig. 3.4B-E, which are less than 30% fully expanded, functional transfer cells would be absent, while in the leaf in Fig 3.4F (at approximately 67% fully expanded) rapid
maturation of transfer cells would be occurring. It is not until the final developmental stage sampled (Fig. 3.4G) that an additional vein order is observed; this 8th order consisted of terminal veinlets that extend into individual areoles. It is interesting to note that the leaves remain folded in half along the midrib during these early stages, and only unfold once about two-thirds fully-expanded, so that maximal exposure to light coincides with the attainment of functional minor veins.

To ensure that both sink and source stages of leaves were sampled for protein analysis, developmental stages prior to emergence from stipules were selected for sink leaf tissue sampling (Fig. 3.4B-C), as it was reasoned that in these leaves photosynthesis would be sub-optimal. The first fully expanded leaves (Fig. 3.4G), with functionally-mature leaf minor veins and fully differentiated transfer cells were used for source leaf tissue.

3.3.3 SDS-Page and Immunoblotting of Sucrose Transporters in Pea Leaves

The Ponceau stain (Fig. 3.5A) is included to show the loading and separation of protein from the samples. The 55 kDa band that is prominent in the source leaf sample (and, to a lesser extent, the sink leaf) is quite possibly the large subunit of rubisco (Fig. 3.5A; Spreitzer, 1993). As total microsomal membrane was collected from the plant samples, the thylakoid membranes from disrupted chloroplasts would be included in the leaf samples, particularly that of the source leaf. There are several reports of a close association between rubisco and thylakoid membranes - it appears that complexes of carbon dioxide-fixing enzymes may be bound to the stromal face of the thylakoid membranes (Suss et al., 1993). Thus, the presence of the 55 kDa band is likely a result of the inclusion of rubisco along with thylakoid membranes in these samples.

Antibodies raised against PsSUT1 cross-reacted with a band of approximately 47 kDa (Fig. 3.5B) in the source leaf tissue only. Although this size is smaller than the predicted molecular mass of 55 kDa, it is similar to those reported previously for several sucrose transporters, including StSUT1 at 47 kDa (Lemoine et al., 1996) and RcSUT1 at 45 kDa (Bick et al., 1998), and corresponds to previous immunoblotting of PsSUT1 (Tegeder et al., 1999) in seed coats and cotyledons. No bands were observed in either
source or sink leaf extracts when probed with PsSUF1 (Fig. 3.5C) and PsSUF4 (Fig. 3.5D) antibodies, the same as that observed for the pre-immune control (Fig. 3.5E).

Figure 3.5. SDS PAGE and Western immunoblot analysis of PsSUT1, PsSUF1 and PsSUF4 protein expression in developing pea leaves. Total microsomal protein was extracted from tissue as described by Ripp et al. (1988), and 30 µg protein loaded per lane. **A.** Ponceau stain of protein transferred to nitrocellulose membrane, showing clear separation of protein bands. **B - E.** Western immunoblot of membrane probed with **B.** PsSUT1 antibodies; **C.** PsSUF1 antibodies; **D.** PsSUF4 antibodies and **E.** Non-immune control serum. For B-E, antibody binding was visualised by probing with an alkaline-phosphatase conjugated anti-rabbit secondary antibody raised in goat, and undergoing a BCIP/NBT colour reaction.

3.3.3.1 Anatomy of a fully expanded source leaf

As the Western blot indicated the presence of PsSUT1 in source leaves (Fig. 3.5B), and intracellular localisation has established that it is plasma membrane-located (Fig. 3.3B), investigation of the cellular localisation of this transporter was warranted. In plant species that are “apoplastic loaders” such as *P. sativum* (Wimmers and Turgeon, 1991; Amiard et al., 2005), a symplasmic discontinuity occurs between phloem SE-CC complexes and surrounding phloem parenchyma and bundle sheath cells of the minor
veins. This was originally observed as a paucity of plasmodesmatal connections between these two regions (Gamalei, 1985; 1989). The anticipated route of sucrose movement from the site of sucrose synthesis in photosynthetic mesophyll cells to the collection phloem involves efflux from surrounding cells upstream from, or adjacent to, phloem, movement through the cell wall matrix, and subsequent uptake into SE-CC complexes. A model of the putative route of sucrose transport from the mesophyll cells to the collection phloem in leaf minor veins was constructed (Fig. 3.6). The plasmodesmatal frequencies observed for pea leaf minor veins between adjoining cell types (Wimmers and Turgeon, 1991) were plotted (Fig. 3.6B) to delineate the symplasmic discontinuity between the photosynthetic regions of the leaf and the SE-CC complexes. Sites of possible sucrose efflux (E1 in Fig. 3.6A, E2, E3 in Fig. 3.6B) and uptake (U1, U2 in Fig. 3.6B) were identified, with these sites the focus of examination for immunolabelling by SUT/SUF antibodies (reported in Section 3.3.4). Given that PsSUT1 is a plasma membrane-localised sucrose proton symporter, and that SUT1 members have been previously reported to mediate sucrose uptake into the SE-CC complexes, it is likely that PsSUT1 is represented in this model by the transporters U1 and/or U2.

For histological and immunological study, veins with two to four sieve elements were typically chosen to represent minor veins (Fig. 3.7C), as these higher order (6-8) veins were in most intimate contact with the mesophyll cells.

Vascular bundles were located in the mid-plane of the leaf, abutting both the palisade and spongy mesophyll layers (Fig. 3.7A). Both major and minor veins were completely enclosed within a ring of bundle sheath cells that are in intimate contact with adjacent mesophyll (Fig. 3.7B). This presumably allows a more direct path from sites of photosynthesis to sites of phloem loading in minor veins. Wimmers and Turgeon (1991) report a high plasmodesmatal frequency between bundle sheath and mesophyll cells and so (assuming that the plasmodesmata are functional) these cells are symplasmically connected (Fig. 3.6B). Additionally, due to the abundance of minor veins throughout the leaf, individual mesophyll cells are in close proximity to a nearby vein, with never more than about 5 intervening cells between any mesophyll cell and the bundle sheath (Fig. 3.7A).
Figure 3.6. A. Generalised anatomy of minor vein and surrounding tissue in source leaves of *P. sativum*, showing the apoplastic route of sucrose movement from mesophyll to collection phloem.

bs- bundle sheath; cc- companion cell; cwm- cell wall matrix; ia- intercellular airspace; pm- palisade mesophyll; pp- phloem parenchyma; se- sieve element; sm- spongy mesophyll; x- xylem element; xp- xylem parenchyma ★- major sites of sucrose synthesis; — symplasmic continuity via plasmodesmata; E1- possible site of sucrose efflux from mesophyll cells.

B. Detail of minor vein anatomy, plasmodesmatal frequencies and possible sites of sucrose efflux (E2, E3) and uptake (U1, U2) to the sieve element-companion cell complexes.

Numerical values are the number of plasmodesmata reported per µm² of cell wall interface between cell types in pea minor veins by Wimmers & Turgeon (1991). Pink circles- sites of sucrose efflux across plasma membrane; purple circles- sites of sucrose uptake into se-cc complexes.
Figure 3.7. Anatomy of fully expanded source leaf of *P. sativum*. Light micrographs of a 1 µm thick transverse section stained with toluidine blue.

A. Low magnification micrograph of leaf showing arrangement of major and minor veins. Bar = 100 µm.

B. Section through a minor vein showing location in relation to leaf dermal and ground (mesophyll) tissue. Bar = 50 µm.

C. Cellular detail of pea leaf minor vein. Wall ingrowth material (arrows) has been deposited within companion cells; note however that this does not extend along walls adjacent to the sieve elements. Bar = 5 µm.

bs- bundle sheath; cc- companion cell transfer cell; le- lower epidermis; MV- leaf major vein; mv- minor vein; pm- pallisade mesophyll; pp- phloem parenchyma; se- sieve element; sm- spongy mesophyll; ue- upper epidermis; x- xylem element; xp- xylem parenchyma.
Within the minor veins, xylem and phloem are arranged collaterally, with xylem overlaying phloem (Fig. 3.7C). In the minor veins sampled, it was observed that, in the majority of cases, there was one companion cell per sieve element; however two companion cells per sieve element were occasionally seen. This differs from minor veins of Arabidopsis, where there are normally two companion cells per sieve element (Haritatos et al., 2000). In pea leaf, the SE-CC complexes were adjacent to both phloem parenchyma and bundle sheath cells, particularly in smaller veins of only a few SE-CC complexes. In pea minor veins, companion cells usually develop wall ingrowths, with the increased plasma membrane surface area supporting high rates of solute flux along this apoplastic route (Wimmers and Turgeon, 1991; Amiard et al., 2005; Adams et al., 2007). It was noted that transfer cell wall ingrowths did not form on the wall adjacent to the partner sieve element (Fig. 3.7C).

Calcofluor white staining was carried out (Fig. 3.8A) as it allows clear visualisation of cell walls. Calcofluor fluoresces when bound to cellulose, with fluorescence intensity correlating with cellulose content of cell walls (Galbraith, 1981). All of the cells of the vascular bundle showed a greater intensity of wall staining than the surrounding bundle sheath cells.

Mature sieve elements do not have a vacuole. DAPI staining (Fig. 3.8B) highlights the location of sectioned nuclear material; a comparison of the calcofluor-stained and DAPI-stained sections confirms sieve element identity as those cells which are enucleate and usually closely associated with a partner companion cell. Note that not every companion cell exhibited DAPI-staining, as this was contingent on the 1 µm thick plane of the section passing through the nucleus.

### 3.3.4 Immunolocalisation of SUTs/SUFs in Leaf Minor Veins

The PEP2 antibody (Bagnall et al., 2000), raised against highly conserved regions common to all SUT clades, was used to determine the location and extent of sucrose transporters within the target tissue. PEP2 has identities to PsSUT1, PsSUF1 and PsSUF4 of 85, 84 and 86% respectively over the same region of sucrose transporter amino acid sequence, and so it is anticipated that PEP2 should cross-react with our transporters of interest. In addition, it may highlight the location of any other sucrose transporters present in *P. sativum* that are present in these tissues but have not been cloned.
**Figure 3.8. Minor vein from mature leaf of *P. sativum*, showing immunolocalisation of sucrose transporters.** Light and fluorescence micrographs of 1 µm thick transverse sections.

**A.** Calcofluor white stained section, showing extent of cell walls.

**B.** DAPI-stained section, showing nuclear material (arrows) in companion cells, phloem parenchyma and bundle sheath cells, but not enucleate sieve elements. A and B: bar = 10 µm.

**C.** Light micrograph image corresponding to D.

**D.** Immunolocalisation of sucrose transporters in pea leaf minor vein using the generic anti-SUT antibody PEP2. Antibody binding was visualised using a FITC-conjugated anti-rabbit secondary antibody raised in goat. Labelling of companion cells and sieve elements is apparent. Dashed lines in C and D delineate cell boundaries.

**E.** Non-immune serum control, using column-purified pre-immunisation rabbit serum. C-E: bar = 5 µm.

bs- bundle sheath; cc- companion cell; pp- phloem parenchyma; se- sieve element; x- xylem element; xp- xylem parenchyma.
A. Calcofluor White

B. DAPI

C. Light

D. PEP

E. PEP pre-immune
In leaf minor veins, immunolocalisation with PEP2 labelled both sieve elements and companion cells (Fig. 3.8D). These transporters are likely to be those involved with sucrose uptake from the apoplasm by SE-CC complexes presented in the minor vein loading model (purple circles, Fig. 3.6B). Note that there was no labelling of the putative efflux sites (pink circles, Fig. 3.6A,B) in the bundle sheath or phloem parenchyma cells by PEP2. The labelling of the companion cells appears somewhat diffuse, and may be due to the invagination of plasma membrane around transfer cell wall ingrowths. Some non-specific labelling of structures in the bundle sheath cells is probably due to antibodies non-specifically binding to starch grains in sectioned chloroplasts.

Immunolabelling of PsSUT1, PsSUF1 and PsSUF4 was restricted to the sieve elements only (Fig. 3.9B-D). So although PEP2 may have cross-reacted with PsSUT1, PsSUF1 and PsSUF4 in the sieve elements, it appears that there is an additional sucrose transporter located in companion cells (Fig. 3.8D). The Western blot (Fig. 3.5B-E) indicated that only PsSUT1 was present in leaves. The appearance of the facilitators suggests that the level of protein in the Westerns for these transporters was below the limits of detection of the antisera raised against them. Rather, it is surprising to find facilitators on the SE plasma membrane, as our model predicts that their mechanism of transport would see them on the bundle sheath and/or phloem parenchyma cells. In particular, the co-localisation of both the facilitators and PsSUT1 on the same membrane creates a situation where a futile cycling of sucrose first in (by PsSUT1) and then out (by leakage through the facilitators) of the sieve element may exist.

Although the facilitators PsSUF1 and PsSUF4 do not appear to be playing a role in sucrose efflux at the leaf minor vein, these two transporters (along with PsSUT1) were cloned from tissues functionally committed to efflux. To further investigate the potential roles of these transporters protein profiles localisation studies were carried out in the tissues of developing seed coats.
Figure 3.9. Immunolocalisation of PsSUT1, PsSUF1 and PsSUF4 proteins in minor veins of *P. sativum* source leaves. Light and fluorescence micrographs of 1 µm thick transverse sections, showing FITC- labelled immunolocalisation and corresponding light micrograph, for A. Pre-immune serum control; B. PsSUT1; C. PsSUF1 and D. PsSUF4. Labelling by all three anti SUT/SUF antibodies was restricted to sieve elements. Dashed lines in the light and FITC images delineate cell boundaries. Bar = 5 µm.

bs- bundle sheath; cc- companion cell; pp- phloem parenchyma; se- sieve element; x- xylem element; xp- xylem parenchyma.
Light  FITC

A. Pre-immune

B. PsSUT1

C. PsSUFI

D. PsSUFI4

FITC

Light
3.3.5 SDS-Page and Immunoblotting of Sucrose Transporters in Developing Seed Coats

The 12 daf seeds sampled for Western blotting (Fig. 3.10) were near the transition between the pre-storage and storage phases of seed development, with a relative water content (RWC) for the entire seed of approximately 80 – 85%. At this point, rapid cell division rates are declining and rapid cell expansion is occurring, with the seeds having a moderate absolute growth rate (measured as dry matter accumulation of cotyledons in Fig. 1A, Zhou et al., 2009). The cotyledons are very small (1-1.5 mm in length) and there is liquid endosperm present within the embryo cavity; this stage corresponds with seeds of Stage 23 of Marinos (1970). The cells of the seed coat are also rapidly expanding, and the inner layers would be intact, and not subject to crushing from the cotyledons, as seen in older seeds. Although the seed coat would be a major source of nutrients for the developing cotyledons and embryonic axis, as indicated by the gains in dry matter, it would not be the only one, with endosperm reserves also being consumed. At this point in development, the osmotic concentration of solutes in the liquid of the embryonic sac is over 900 mM (Marinos, 1970) and of this, approximately 170 mM is sucrose, and a further 10 mM hexoses (Rosche et al., 2005).

In contrast, at 20 daf the seeds are at early-mid storage phase, with cotyledons rapidly accumulating storage products, including starch and storage proteins (Rosche et al., 2005), and are at a maximal absolute growth rate. As endosperm has been depleted at this stage, nutrient needs of the embryo are met solely through export from the seed coat. As such, transporters responsible for this export should be at their peak in terms of activity and expression levels. However, the inner cell layers may be crushed to some degree by the abutting developing embryo as it continues to expand (van Dongen et al., 2003).
Figure 3.10. SDS PAGE and Western immunoblot analysis of PsSUT1, PsSUF1 and PsSUF4 protein expression in developing pea seed coats at 12 and 20 daf. Total microsomal protein was extracted from tissue as described by Ripp et al., (1988) and 30 µg protein loaded per lane.

A. Ponceau stain of protein transferred to nitrocellulose membrane, showing clear separation of protein bands. B - E. Western immunoblot of membrane probed with B. PsSUT1 antibodies; C. PsSUF1 antibodies; D. PsSUF4 antibodies and E. Non-immune control serum. Note two distinct bands for PsSUT1 in 20 daf seed coats (arrowheads). For B-E, antibody binding was visualised by probing with an alkaline-phosphatase conjugated anti-rabbit secondary antibody raised in goat, and undergoing a BCIP/NBT colour reaction.

All three SUT/SUF proteins were present in seed coat tissues, with a decline in the amount of protein as seed coats aged from 12 to 20 daf (Fig. 3.10B-D). PsSUF1 showed a weaker signal than PsSUT1 and PsSUF4 at both developmental stages.

Although all three of the pea transporters had the same predicted molecular mass of 55 kDa, the three antibodies labelled bands of different sizes. For PsSUT1, a 47 kDa band was labelled in both 12 and 20 daf seed coat extracts (Fig 3.10B). In addition to the 47 kDa band, two slightly smaller bands (of about 45 and 42 kDa) were labelled (noting arrowheads), with a much reduced intensity, as well as a faintly labelled band at
about 48 kDa. These smaller bands possibly represent partial protein degradation, while the 48 kDa band may be incompletely denatured protein that migrated more slowly than the bulk of the PsSUT1 protein. Anti-PsSUF1 antibodies were shown to label a single band of approximately 45 kDa (Fig. 3.10C). Antibodies raised against PsSUF4 labelled two bands at approximately 41 and 42 kDa, with the more abundant of these being the 41 kDa band (Fig. 3.10D). This is similar in size to a transporter identified in a sugar beet plasma membrane vesicle extract labelled with an AtSUC1 antibody (Sakr et al., 1997). Again, the slightly larger 42 kDa protein band may represent a small pool of PsSUF4 that has been incompletely denatured prior to SDS-PAGE separation. No bands were detected in either developmental stage using pre-immune serum (Fig. 3.10E).

In terms of transporter abundance throughout development, it appears that there is a reduction in abundance of both PsSUF1 and PsSUF4 in the 20 daf seed coats relative to the younger tissues. It is important to be mindful of the changes in relative membrane surface areas of the different cell types as the seed coats expand. In particular, it can be seen from micrographs in van Dongen et al. (2003) that much of the cell expansion between 10 and 20 days occurs within the thin-walled parenchyma layers, so any proteins that are restricted to other tissues (such as the vasculature) would appear to decrease due to changes in relative membrane quantities. However, for proteins that are well represented in the parenchyma layers (as PsSUF1 and PsSUF4 appear to be; see immunolocalisation in Section 3.3.7), the observed effect tends to indicate that both of these proteins decline in abundance as seed development progresses. Despite this small decline in facilitator abundance, immunolocalisation studies focused on the 20 daf seed coats, as at this stage of development seed absolute growth rates are at a maximum, hence maximal rates of sucrose efflux by seed coats is occurring.

### 3.3.6 Model of Sucrose Movement within Developing Seed Coats

Sucrose initially enters the developing seed coat from the pod wall and through the funicle via the vascular strand. In pea seed coats, the vasculature consists of a single chalazal vein with two lateral phloem strand branches (Hardham, 1976); the arrangement is similar in broad bean (Offler et al., 1989). Sucrose is transported radially around the seed coat through the chalazal vein over a considerable distance; in broad
bean this path length is in excess of 40 mm (Offler and Patrick, 1993). Beyond the chalazal vein, sucrose moves via lateral transfer to the non-vascular regions of the seed coat.

The extent of the symplasmic pathway of phloem unloading in pea seed coats extends radially from the chalazal vein and through the chlorenchyma and thin-walled parenchyma and into the inner parenchyma layers (Tegeder et al., 1999; van Dongen et al., 2003). So, sucrose may leave the phloem symplasmically, and move throughout the chlorenchyma and thin-walled parenchyma both radially and laterally before being effluxed from the thin-walled parenchyma transfer cells (Fig. 3.11). The bulk of movement occurs via the radial rather than lateral pathway, with 80% of photoassimilate movement occurring via the more direct radial pathway in broad bean (Offler et al., 1989). In the cells responsible for release, the thin-walled parenchyma transfer cells, it is possible that the facilitators mediate sucrose efflux. Evidence supporting the energy-independent efflux of sucrose has been presented for both pea (de Jong et al., 1996) and broad bean (Fieuw and Patrick, 1993).
Figure 3.11. A model showing the pathway of sucrose movement within a developing seed of *P. sativum*. Boxed regions show A. vascular and B. non-vascular regions examined for sucrose transporter expression (subsequent plates), while arrows show possible routes of sucrose movement from source maternal seed coat tissues to sink filial tissues. cot - cotyledon; cv - chalazal vein; sc - seed coat.
3.3.6.1 Seed coat anatomy

The outer cell layer of the seed coat consists of a single layer of dark-staining palisade or macrosclereid cells, underlain by a single layer of osteosclereid hypodermis. Chlorenchyma cells, which are chloroplast-containing parenchyma, are located beneath the hypodermis, occupying five to six layers (Fig. 3.12). At the 20 daf point in development, the parenchyma cells of the seed coat are highly vacuolated, providing sucrose storage capacity to buffer diurnal variations in sucrose delivery to the developing pod and seed from the plant, circumventing the need for storage as starch.

Seed coat anatomy studies and immunolabelling of sucrose transporters were carried out in both the vascular (Fig. 3.12 - 3.14) and non-vascular regions of the seed coat (Fig. 3.15 - 3.17) and will be presented in turn.

Anatomical studies of the chalazal seed coat region (Fig. 3.12) revealed the cellular detail of vascular tissue, including the arrangement of sieve elements (with the occasional companion cell) within the phloem (Fig. 3.12B). The identity of companion cells and enucleate sieve elements was established by DAPI staining of companion cell nuclei and Calcofluor white staining of cell walls (Fig. 3.14A, B). The cells immediately underlying the chalazal vein consisted of 4 to 5 layers of parenchyma cells, having thin walls and little other detail apparent when stained with toluidine blue. In some publications (see van Dongen et al., 2003 for pea, Yeung and Cavey, 1990 for bean), these cell layers have been referred to as branched parenchyma, due to their irregular shape and appearance of intercellular spaces. In soybean, this layer is described as aerenchyma (Thorne, 1981), while in broad bean, Johansson and Walles (1994) described this layer as being small parenchyma with prominent intercellular spaces. When compared to other examples of branched parenchyma, the degree of irregularity observed in this cell layer (Figs. 3.12A, C; 3.13B) is much less pronounced, probably better described as being lobed rather than branched. As such, the descriptor of thin-walled parenchyma has been adopted.

The inner layers of thin-walled parenchyma often undergo modification into transfer cells by the deposition of additional wall material, reflecting their putative role in sucrose efflux. However, these modifications could not be visualized at a light microscope level (Figs. 3.12C; 3.13B). The non-vascular region anatomy (Fig. 3.13) showed features identical to those observed for non-vascular tissues surrounding the chalazal vein (Fig. 3.12A, C).
**Figure 3.12. Anatomy of seed coat of a developing *P. sativum* seed, sectioned through the chalazal vein.** Light micrographs of a 1 µm thick transverse section cut through the vascular strand and stained with toluidine blue.

**A.** Low magnification micrograph showing location and extent of chalazal vein within the ground (parenchyma, collenchyma) tissues. Note that the epidermal tissue layer and underlying thin-walled parenchyma proximal to the developing cotyledons has been crushed (see also C). Bar = 100 µm.

**B.** Vascular tissues showing the location of sieve elements (arrows). Inset shows cellular detail of vascular tissues. B inset: bar = 10 µm.

**C.** Thin-walled parenchyma cells located on inner layers of the seed coat, showing evidence of crushing of the innermost layers of cells. The layers functioning as putative sites of efflux show modification as thin walled parenchyma/transfer cells at the electron microscopy level (see Tegeder et al., 1999). B, C: bar = 50 µm.

c- chlorenchyma; cc- companion cell; h- hypodermis (osteosclereids); le- lower epidermis; p- palisade (macrosclereids); ph- phloem; pp- phloem parenchyma; se- sieve element; twp- thin-walled parenchyma; vb- vascular bundle (chalazal vein); x- xylem.
Figure 3.13. Anatomy of the non-vascular region of developing P. sativum seed coat. Light micrographs of a 1 μm thick transverse section stained with toluidine blue. (A) Bar = 200 μm. (B) Detail of thin-walled parenchyma layers, showing evidence of crushing of the innermost layers of cells. Bar = 50 μm. twp- thin-walled parenchyma; le- lower epidermis; vb- vascular bundle; ph- phloem; p- palisade; h- hypodermis; c- chlorenchyma.
3.3.7 Immunolocalisation of SUTs/SUFs in Developing Seed Coats

Seed coat sections through the chalazal vein were stained with calcofluor white (Fig. 3.14A) to delineate cell walls, while DAPI was used (Fig. 3.14B) to stain nuclei (noting arrows) and so distinguish between companion cells and enucleate sieve elements and xylem elements. Immunolocalisation of sucrose transporters using PEP2 antibody showed labelling of sieve elements, as well as some non-specific labelling of what are possibly starch grains in phloem parenchyma cells (starred in Fig. 3.14D, E). Some labelling of phloem parenchyma plastids was also observed in the pre-immune serum, suggesting that this was non-specific binding of the secondary antibody in these cells.

The symporter PsSUT1 was found to localise to sieve elements within the chalazal vein (Fig. 3.15B). Interestingly, the observed distribution of PsSUT1 in what is presumably the plasma membrane was punctate. The PsSUT1 antibody did not label any other cells of the seed coat. When the vascular tissues were probed with the anti-SUF probes, PsSUF4 was also found to localise to sieve elements of the chalazal vein (Fig. 3.15D), with a similar punctate pattern of labelling to that seen for PsSUT1. This presents the situation where a sucrose symporter and sucrose facilitator are present in the same cells, with potentially opposing functions. PsSUF1 labelling was not observed for any cells in the chalazal vein (Fig. 3.15C).

In the non-vascular regions of the seed coat, immunolabelling with PEP2 (Fig. 3.16) showed an abundance of sucrose transporters throughout thin-walled parenchyma/transfer cell layers. In addition, subtle labelling of a second membrane within the cells, possibly the tonoplast, was observed (Fig. 3.16C, noting arrowheads).

Upon probing with the specific SUT/SUF antibodies, it was found that PsSUF4 showed a uniform labelling of plasma membrane throughout the thin-walled parenchyma, (Fig. 3.17D) reflecting the pattern observed for PEP2 in this region (Fig. 3.16). In addition, there was subtle labelling of PsSUF1 in cells that may have plasma membrane amplification due to transfer cell development (Fig. 3.17C and insert). No labelling by PsSUT1 was apparent (Fig. 3.17B), nor was there any specific labelling of other intracellular structures by PsSUT1, PsSUF1 or PsSUF4. Thus, the transporter labelled by PEP2 on what may be the tonoplast (Fig. 3.16C) may be an uncloned SUT family member.
Figure 3.14. Vascular tissue of a developing seed coat of *P. sativum*, showing immunolocalisation of sucrose transporters. Light and fluorescence micrographs of 1 µm thick transverse sections.

**A.** Calcofluor white staining showing extent of wall material.

**B.** DAPI staining of nuclear material, as indicated by arrows, can be seen in many of the companion cells, but not in the enucleate sieve elements.

**C.** Light micrograph image corresponding to D.

**D.** Immunolocalisation of sucrose transporters in seed coat vascular tissue using the generic anti-SUT antibody PEP2. Antibody binding was visualised using a FITC-conjugated anti-rabbit secondary antibody raised in goat. Diffuse plasma membrane labelling of all cells, including companion cells and vascular parenchyma was observed, while sieve elements were labelled more intensely. Dashed lines in C and D delineate cell boundaries.

**E.** Non-immune serum control, using column-purified pre-PEP2 immunisation rabbit serum.

A-E: bar = 10 µm.
A. Calcofluor White

B. DAPI

C. Light

D. PEP2

E. Pre-immune
Figure 3.15. Immunolocalisation of PsSUT1, PsSUF1 and PsSUF4 within vascular tissue of developing *P. sativum* seed coat. Fluorescence micrographs of 1 µm thick transverse sections through the chalazal vein.

**A** - **D.** Immunolocalisation of sucrose transporter protein with SUT/SUF primary antibodies detected by binding with FITC- conjugated secondary antibody.

**A.** Representative pre-immune control, using column-purified pre-SUT/SUF immunisation rabbit serum.

**B.** Labelling by anti-PsSUT1 labelling was restricted to sieve elements, and was punctate (inset) rather than uniform.

**C.** There was no apparent labelling of vascular tissue by anti-PsSUF1.

**D.** Punctate labelling of sieve elements was seen in the case of PsSUF4, suggesting that PsSUT1 and PsSUF4 (but not PsSUF1) co-localise. Some non-specific labelling of organelles (possibly chloroplasts) in chlorenchyma was seen for PsSUF4 (starred). c – chlorenchyma; cc - companion cell; se - sieve element; vp - vascular parenchyma.

A-D: bar = 10 µm.

B inset: bar = 2.5 µm.
Figure 3.16. Thin-walled parenchyma cell layers of the non-vascular region of developing seed coat from *P. sativum*, showing immuno-localisation of sucrose transporters. Fluorescence micrographs of 1 µm thick transverse sections.

A. Calcofluor white stain showing walls of thin-walled parenchyma cells located on the inner layers of the seed coat. The most innermost layer of cells have been progressively crushed as the abutting cotyledon (not shown) expands. Bar = 50 µm.

B. Non-immune serum control, using column-purified pre-PEP2 immunisation rabbit serum. Bar = 20 µm.

C. Immunolocalisation of sucrose transporters in thin-walled parenchyma using the generic anti-SUT antibody PEP2. Antibody binding was visualised using a FITC-conjugated anti-rabbit secondary antibody raised in goat. Plasma membrane of thin-walled parenchyma/ thin-walled parenchyma transfer cells was labelled extensively. Note labelling also of a membrane, possibly the tonoplast (arrows) with these cells. Bar = 20 µm.
Figure 3.17. Immunolocalisation of PsSUT1, PsSUF1 and PsSUF4 proteins in thin walled parenchyma of the non-vascular region of a developing *P. sativum* seed coat. Fluorescence micrographs of 1 µm thick transverse sections.

A- C. Immunolocalisation of sucrose transporter protein with SUT/SUF primary antibodies detected by binding with FITC-conjugated secondary antibody. A. Pre-immune serum control. B. No labelling was detected for PsSUT1. C. Very low levels of labelling of thin-walled parenchyma cell plasma membrane was observed for PsSUF1. The extent of this labelling can be seen in C inset (arrows). D. Labelling throughout the thin-walled parenchyma cell layers were observed for PsSUF4, indicated with arrows. No labelling of internal cell structures was observed.

A-D: bar = 50 µm. C insert: bar = 20 µm.
3.4 Discussion

The data presented in this chapter examines the potential contribution of cloned sucrose transporters (two facilitators and a sucrose proton symporter) to sucrose loading and unloading events in planta. The sub-cellular and cellular localisation in both leaves and developing seed coats will be examined in turn to assess the likely physiological significance of these transporters.

3.4.1 Sub-cellular Localisation of SUT/SUFs

The sub-cellular localisation of sucrose transporters will impact on their physiological function. Previous localisation studies reported in the literature have shown that sucrose transporters (all characterised as sucrose proton symporters) can be either plasma membrane or tonoplast proteins.

This study clearly showed that the three transporters studied, including the SUT4 clade member PsSUF4, were plasma-membrane localised (Fig. 3.3). Thus, it appears that the facilitators play a role in the exchange of sucrose between the apoplasm and symplasm. In the case of PsSUT1, its likely physiological role is the energised loading of sucrose into the cell from the apoplasm, which is similar to that reported for other SUT1 clade members.

In the case of SUT1 family proteins, there have not been any reported as being tonoplast-localised to date. Instead, SUT1 clade members, such as AtSUC9, have been shown to be plasma membrane localised (Sivitz et al., 2007). So, it is not surprising that PsSUF1 and PsSUT1 have been identified in this study as plasma membrane transporters.

The most similar SUT4 clade member to PsSUF4 that has undergone sub-cellular localisation study to date is LjSUT4 (Reinders et al., 2008). LjSUT4, like the monocot SUT4 clade member HvSUT2 (Weschke et al., 2000), AtSUT4 (Endler et al., 2006) and PtaSUT4 from poplar (Payyavula et al., 2011) appears to be tonoplast-localised. Although this legume family member (and closest studied relative to PsSUF4) is tonoplast-localised, this does not appear to be a legume-family wide trait. So, PsSUF4 is the first non-Solanaceae SUT4 shown to be plasma membrane localised. Other cloned SUT4s that have yet to be investigated in terms of membrane localisation include DcSUT1 (Shakya and Sturm, 1998), VvSUC11 (Davies et al., 1999) and OsSUT2 (Aoki et al., 2003). An apparent tonoplast targeting sequence has been recently
identified in the C-terminus region of the inositol transporter group of proteins in plants. This sequence is a di-leucine motif (Wolfenstetter et al., 2012). Perhaps not surprisingly, no such motifs were identified in the C terminus of PsSUT1, PsSUF1 or PsSUF4 (Fig. 3.4).

While the arrangement of PsSUT1 and PsSUF1 in the plasma membrane was fairly uniform and unremarkable, PsSUF4 was more punctuate (Fig. 3.3D). This observed punctate distribution could be accounted for by the finding that SUTs may be contained within plant plasma membrane micro-domains (Krugel et al., 2008). In addition, PsSUF4 labelled a perinuclear ring, and so appears that this fusion protein is expressed at a high level, to a point that it could be visualised in the endomembrane system prior to plasma membrane targeting. The combination of a punctate plasma membrane distribution and labelling of the perinuclear ring was similar to that for StSUT4 when expressed in tobacco (Chincinska et al., 2008). Likewise, LeSUT4 localisation in yeast also labelled membranes in the perinuclear region (Chincinska et al., 2008). It can be argued that plant phylogenetic relationships are not a clear predictor of SUT4 localisation. While the solanaceous SUT4 members, StSUT4 and LeSUT4, appear to be plasma-membrane localised (Chincinska et al., 2008), a recent report places the solanaceous NtSUT4 on the tonoplast (Okubo-Kurihara et al., 2011). Similarly, the legume LjSUT4 (Reinders et al., 2008) was tonoplast-localised, whereas the closely related PsSUF4 (this study) was plasma-membrane localised. To further complicate the picture in the legume family, there may be more than one paralog of SUT4 found in some species. There are two SUT4 family members in soybean (Payyavula et al., 2011) that have been identified through whole genome sequencing, while only one has been found to date in Medicago truncatula, as well as in non-leguminous species that have been sequenced, such as Arabidopsis, poplar (Payyavula et al., 2011) and rice (Aoki et al., 2003). It would be interesting to see whether both SUT4 paralogs in soybean are functional, and if so whether they localise to different membranes. Furthermore, as more plant species are fully sequenced and annotated, including members of the legume family, it is possible that more species will be found to have multiple SUT4 paralogs with differing physiological functions.

Localisation studies of the novel sucrose transporters were carried out to elucidate their in planta function. Previous investigation (Chapter 2) has shown that two of the transporters are sucrose facilitators (PsSUF1 and PsSUF4), and the third one (PsSUT1) a sucrose-proton symporter. As the sub-cellular localisation studies have shown these to
be plasma membrane transporters, it would be expected that the two facilitators would be found in tissues where sucrose movement is required across membranes down a concentration gradient. Such locations investigated include the mesophyll-minor vein interface of source leaves, and the sucrose effluxing layers of tissue within the maternal seed coat of a developing seed. The putative function of sucrose transporters in leaves (both sink and source) and developing seed coats will be examined in turn.

### 3.4.2 Sucrose transporters in sink leaves

Delivery of sucrose to sink leaves is generally considered to be symplasmic (Oparka and Turgeon, 1999). The absence of SUT/SUF signal from young leaf tissue (Fig. 3.5 B-D) is not surprising, given that the vascular tissue is still differentiating (as observed in the leaf venation studies in Fig. 3.4) and the sucrose transporters were restricted to sieve elements in source leaf vascular tissue (Fig. 3.9) and companion cells, in the case of the unidentified transporter labelled by PEP2 (Fig. 3.8D).

There is limited evidence for sucrose transporter expression in sink leaves in previous studies. GUS staining has shown that AtSUC9 is expressed in sink tissues, including the provascular tissue and mesophyll of sink leaves (Sivitz et al., 2007). However, SUT expression can commence as leaves undergo the sink-source transition. In Arabidopsis, for instance, AtSUC2 promoter-GUS fusion plants exhibited developmentally-dependent labelling of phloem in leaf tissue, with young leaves of Arabidopsis seedlings showing scant labelling (Truernit and Sauer, 1995). As leaf development progressed, expression of AtSUC2 in phloem of leaf veins progressed basipetally, reflecting phloem differentiation during sink-source transition of leaf development (Truernit and Sauer, 1995). Instead, sucrose transporter expression reports for sink leaves are predominately SUT2 clade members. For instance, PmSUC3 (a SUT2) is expressed in sieve elements of sink leaves in Plantago major whereas the SUT1 member PmSUC2 was restricted to petiole vascular bundles, and absent from sink leaves (Barth et al., 2003).

Functionally, it is possible that neither SUT family facilitators nor symporters are required for sucrose delivery to young sink leaf tissues (Tegeder et al., 2012). It appears that a system is in operation that relies on facilitated sucrose release by SBP, which is localised to sink leaf phloem (Contim et al., 2003), and subsequent cleavage by cell
wall invertases (Kocal et al., 2008) to maintain the sucrose concentration gradient to drive facilitated efflux.

However, the possibility remains that the absence of bands for these transporters may merely represent a dilution of the protein of interest, as seen for source leaves (further discussed below). Given that sucrose transporter expression is restricted to the phloem of minor veins and metaphloem of major veins, there are probably only very low levels of SUT/SUF proteins (below the level of detection via Westerns) in young sink leaves. At the transcript level, a high level of PsSUF4 expression was reported for sink leaves, while minimal transcript of PsSUF1 was observed using SUF-specific primers (Zhou et al., 2007). For PsSUT1 there was little transcript in sink leaves using a partial PsSUT1 probe (covering approximately a third of the coding region) (Tegeder et al., 1999). However, given the high homology between PsSUT1 and the two facilitators, it is possible that this probe recognized the RNA for more than one of these transporters.

There was a discrepancy between the findings of the Western blots for source leaf tissue (Fig 3.5), where PsSUF1 and PsSUF4 bands are absent, and their immunolocalisation in leaf minor veins, where antibody label for both of these proteins is clearly visible in sieve elements (Fig. 3.9C, D). As previously mentioned, this is most likely a result of the target proteins (PsSUF1 and PsSUF4) being diluted in the Western protein isolation below the limit of detection.

### 3.4.3 Sucrose Efflux in Source Leaves

The pea source leaf minor vein configuration has been classified as an apoplasmic (type 2B) loader, having very low plasmodesmatal frequencies between the SE-CC complexes and surrounding cells (Gamalei, 1989). Therefore it is theorized that in this species, both efflux and uptake membrane transport steps occur (see Fig. 3.6, noting circles). As can be seen from the model (Fig 3.6), there are three sites from which sucrose efflux could occur. These are at the plasma membranes of the mesophyll, bundle sheath or phloem parenchyma cells.

The generalist antibody PEP2 was used to probe the cells in these regions for SUT homologs that may possibly be involved in sucrose unloading. However, no evidence of sucrose transporters was found in the mesophyll, bundle sheath or phloem parenchyma cells (Fig. 3.8D). There has not, to date, been any direct molecular evidence of SUT1 activity in mesophyll cells, despite the limitations to apoplasmic movement of sucrose after efflux from the mesophyll plasma membrane. However, observations suggest that
SUT1 activity exists. Lemoine et al., (1996) created potato lines with companion cell-specific StSUT1 antisense, and were still able to detect sucrose transport activity in plasma membrane vesicles derived from source leaves. This led them to conclude that sucrose transporters are present at low levels, on mesophyll cell plasma membranes, and that they play a role in sucrose retrieval. An alternate role of SUT1 proteins in mesophyll cells could be in the efflux of sucrose, with the symporter working in reverse mode given the strong outward directed concentration gradient (a 10 to 100 fold difference - Ntsika and Delrot, 1986; Lohaus et al., 2001). If this was thermodynamically achievable however, it would again result in sucrose having to diffuse through the apoplasm against the transpirational stream.

In apoplastic loading species such as pea it appears more likely that sucrose moves via plasmodesmatal connections towards the vascular bundles; high densities of plasmodesmata between adjacent mesophyll cells and between mesophyll and bundle sheath cells (0.79 and 1.01 plasmodesmata µm² of cell wall respectively; Wimmers and Turgeon, 1991 and see Fig. 3.6B) in pea provide the symplasmic connectivity required. That would then make the likely site of efflux at the phloem parenchyma and/or bundle sheath cells in pea.

The feasibility of SUT reversal mediating sucrose efflux from the mesophyll or bundle sheath cells can be explored from a thermodynamics perspective. For a mesophyll cell membrane potential of -110 mV (Elzenga et al., 1995; Shabala et al., 2000), a trans-membrane pH difference of 1 and an apoplastic sucrose concentration of 1.5 mM (Lohaus et al., 2001), the Nernst equation (Equation 2-2) predicts that an intracellular sucrose concentration of 478 mM would be required to drive reversal of a sucrose symporter to mediate sucrose efflux from the mesophyll or bundle sheath cells. As mesophyll cytoplasmic sucrose concentrations are reported to be 20 - 200 mM (Ntsika and Delrot, 1986), this reversal seems unlikely from a thermodynamic point of view. Further, H⁺-sucrose transporters of the SUT family appear to be absent from these membranes, with no label detectable for either PsSUT1 or PEP2 antibodies (Fig. 3.8D, 3.9B). Thus the possibility remains that a non-SUT (and possibly novel) family of sucrose transport proteins is mediating this efflux.

A most apparent candidate for the efflux of sucrose in leaf minor veins is one or members of the SWEET sucrose facilitator family, given the localisation of AtSWEET11 and AtSWEET12 to what is most likely the phloem parenchyma in Arabidopsis source leaves (Chen et al., 2012).
The sucrose binding protein (SBP), characterised as a sucrose facilitator in yeast (Overvoorde et al., 1996), is another possible candidate. In soybean leaves, it is localised to the paraveinal mesophyll cell plasma membranes (Lansing and Franceschi, 2000). Pea is considered to have an attenuated paraveinal mesophyll morphology (Kevekordes et al., 1988), with some extended bundle sheath cells present (starred in Fig. 3.6A, B) that act to increase the connectivity between the spongy mesophyll and minor veins. Similarly, in Arabidopsis, the sucrose transporter AtSUT2 (same as AtSUC3) localised to bundle sheath cells surrounding the phloem in source leaf minor veins (Meyer et al., 2000). However, in pea, there was no apparent labelling of sucrose transporters by either general or specific antibodies in the bundle sheath cells. It would, however, be interesting to carry out immunolocalisation in the bundle sheath using an SBP antibody, given their apparent role in bridging between the mesophyll and phloem, and the capacity for the system to support facilitated release of sucrose to the apoplasm due to the steep outward-directed sucrose gradients. As the observed rates of sucrose efflux into the apoplasm are 6 orders of magnitude greater than that possible via diffusion across the plasma membrane alone, efflux must be carrier mediated (Giaquinta, 1983).

For the uptake of sucrose into SE-CC complexes, it appears that all three of the transporters - the symporter PsSUT1 and facilitators PsSUF1 and PsSUF4 may be involved, with all three localising to sieve elements (Fig. 3.9). In addition, an uncloned sucrose transporter appears to be present in companion cells, as indicated by the PEP2 label (Fig. 3.8D). There have been other reports of SUT proteins localising to companion cells in apoplastic loading species. For instance, NtSUT1 in tobacco (Schmitt et al., 2008), PmSUC2 in plantago (Stadler et al., 1995) and AtSUC2 in Arabidopsis localised to the companion cells of leaf minor veins. It has been suggested that SUT proteins in Solanaceous plants (Kühn et al., 1997; Barker et al., 2000; Kühn et al., 2003; Hackel et al., 2006) may have misattributed immunolabelling to the sieve elements when the labelling was actually due to an antibody (against a sieve element epitope) present in unpurified rabbit serum (Schmitt et al., 2008). In this study however, the use of affinity purified antibodies and pre-immune serum controls has eliminated this artifact, thus any labelling of the sieve elements is due to SUT or SUF protein presence.

In pea, despite transcript abundance of PsSUF4 in source (as well as sink) leaves (Zhou et al., 2007) no transporter protein was discernable in source nor sink leaf
Western blots (Fig. 3.5D), and labelling was restricted to the sieve elements of minor veins (Fig. 3.9D). Similarly, PsSUF1 antibody failed to label protein in both sink and source leaf samples (Fig. 3.5C). However, expression studies show only low levels of transcript in both of these organs (Zhou et al., 2007). For PsSUT1 source leaves, a low level of protein was detected in the Western blot (Fig. 3.5B). It is not clear whether the appearance of PsSUT1 and not PsSUF1 or PsSUF4 is related to the relative abundance of these proteins in source leaf tissue, or whether it reflects different efficiencies of the antibodies raised against the peptides.

Both SUT1 and plasma-membrane localised SUT4 clade members have been extensively reported to be SE-localised, particularly in solanaceous species. In tomato, immunolocalisation revealed the presence of LeSUT1 (Kühn et al., 1997) and LeSUT4 (Weise et al., 2000) in sieve elements. Similarly, SE-localisation of SUT4 clade members, including StSUT4 and LeSUT4 in stems has been reported (Weise et al., 2000), although expression studies indicate that LeSUT4 is more commonly associated with sink regions.

Being located on the sieve elements of minor veins in source leaves is suggestive of a role in sucrose loading of phloem from the apoplasm. However, when functionally characterised in yeast (Chapter 2), both PsSUF1 and PsSUF4 act as sucrose facilitators, transporting sucrose across the plasma membrane at rates linearly related to the concentration gradient. In sieve elements, it could be envisaged that the high luminal sucrose concentration (estimated at 0.5 - 1 M across a range of species; Lohaus et al., 1994) would drive sucrose movement out of the sieve elements. In addition to the two SUFs, it is possible that the sucrose facilitator SBP is also present in these sieve elements, as SBP localises to sieve elements in the minor veins of source leaves of spinach and soybean (Warmbrodt et al., 1989; Contim et al., 2003 but see Grimes et al., 1992 where companion cell localisation was observed). Juxtaposed to these facilitators, in the same sieve element is the symporter PsSUT1 (Fig. 3.9B), which like other SUT1 members may mediate the energy-dependent loading of sucrose into the sieve elements. This creates a situation where futile cycling of sucrose could occur. However, kinetic studies of these transporters (Chapter 2) show they have differing affinities for sucrose. PsSUT1 has a higher affinity, with an apparent Km of 1.5 mM, whereas PsSUF1 and SUF4 have lower affinities of 100 and 40 mM respectively. These differing affinities, in conjunction with their co-localisation in the sieve elements of leaf minor veins, are worth examining further in light of previously published tissue studies.
There appear to be two distinct carrier-mediated mechanisms for sucrose loading into the phloem of source leaves. For instance, in *Beta vulgaris* leaf discs, Maynard and Lucas, (1982a; 1982b) described a high affinity low capacity (HALC) \( \text{H}^+ \)-sucrose symporter with an apparent \( K_m \) value of about 1 mM and a non-saturable system that appeared to be energy-independent. Delrot and Bonnemain (1981) reported biphasic sucrose uptake for broad bean leaves (with the lower epidermis removed). Sucrose uptake was pH-independent at concentrations above 20 mM, whereas at below 5 mM a pH-sensitive mechanism was detected. The apparent \( K_m \) value for this system was about 5 mM at pH 5.5; this is comparable to the 1.5 mM apparent \( K_m \) value observed for PsSUT1 at pH 4.5 in Chapter 2 (Table 2.2). Also, both the \( \text{H}^+ \)-sucrose symporter in leaves of *B. vulgaris* (Maynard and Lucas, 1982b) and PsSUT1 uptake in yeast (Fig. 2.8) are competitively inhibited by maltose. Thus, PsSUT1 may account for the observed HALC in tissue studies. Likewise, PsSUF1 and PsSUF4 may represent the low affinity high capacity (LAHC) system also reported in these studies.

There are other mechanisms worth considering in the scenario of symporter and facilitator co-localisation that may mitigate futile cycling of sucrose. A similar situation that has been identified at the tonoplast of mesophyll cells, where the sucrose-proton antiporter AtTMT1 and sucrose-proton symporter AtSUC4 co-localise to the tonoplast membrane of mesophyll cells and move sucrose in opposing directions (Schultz *et al.*, 2011). In this case, the central loop of AtTMT1 is phosphorylated by a protein kinase, stimulating transporter activity (Wingenter *et al.*, 2011). This way, the transporters may be able to activate and deactivate to meet the short-term storage demands for the vacuole throughout the diurnal cycle. Although the situation differs in SE-CC complexes, with the cells committed to sucrose loading (rather than reversible flows of sucrose across the membrane), a similar capacity to activate/deactivate transporters, perhaps in conjunction with the operation of a dual HALC/LAHC loading system may exist.

Another possibility to consider is that the sucrose transporters are not necessarily active in transport function. Oligomerisation of 12 TMD transporter proteins appears to be a common feature of this group (for a review, see Veenhoff *et al.*, 2002). Hetero-oligomerisation of sucrose transporters has been reported previously (Reinders *et al.*, 2002b) where SUT1, SUT2 and SUT4 from potato have been shown to form complexes when heterologously expressed in yeast. This raises the question of what the impact of such interactions could mean for their *in planta* function. Significantly, Reinders *et al.*
(2002a) reported that StSUT1 and the plasma-membrane localised StSUT4 (as well as StSUT2) localised to the same sieve element, identical to what we have observed in our study. The difference is that both StSUT1 (Riesmeier et al., 1993) and StSUT4 (Weise et al., 2000) have been characterised as being sucrose-proton symporters in a yeast heterologous expression system, whereas we have characterised only PsSUT1 as a symporter. However, it is possible that, due to transporter interactions and hetero-oligomerisation, that the facilitators do not retain the energy-independent transport function established for them in vitro.

It is possible that there are regulatory interactions occurring between the different co-localised sucrose transporters. Several observations have lead to speculation that plasma-membrane localised SUT4 protein may be a negative regulator of SUT1 (Chincinska et al., 2008; Liesche et al., 2011). Knock-down transgenics of PtaSUT4 exhibited a slowed export from source leaves (Payyavula et al., 2011), whereas the opposite effect was observed for the knock-down of StSUT4 (Chincinska et al., 2008). In this case, sucrose export from source leaves to sink organs was enhanced, which is what would be expected for over-expression of the phloem loader SUT1, leading to speculation that SUT4 may act as a negative regulator of SUT1. Indeed, the effects of StSUT1 inhibition (Riesmeier et al., 1994) and StSUT4 over-expression appear analogous, with the accumulation of sugars and starch in source leaves (Liesche et al., 2011). It appears that the regulation of SUT1 by SUT4 occurs at a post-translational level, as it was noted that the abundance of StSUT1 was unaffected in StSUT4-RNAi plants; despite enhanced sucrose export (Chincinska et al., 2008).

SUT1 has been reported to occur in micro-domains or lipid rafts (Kruegel et al., 2008), increasing the likelihood of interaction with other proteins. To date, few studies have been carried out on the identification of proteins present within plant plasma membrane micro-domains. To obscure things further, the validity of the techniques used in isolating lipid rafts (by the extraction of detergent-resistant membrane fractions) has recently come into question (Tanner et al., 2011). Proteomic analysis of detergent-resistant membrane fractions from Medicago truncatula root plasma membranes has shown the presence of transporter proteins, including hexose-proton symporters, plasma membrane H⁺-ATPases and aquaporins (Lefebvre et al., 2007). Interestingly, these micro-domains also contained a suite of protein kinases, and a complete redox system. However, these findings now need to be corroborated using more robust methods. If such protein complexes are confirmed to exist in planta, it seems possible that the
different SUT clade members may occur together within these micro-domains. Here, the SUTs could interact with other proteins, such as kinases and peroxidases, which have the capacity to regulate SUT activity. It becomes apparent that the regulation of sucrose transporter activity is very complex; their deduced function determined by isolated expression in yeast may not resemble that seen in planta, where extensive interaction with other SUTs and proteins can occur.

### 3.4.4 Function of Seed Coat SUT/SUFs

Sucrose is delivered to pea seed coats in sieve elements incorporated into the chalazal vein and two lateral veins. The chalazal vein extends a considerable circumferential distance within the seed coat - in broad bean at a comparable developmental stage, the vein length is over 40 mm (Offler and Patrick, 1993). It is along the chalazal vein, in the sieve elements, where both PsSUT1 and PsSUF4 localised, to the same cells (Fig. 3.14A, B) in many ways similar to the minor vein situation discussed above for source leaves. Although the direction of sucrose flow has changed in the seed coat, with sucrose being unloaded rather than loaded, a putative facilitator and symporter are once again co-localised to sieve elements. There are several possible scenarios for the role of these transporters, and these will be examined in turn.

PsSUT1 labelling was restricted to the chalazal vein, being present in what appear to be sieve elements (Fig. 3.14B), with this accounting for the detection of this transporter in both 12 daf and 20 daf seed coats (Fig. 3.10B). This transporter may account for the sucrose retrieval mechanism detected by Ritchie et al. (2003) in populations of protoplasts derived from broad bean seed coat cells. The cell populations studied were those that had fewer thin-walled parenchyma transfer cells relative to other cell types. As the majority of this premature sucrose leakage is from the sieve elements and vascular parenchyma cells (Wang et al., 1995) it would be expected that the cells mediating retrieval would be in close proximity. Tegeder et al. (1999) reported the presence of PsSUT1 transcript in pea seed coat, and proposed that it was acting to retrieve sucrose leaked to the apoplasm along the delivery pathway. The localisation of PsSUT1 to sieve elements in the chalazal vein adds strength to this hypothesis.

An alternate role for PsSUT1 in the sieve elements is in the unloading of sucrose, working in reversal mode, as proposed by Carpaneto et al. (2005). The delineation of the symplasmic domain in pea seed coats using fluorescent tracers (Tegeder et al., 1999;
van Dongen et al., 2003) suggest that there is no intervening apoplastic step from the sieve element right through to the thin-walled parenchyma transfer cell layers. Additionally, calculations of the maximal possible sucrose efflux to the apoplast by SE-CC complexes (based on membrane surface areas and reported values for maximal active transport rates for sugars) show that they are capable of supporting only 5% of the sucrose flux required to support cotyledon growth in broad bean (Offler and Patrick, 1993). In contrast, when the additional surface area generated by wall ingrowths is taken into account, the thin-walled parenchyma transfer cells have sufficient capacity for sucrose flux to act as the sole site of sucrose efflux (Offler and Patrick, 1993). Thus, a physiologically significant role in sucrose efflux via reversal for PsSUT1 in the SE-CC complexes is unlikely.

In addition to PsSUT1, PsSUF4 co-localised to the same cells (Fig. 3.14D). This transporter was characterised as a facilitator when expressed in yeast (Chapter 2) and so again (as the case in leaf minor veins) it would appear there is a juxtaposition of leakage by PsSUF4 and retrieval by PsSUT1 in these sieve elements. However, given the punctuate arrangement of both of these transporters in this location, and the sub-cellular localisation of both PsSUF4 and PsSUT1 to the plasma membrane, it may be possible that these transporters are acting as part of a larger complex, as proposed for the sieve elements of leaf minor veins above. At the very least, it may be a SUT1-SUF4 hetero-oligomer, which is likely to change its transport characteristics. The possibility that SUF4 could be acting as a regulator of SUT1 (rather than a transporter in its own right) also exists, similar to the case seen in sieve elements of source leaves (Chincinska et al., 2008).

In the seed coat, there is scope for the proposed SUT1-SUF4 complex to carry out a dual function, if it had the capacity to sense apoplastic sucrose concentrations. When apoplastic sucrose rises above a given point, PsSUT1-mediated retrieval from the apoplast could occur, ensuring sucrose is retained within the sieve elements for delivery further along the circumferential pathway. When the sieve elements along the circumferential pathway and/or the plasmodesmatal conductances between the vascular parenchyma-thin walled parenchyma (shown to be a bottleneck in the pathway - Offler and Patrick, 1993) are at capacity, SUF4 could facilitate release from the sieve elements to the apoplast, thus providing a dual pathway of sucrose movement. The lag time from transcription through to protein translation is several hours (Vaughn et al., 2002); and so the regulation of protein activity rather than at the transcription level would
provide more immediate control of sucrose transport ability. This would be particularly useful in the case of sieve element-localised transporters (such as the case here) where the enucleate sieve element relies on the companion cell for transcription of transporters (Kühn et al., 1997).

3.4.4.1 Post phloem pathway and release to the seed apoplastic space

The post-phloem sucrose movement to the site of efflux can be conceptualized as two symplasmic routes through the tissues - one is a radial route, where sucrose moves out through the cells of the chalazal vein.

Calculated sucrose flux required through plasmodesmata at the vascular parenchyma/thin-walled parenchyma interface (based on plasmodesmatal frequencies and diameters) exceeds the maximum plasmodesmatal transport rates reported, hence a restriction to the symplasmic pathway may occur at this point (Offler and Patrick, 1993). However, no evidence of sucrose transporters (the three studied or any others homologous to SUTs) was found in the vascular parenchyma cells.

A proportion of the sucrose moving along the symplasmic, post-phloem pathway would be leaked to the apoplast by passive diffusion. Although it could be expected that a symporter could act as a retrieval mechanism, there was no evidence of PsSUT1 in the post-phloem pathway cells of ground or thin-walled parenchyma (Figs. 3.14A, 3.17A). It is not until later along the pathway, at the inner layers of thin-walled parenchyma, that the facilitators, PsSUF1 and PsSUF4 (Fig. 3.17B, C) localise. Harrington et al. (1997a) show that another putative facilitator SBP, immunolocalises to the innermost seed coat cell layers. H^+-ATPases are also distributed more extensively throughout these layers, commenting on their role as active sites of nutrient efflux.

3.4.5 Sucrose Delivery across Seed Coat Development

PsSUF4, and to a lesser extent PsSUF1 are present in the thin-walled parenchyma efflux layers (Fig 3.17B, C). When the location of PsSUF4 in thin-walled parenchyma, the ability of PsSUF4 to efflux sucrose (Fig. 2.11), and the apparent LAHC transport system (K_m = 40 mM; Chapter 2) are taken together, it appears that PsSUF4 may account for much of the energy-independent transport activity reported previously for seed coat sucrose efflux. For instance, for broad bean, it was estimated that half of sucrose release was by an energy-independent mechanism (Fieuw and Patrick, 1993). Similarly, de Jong et al. (1996) described energy-independent sucrose release from pea.
seed coats, although they concluded that this occurred by a non-selective pore, rather than a sucrose carrier such as PsSUF4. PsSUF1 is also located in thin-walled parenchyma (Fig 3.17C) although it appears less extensive than PsSUF4. It is difficult to determine whether this is due to a lower abundance of protein, or less efficient labelling by the anti-PsSUF1 antibody. The signal detected in the Western blot (Fig 3.10) showed a similar phenomenon, with a lesser signal for PsSUF1 for both pre-storage (12 daf) and storage phase seed coats. Both PsSUF1 and SUF4 are facilitators with high apparent \( K_m \) values for sucrose (100 and 40 mM respectively; Chapter 2), when expressed heterologously in yeast. The presence of an energy-independent, sulfhydryl-insensitive transport mechanism functioning under high external sucrose concentrations reported for broad bean seed coats, with an apparent \( K_m \) value of 550 mM (Ritchie et al., 2003), resemble the transport function of PsSUF1 and PsSUF4 (with apparent \( K_m \) values of 100 and 40 mM respectively), which have now been shown to localise to these sites of efflux. It is also worth noting that PsSUT1 is absent from these cell layers, adding support to the theory that they are playing a role in the retrieval of sucrose leaked prematurely from the vascular tissues.

The rates of pea embryo biomass gains reach maximal levels at around 20 daf (Zhou et al., 2009). Hence, if the SUFs (PsSUF4, also PsSUF1) are the main transporters responsible for sucrose efflux, their expression and activity should be at a peak at 20 daf. Yet, higher levels of PsSUF4 were observed in Western blots of 12 daf seed coats (Fig. 3.10D). This observation raises the question of whether the relative contributions of different modes of efflux may vary across development.

For instance, SUF-mediated efflux could be more prominent earlier in development, such as through the pre-storage phase (up until approximately 12 daf in pea and 25 daf in broad bean- Briarty et al., 1969; Lanfermeijer et al., 1989 respectively). During this time in development, the embryo is still expanding within the confines of the seed coat, and the inner layers of the seed coat parenchyma are still intact. In addition, there are high levels of extracellular invertase activity (Weber et al., 1995), which creates a large outward-directed sucrose concentration gradient to drive facilitated diffusion. As the embryo continues to expand, the inner seed coat layers are crushed and lysed, cell wall bound invertase expression decreases markedly (Weber et al., 1995) and so their contribution to maintaining the outward directed sucrose gradient by cleavage of sucrose into hexoses is diminished. To compensate for the collapse in trans-membrane sucrose gradient, energized sucrose release, mediated by sucrose-proton antiporters,
may be required. Evidence does exist supporting a role for antiporters in sucrose efflux from seed coats. Inhibitor studies of whole seed coats in broad bean (Wolswinkel and Ammerlaan, 1983; Fieuw and Patrick, 1993) and pea (Wolswinkel et al., 1983; Minchin and Thorpe, 1990) showed that at least part of sucrose release was mediated by energy-dependent carriers. In broad bean whole seed coat studies (of developmental stage comparable to the 20 daf seed coats used in our study), the sensitivity of sucrose efflux to the protonophore CCCP and the ATPase inhibitor EB indicated that sucrose antiporter-mediated transport may account for as much as 50% of observed sucrose efflux (Fieuw et al., 1993). Immunolocalisation has revealed that H⁺-ATPases are abundant in these cell layers (Harrington et al., 1997a) and could maintain the inward-directed proton gradient needed to support sucrose-proton antiport. In addition, the membrane potentials of seed coat parenchyma cells are measured to be approximately -50 mV (van Dongen et al., 2001), further contributing to the inward-directed proton motive force. A similar sucrose antiport mechanism was detected in French bean seed coats, (Walker et al., 1995). Whether this antiport mechanism is mediated by a SUT family protein remains to be seen, and the development of an experimental system to examine this further is presented in Chapter 4.

Changes in the mechanism of sucrose efflux across development could account for conflicting descriptions of the mode of sucrose delivery in different studies. It is worth noting that the seed coats studied by different research groups were of different developmental stages. van Dongen et al. (2001), in presenting evidence for non-selective pores in the release of sucrose in pea, used seed coats from seeds where the embryos were at 60% RWC. This approximates a seed coat age of 37 daf according to the relationship between cotyledon relative water content and age (Lanfermeijer et al., 1989), at which seed absolute growth rates have declined. Similarly, de Jong et al. (1996) used seeds of the same age, and were unable to detect energized transport for sucrose release. In contrast, studies by Fieuw and Patrick (1993) used younger seed coats in the linear phase of dry weight gain (at a RWC of ~80%), where dry weight gains are maximal. As such, the flux of sucrose from the seed coat would vary considerably. In addition, the cells mediating the efflux may vary between these two developmental stages - by 30 daf, micrographs of the chalazal layer and underlying cells in pea show that more than half of the layers of thin-walled parenchyma transfer cells (referred to as branched parenchyma in their paper) have been crushed and lysed by the expanding embryo (van Dongen et al. 2003). It would be informative to conduct an
immunohistochemical study on the expression of our two facilitators, as well as SBP and ATPases, across a developmental sequence to gain a more accurate perspective on the contributions of active and facilitated sucrose efflux to seed growth.
CHAPTER 4

Development of a System to Allow the Functional Characterisation of Sucrose Efflux Mediated by Novel Transporters
4.1 Introduction

In Chapter 2, the novel sucrose transporters PsSUT1, PsSUF1 and PsSUF4 from pea, and PvSUT1 and PvSUF1 from French bean were characterised in a heterologous yeast system (see Fig. 4.1A). In this experimental system, varying concentrations of sucrose, sulfhydryl modifying inhibitors, different sugar species and varying pH levels were applied to the extracellular face of the sucrose transporters. It was concluded that two of the transporters (PsSUT1 and PvSUT1) were sucrose-H⁺ symporters, while three (PsSUF1, PsSUF4 and PvSUF4) were energy-independent sucrose facilitators.

Immunolocalisation studies (Chapter 3) revealed that the plasma membrane localised facilitators PsSUF4 and (to a lesser extent) PsSUF1 are present in seed coat tissues committed to solute efflux. These transporters localised to thin walled parenchyma transfer cells positioned adjacent to the seed apoplastic space abutting the developing embryo (Fig. 3.17 and see Fig. 4.1A).

Heterologous expression of sucrose transporters in whole yeast cells is an experimental system commonly used for characterising their influx properties (Frommer and Ninnemann, 1995). However, it is not ideal for transporters that are putative sucrose effluxers. When comparing transporter orientation relative to sucrose flux across the membrane (Fig. 4.1A), the limitation becomes apparent. That is, the cytoplasmic face of the transporter is the binding site for sucrose efflux. However, in the whole yeast cell, only the extracellular face of the transporter is readily accessible for study.

A more appropriate system to enable study of the cytoplasmic face of effluxers would be one where the transporter is embedded in a membrane re-oriented to be inside-out. Proteoliposomes are one such system used to characterise transporter proteins such as the bacterial multidrug resistance proton/drug antiporters from Staphylococcus aureus (Grinius and Goldberg, 1994) and E. coli (Yerushalmi et al., 1995). To generate proteoliposomes, the transporter of interest is expressed, tagged, extracted, purified (usually via an affinity column) and reconstituted within phospholipids. Several freeze-thaw cycles are then carried out to re-orient proteoliposomes to an inside-out configuration. Proteoliposomes have been used with a variety of solute transport detection systems including radioisotope, electrophysiology and fluorescence measurements (e.g., Grinius and Goldberg, 1994; Yerushalmi et al., 1995). However, proteoliposome generation is time consuming and resource-intensive.
Figure 4.1. **Experimental systems for examining efflux characteristics of sucrose transporters.** Comparison of the orientation of sucrose facilitators (SUFs) functioning:

**A. *In vivo*** as seed coat facilitators, where sucrose efflux occurs from thin-walled parenchyma transfer cells located in the inner seed coat layers, and **B. *in vitro*** characterisation systems including whole yeast cells and inside-out secretory vesicles. Note that the orientation of the transporter protein in yeast secretory vesicles, where sucrose is presented to the cytoplasmic face, most closely represents the case *in vivo*.

**C.** Examples of experimental conditions imposed on plasma membrane vesicles to determine the energisation, if any, of sucrose efflux by sucrose transporters. Vesicle swelling under isotonic conditions with sucrose in the bathing media indicates facilitated sucrose transport. In the presence of ATP, which acidifies the vesicle lumen via ATPase activity, vesicle shrinkage due to sucrose efflux indicates symport, whereas vesicle swelling due to sucrose uptake indicates antiport.
A system that has gained popularity recently takes advantage of the sec6-4 mutant yeast (Novick et al., 1980). The mutation blocks the endomembrane secretory pathway causing inside-out membrane vesicles to amass in the cytoplasm (Walworth and Novick, 1987). The potential of using the sec6-4 mutant for amassing tightly sealed secretory vesicles to study membrane transporters was realised by Nakamoto et al. (1991). These authors placed their transporter of interest (a yeast plasma-membrane H⁺-ATPase) under the control of a heat-shock promoter, resulting in inside-out membrane vesicles highly enriched in this transporter. This system is becoming widely utilised to study a range of transporters including ATPases (Soupene et al., 2008; Petrov, 2009; Petrov, 2010), aquaporins (Kaufmann et al., 2005; Karlgren et al., 2005; Fischer and Kaldenhoff, 2008; Schnurbusch et al., 2010; Otto et al., 2010), calcium channels (Zhou et al., 2010), antimicrobial drug effluxers (Ruetz and Gros, 1994; Golin et al., 2007) and metal transporters (Gueldry et al., 2003; Lazard et al., 2011). However, there are no previous reports of its use in the characterisation of sucrose transport in the literature.

The sec6-4 yeast mutant potentially provides an ideal system for accessing the cytoplasmic face of our putative sucrose efflux facilitators (Fig. 4.1A). In addition, this system makes it possible to test whether sucrose-H⁺ symporters may function as effluxers under conditions where the potential energy in the outward-directed transmembrane sucrose gradient exceeds that of the inward-directed pmf. The study described in this chapter aimed to develop the sec6-4 secretory vesicle system, and adapt it to enable the characterisation of sucrose transporters.

For solute transporters, osmotic shrinkage or swelling of vesicles (resulting from solute movement) can be used as an indicator of membrane transport. Stopped-flow fluorimetry, which measures vesicle shrinkage or swelling by detecting changes in the light scattering properties of a vesicle suspension, enables real-time measurement of osmotic water movement (Solomon, 1989). In the case of sucrose transporters, the combination of the sec6-4 vesicle production system (see above) and detection of solute movement by stopped-flow fluorimetry provides a promising system for characterising sucrose transport. For instance, in isotonic conditions with sucrose in the bathing media only, swelling of vesicles indicates facilitated sucrose transport. Endogenous ATPases can be manipulated to determine whether sucrose movement is energised, with the addition of ATP to acidify the vesicle lumen. With equimolar sucrose inside and outside the vesicles, vesicle shrinkage due to sucrose efflux from vesicles indicates symport, whereas vesicle swelling due to sucrose uptake indicates antiport (Fig.4.1C).
addition, transporter kinetics of the cytoplasmic face for sucrose can be determined by concentration-dependent uptake studies, providing an understanding of transporter affinity and capacity for sucrose efflux. A major benefit of stopped-flow fluorimetry detection is that it replaces the use of radioisotopes, which can be hazardous and expensive. Stopped-flow fluorimetry is very sensitive to small transport fluxes due to the small vesicle volumes and hence can be carried out over short timeframes. This enables collection of real-time transport data sets to determine unidirectional transport rates that allow more precise estimates of transporter substrate affinities for efflux.

A particular requirement in adapting the sec6-4 mutant yeast system to study sucrose transport is to ensure that any osmotic shrinkage or swelling of the membrane vesicles is attributable only to transporter-mediated sucrose movement. Thus, other potential pathways for sucrose transport in the isolated membrane vesicles must be excluded. In this context, most laboratory yeast strains have the ability to utilise sucrose as a sole carbon source through hydrolysis by an extracellular invertase with subsequent uptake of the hydrolysis products by hexose transporters. This ability is attributable to the original sources of laboratory yeast strains being derived from baking and brewing isolates, both processes that rely on sucrose utilisation. In addition, one strain of yeast, which is the progenitor of several important laboratory yeast strains (including S288C, the yeast strain used for genome sequencing), was originally isolated from rotting figs (Mortimer and Johnston, 1986). S288C is the progenitor of the yeast strain SY1 used in this study.

In this study, we modified the established sec6-4 membrane transporter system to accommodate characterisation of sucrose transporters. To do this, the metabolism and transport of sucrose by enzymes and transporters endogenous to the sec6-4 system was averted using genetic approaches. The initial approach involved using antibiotic (kanamycin or bleomycin) resistance gene knockout of the endogenous invertase gene. A subsequent and successful approach involved incorporating the sec6-4 mutation into SUSY7 yeast, which has an invertase and maltose transporter-free genetic background.
4.2 Materials and Methods

4.2.1 Yeast Strains and Plasmids

The yeast strains and plasmids used for this study are presented in Table 4.1 and Fig. 4.2. The sec6-4 mutant yeast strains used were provided by Prof. Wolf Frommer (Carnegie Institute, Stanford University, USA- SY1-F) and by Prof. Carolyn Slayman (Dept. of Genetics, Yale University School of Medicine USA- SY1-S). The plasmid pRN90 was supplied by Prof. Slayman. The plasmid pSK-sec6-4-URA3 (Lamping et al., 2005) was kindly provided by Dr. Erwin Lamping (University of Otago, New Zealand). Plasmids pUG6 (Güldener et al., 1996) and pUG66 (Güldener et al., 2002) were sourced from the European Saccharomyces cerevisiae Archive for Functional Analysis (EUROSCARF), University of Frankfurt, Germany.

Table 4.1. Yeast strains and plasmids used or developed in this study.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUSY7/ura3</td>
<td>MATa leu2Δ::p1282A2Susy suc2Δ::URA3 mal0 GAL trp1-92 ura3</td>
<td>Riesmeier et al., 1992; Barker et al., 2000</td>
</tr>
<tr>
<td>SY1-F</td>
<td>MATa sec6-4 GAL suc2Δ::URA3 leu2-3,112 his4-619</td>
<td>Wolf Frommer, Stanford, USA</td>
</tr>
<tr>
<td>SY1-S</td>
<td>MATa sec6-4 GAL ura3-52 leu2-3,112 his4-619</td>
<td>Nakamoto et al., 1991</td>
</tr>
<tr>
<td>s6s7</td>
<td>SUSY7/ura3 sec6-4</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUG6</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt; gene conferring resistance to the antibiotic G418 (kanamycin), flanked with loxP sites allowing marker removal by expressing cre recombinase. E. coli selectable marker is Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Güldener et al., 1996; EUROSCARF</td>
</tr>
<tr>
<td>pUG66</td>
<td>Blc&lt;sup&gt;R&lt;/sup&gt; gene conferring resistance to the antibiotic phleomycin, flanked with loxP sites allowing marker removal by expressing cre recombinase. E. coli selectable marker is Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Güldener et al., 2002; EUROSCARF</td>
</tr>
<tr>
<td>pSK-sec6-4-URA3</td>
<td>pBluescriptIISK(+) containing XhoI/KpnI fragment of sec6-4 with modified EcoRV site and URA3 selectable marker</td>
<td>Lamping et al., 2005</td>
</tr>
</tbody>
</table>
Figure 4.2. Maps of plasmids used in generating a sucrose efflux characterisation system. A. pUG6 contains the kanamycin resistance gene flanked by LoxP recombination sites. B. pUG66 has a similar arrangement with the bleomycin resistance gene. Both pUG6 and pUG66 were used as PCR template to generate invertase disruption fragments. C. pSK-sec6-4-URA3 contains the sec6-4 mutant gene, with a PstI cut site to facilitate integration into the yeast genome, and a destroyed EcoRV site to enable screening.

4.2.2 Yeast Culture

Yeast cells were cultured in yeast extract peptone media or plates, supplemented with 2% glucose (YPG) or sucrose (YPS). Liquid cultures were grown at 30 °C on a rotary shaker circulating at 250 rpm.

4.2.3 Disruption Fragment Generation

The generation of SUC2-targeted KanR disruption fragments were generated according to Güldener et al., 1996). Briefly, primers were designed (Table 4.2) with a region homologous to 41 bp at either the 5’ (forward primer) or 3’ (reverse primer) end
of the \textit{SUC2} gene. For each primer, a region with 19 bp homologous to the KanMX cassette of the plasmid pUG6 was included. Thus, PCR using pUG6 as a template amplified the KanMX cassette, including the kanamycin resistance gene, with a 41 bp region at each end of the PCR product homologous to the target gene. Fragments were gel purified prior to transformation.

\textbf{Table 4.2. DNA oligomers used in this study.}

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ – 3’</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>KANdisF</td>
<td>CAAGCAAAAAAAGCTTTTCTTTCACTAAGGTGACAGGTGACAACTTTAAT</td>
<td>41bp from SUC2 upstream region plus 19bp from KanMX cassette (underlined)</td>
</tr>
<tr>
<td>KANdisR</td>
<td>TCTTTTGAAAAAAATAAAAAAAGCAGGTGACAGGTGACAACTTTAAT</td>
<td>41bp upstream of SUC2 stop region plus 19bp from KanMX cassette (underlined)</td>
</tr>
<tr>
<td>KANinfF</td>
<td>CTTCCGACCATCAAGCATTTCACAGCACTTTAAT</td>
<td>Internal primers to confirm presence of Kan\textsuperscript{R} gene</td>
</tr>
<tr>
<td>KANinR</td>
<td>TTAACAGGCCCAGCATTACCACTTTAAT</td>
<td></td>
</tr>
<tr>
<td>BLEinfF</td>
<td>CTTCCGACCATCAAGCATTTCACAGCACTTTAAT</td>
<td>Internal primers to confirm presence of Ble\textsuperscript{R} gene</td>
</tr>
<tr>
<td>BLEinR</td>
<td>TTAACAGGCCCAGCATTACCACTTTAAT</td>
<td></td>
</tr>
<tr>
<td>INVinfF</td>
<td>AACACCACAAACATATCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC</td>
<td>Internal primers to confirm presence of SUC\textsubscript{2} gene</td>
</tr>
<tr>
<td>INVinR</td>
<td>TCCATCGTTGAAGTACAATTCCACAGCACTTTAAT</td>
<td></td>
</tr>
<tr>
<td>INVdcfF</td>
<td>ATCCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC</td>
<td>Primer to amplify gene inserted into SUC\textsubscript{2} locus, located 156 bp upstream from ATG of SUC\textsubscript{2} coding region</td>
</tr>
<tr>
<td>INVdcfR</td>
<td>AGCCCTTTAGAATGGCTTGTG</td>
<td>Primer to amplify gene inserted into SUC\textsubscript{2} locus, located +56 bp from STOP of SUC\textsubscript{2} coding region</td>
</tr>
<tr>
<td>SEC6f1927</td>
<td>CTTCCGACCATCAAGCATTTCACAGCACTTTAAT</td>
<td>Internal SEC\textsubscript{6} primers covering modified EcoRV site, to facilitate screening of transformants</td>
</tr>
<tr>
<td>SEC6r2348</td>
<td>TCTCCGACCATCAAGCATTTCACAGCACTTTAAT</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{4.2.4 Yeast Transformation Methods}

For plasmid transformations of yeast cells, the method of Dohmen \textit{et al.} (1991) was adopted. Competent yeast cells were prepared by growing cultures overnight until an \textit{OD}_{600} of 0.8 was reached. 100 mL of yeast culture was harvested by centrifugation at 5000g for 5 min, and resuspending the pellet of yeast cells in 2 mL of 1 M sorbitol, 10 mM bicine-NaOH (pH 8.35), 3% ethylene glycol, 5% DMSO. Competent cells were stored as 200 \textmu L aliquots at -80 °C until required. For transformation, 2 - 5 \mu g plasmid and 50 ng carrier DNA (single stranded DNA from salmon testes, previously denatured by heating to 95°C for 5 min then rapidly cooled on ice) were added directly to the
frozen yeast cells, and mixed vigorously for 5 min at 37°C. 1.4 mL 40% PEG1000, 0.2 M bicine-NaOH (pH 8.35) was added, and the mixture incubated at 30°C for 1 h. Cells were harvested by centrifugation at 16,000g for 30 sec, and the pellet washed with 1.6 mL 150 mM NaCl, 10 mM bicine-NaOH (pH 8.35), before resuspending in 100 µL of the same solution and plated onto appropriate selection plates.

Invertase gene disruptions in yeast were carried out using the one step gene disruption approach (Rothstein, 1983). Briefly, this method relies on chromosomal insertion of a genetic marker based on homologous recombination, with the marker replacing part or the entire target gene, resulting in stable transformation. For these transformations, the lithium acetate protocol of Güldener et al., 1996) was used. Yeast cells were grown overnight in 50 mL volumes to an OD₆₀₀ of 0.8, harvested by centrifugation at 2,500g for 5 min, washed with 10 mL sterile H₂O, harvested, washed with 1.5 mL sterile 100 mM LiAc, 10 mM Tris–HCl, 1 mM EDTA, pH 7.5 and resuspended in 200 µL of the same solution. For each transformation, 50 µL of cell suspension was used. To this, approximately 5 µg of purified DNA fragment, 50 µg denatured salmon testes carrier DNA and 300 µL 40% PEG 4000, 100 mM LiAc, 10 mM Tris–HCl, 1 mM EDTA, pH 7.5 were added, and incubated with gentle shaking at 30°C for 30 min. Cells were then heat shocked in a heating block at 42°C for 15 min to facilitate uptake of fragment DNA. 800 µL sterile H₂O was added before harvesting the cells at 16,000g for 10 sec, resuspending them in 200 µL of H₂O and plating them onto selection plates. For kanamycin selection, Geneticin® G418 (Sigma A1720) at 200 mg/L was used with YPG plates.

4.2.5 Sequencing

Gel purified fragments were blunt-end ligated with T4 DNA ligase into the sequencing plasmid pGEM®-T Easy (Promega), using their standard protocol. Sequencing was carried out by the Australian Genome Research Facility (Brisbane, Queensland) using T7 and SP6 primers.

4.2.6 Invertase Activity Assay

The invertase activity of yeast strains was assayed as follows. Yeast cells were grown to an OD₆₀₀ ~ 1.0, harvested and resuspended in 50 mM sucrose. Following incubation at 30°C for 1 h, the yeast cells were removed by centrifugation (two
successive centrifugations at 3000 rpm for 10 min at 4°C) and an enzyme-linked hexose assay carried out to determine free glucose in the supernatant. For the glucose assay (Boehringer, 1984), 3.1 mL of assay reagent [16 mM triethanolamine, 1.5 mM MgSO₄, 0.006% (w/v) BSA, 0.15 mM dithiothreitol, 2.5 mM ATP, 20 mM NaHCO₃], 5.8 units hexokinase, 2.9 units glucose-6-phosphate dehydrogenase and 2.18 mg NAD+ were added to 20 µL of supernatant. Absorbance was measured at 340 nm following a 15-min incubation, to determine glucose concentration. Data were expressed as a percentage of the invertase activity calculated for untransformed sec6-4 yeast.

4.2.7 Vesicle Isolation

Yeast secretory vesicles were isolated using the method of Coury et al. (1999). Cultures were grown in YPG + 2% glucose on a rotary shaker (250 rpm) at 30°C until OD₆₀₀ ~ 1.0. Cells were harvested at 4000g (5000 rpm in a Beckman SLA1500 rotor) for 5 min and resuspended in fresh media to an OD₆₀₀ of 0.5. Cultures underwent heat shock by growing for 2 h with shaking at 37°C before harvesting 50 mL of culture cells (OD₆₀₀ ~1.0) and resuspending in 1 mL of 10 mM DTT, 100 mM Tris-Cl, pH 9.4. Following a 10 min incubation at 25°C, cells were centrifuged at 4000g (5000 rpm in Beckman SLA1500 rotor) at 4°C for 5 min and resuspended in 1 mL of spheroplast buffer (1.2 M sorbitol, 50 mM K₂HPO₄, pH 7.5, 10 mM NaN₃, 40 mM β-mercaptoethanol). Cell walls digested with lyticase (Sigma L4025, at 100 units/mL spheroplast buffer) for 45 min at 37°C with gentle shaking. Spheroplasts were harvested at 2500g (4000 rpm in SLA1500 rotor) at 4°C for 5 min and resuspended in 1 mL spheroplast - MgCl₂ buffer (1.2 M sorbitol, 50 mM K₂HPO₄, pH 7.5, 10 mM NaN₃, 40 mM β-mercaptoethanol, 10 mM MgCl₂), with Concanavalin A (Sigma C2010) added to 1mg/mL final concentration, and incubated at 0°C for 15 min. Concanavalin A is a lectin which binds to the now-exposed plasma membranes, enabling their removal by centrifugation after cell lysis. Spheroplasts were harvested at 3000g at 4°C for 10 min and resuspended in 770 µL lysis buffer (0.8 M sorbitol, 10 mM TEA, 1 mM EDTA, pH 7.2) to which protease inhibitor cocktail (Sigma P8215) is added at the rate of 3.8 µL/mL lysis buffer. Spheroplasts were ground by 10 pestle strokes in a Dounce homogeniser on ice. Plasma membranes and cellular debris were removed by centrifugation at 20,000g at 4°C for 10 min (13,000 rpm in Sorvall SS-34 rotor). The secretory vesicles were harvested from the supernatant by centrifugation at
120,000g at 4°C for 1 h (59,000 rpm in Beckman TLA 100.4 rotor). The pellet was
resuspended via pipetting in 0.3 M sorbitol, 25 mM Tris-HCl, pH 7.05, with protease
inhibitor cocktail added (Sigma P8215; 3.8 µL/mL). Protein determination was carried
out using the method of Bradford, 1976; also see Section 3.2.4.2).

4.2.8 Proton Pumping Assay

For each reaction, isolated secretory vesicles (50 µg protein) were added to 200 µL
reaction medium, 230 mM sorbitol, 6.25 mM MgSO₄ (to activate ATP), 125 mM KCl,
5 mM bis-tris-propane/HCl pH 6.5 containing 5 µM quinacrine. Relative fluorescence
was assayed over time in a Shimadzu model 5301PC spectrofluorophotometer with an
excitation/emission spectra of 430/500 nm. After several min equilibration, 40 µL ATP
(50 mM Na₂-ATP in reaction medium) was added, to drive proton pumping and so
fluorescence quenching. After at least 5 min of ATP-mediated proton pumping, the
protonophore nigericin (Sze, 1980) was added (to 10 µM final concentration) to
dissipate the trans-membrane proton gradient.

4.2.9 Stopped-Flow Fluorimetry

Vesicles were resuspended (600 µg/mL) in medium containing 10 mM MgSO₄, 5
mM HEPES, 0.3 M sucrose, (or 0.3 M sorbitol for the osmotic gradient experiments),
pH 7 except for the invertase inhibition experiments, where 10 mM MgSO₄, 5 mM Tris,
0.3 M sucrose, pH 7 was used. For the imposed osmotic gradient experiments, bathing
solutions were prepared by the addition of sorbitol to the medium to increase the
osmolality by 100, 200, 300 and 400 mosmol kg⁻¹. When injected against an equal
amount of vesicle-containing medium during the course of the experiments (1 mL each
per experiment), the resulting test solutions generate the specified osmotic gradients
(50, 100, 150, 200 and 300 mosmol kg⁻¹ respectively). The osmolality of all solutions
were verified in a Vapro 5520 osmometer (Wescor, Logan USA) prior to use. 5 mM
ATP was added to all bathing solutions as an energy source.

A stopped-flow fluorimeter (DX.17MV, Applied Photophysics, Leatherhead UK)
was used to measure changes in light scattering due to vesicle shrinkage. Light
scattering was measured at a wavelength of 500 nm at 1 ms intervals, with the
photomultiplier set to 550 v.
Curves of measured light scattering over the first second were fitted to the equation $y = a e^{b t}$, where: $a =$ amplitude, $b =$ instantaneous light scattering rate, $t =$ time since vesicle injection.

Curve fitting was carried out by linear least squares fits, using an online linear regression calculator (http://www.xuru.org/rt/LR.asp), and solved for $b$ to give the initial rate of osmotic swelling induced by the imposed osmotic gradients.
4.3 Results & Discussion

The development of a sec6-4 secretory vesicle system suitable for sucrose effluxer characterisation is reported in chronological order, with each new approach instigated on the findings of the preceding one. There are three main phases of development presented (Fig. 4.3). Firstly, preliminary data obtained by stopped-flow fluorimetry, identified that modifications were required for successful implementation.

Figure 4.3. Phases of development carried out to adapt the sec6-4 secretory vesicle transporter expression system for functional characterisation of sucrose transporters.

The second phase engineered an invertase- and maltose transporter-free sec6-4 yeast strain suitable for vesicle production to study sucrose transport. The final phase details the requirements for an expression vector suitable for use with the newly developed
sec6-4 yeast strain. Initial efforts focused on attempting to knock out invertase from sec6-4 yeast sourced from the Frommer (Phase 1) and then Slayman (Phase 2) labs. Following evidence of multiple invertase genes, a strategy to incorporate the sec6 mutation into an invertase null yeast strain (SUSY7) was adopted (Phase 3), yielding an invertase-free sec6-4 yeast strain suitable for sucrose efflux characterisation.

The sec6-4 yeast used in this study has a temperature-sensitive mutation in one of the genes required for the exocytosis step of the post-golgi secretory pathway in cells (Novick et al., 1980; Potenza et al., 1992). As a result of the mutation, large numbers of inside out membrane vesicles accumulate within the cytoplasm at the non-permissive temperature of 37°C. These mutant yeast cells can be exploited by engineering them to heterologously express a transporter of interest transformed into the yeast on a plasmid (Nakamoto et al., 1991). As the tightly-sealed membrane vesicles are in an inside-out configuration, it becomes possible to access the cytoplasmic face of the transporter for study. This is not possible in more commonly-used heterologous expression systems, including whole yeast cells (Weise et al., 2000) and Xenopus oocytes (Sivitz et al., 2007).

Laize et al. (1995) exploited the sec6-4 mutant for amassing inside-out vesicles to study water movement mediated by the human aquaporin CHIP28, whose expression was placed under the control of a galactose-inducible promoter. These authors then functionally characterised CHIP28 by studying time-dependent vesicle shrinkage detected by light scattering using stopped-flow fluorimetry.

One particular feature of this system that lends itself to the study of proton-coupled transport is that the presence of endogenous H⁺-ATPases can be easily exploited (by the addition of ATP) to generate a trans-membrane (outward-directed) proton gradient (Coury et al., 1999). This may be particularly useful for studying putative sucrose-proton antiporters (see Fig. 4.1C) as the outward-directed proton gradient can be harnessed to drive sucrose uptake into the vesicle causing osmotic swelling. The system is quite sensitive, given the limited volume of the vesicles, and so even modest rates of sucrose uptake will result in the osmotic movement of water into the vesicle, which is detectable using stopped-flow fluorimetry. The success of the system however, relies on any water movement during the course of the experiment being attributable to transporter function only. This then allows study of the transporter kinetics. In the case of sucrose, which is a carbon source for many laboratory yeast strains, the effects of endogenous systems for sucrose transport and metabolism must be removed. In yeast,
invertase is the main sucrose metabolizing enzyme, with the secreted form being trafficked to the plasma membrane for exocytosis via secretory vesicles. Thus, an invertase-null strain of sec6-4 yeast is required to avoid sucrose hydrolysis creating spurious results when measuring vesicle shrinking or swelling.

4.3.1 Phase I: sec6-4 yeast from Frommer Lab

Sec6-4 yeast supplied by Prof. Wolf Frommer (Carnegie Institute, Stanford University, USA) and designated SY1-F, was used for Phase 1 of sec6-invertase free yeast development (Fig. 4.3). Originally sourced from Nakamoto et al., (1991), SY1-F had been subsequently modified by Brigitte Hirner of the (Berlin) Frommer laboratory to be putatively invertase-deficient. This modification was carried out using homologous recombination with a selectable marker (URA3) targeted to replace the SUC2 gene.

4.3.1.1 SY1-F yeast yields functional vesicles

Yeast secretory vesicles were harvested from SY1-F following heat shock. A proton pumping assay (Sze, 1980) was carried out by Dr. Yuchan Zhou, with the assistance of Mr. Nathan Moon, to ensure the vesicles were inside-out and sealed tightly to render them proton impermeable (Fig 4.4). Isolated vesicles were incubated in the membrane-permeable fluorochrome quinacrine. When ATP is added, the active sites of the H⁺-ATPases (which are exposed to the bathing solution in inside-out vesicles) are accessible, with ATP driving vesicle lumen acidification (see Fig. 4.1C). The decreasing pH within the vesicle lumen results in quinacrine quenching, observed as a decrease in relative fluorescence. Upon addition of the protonophore nigericin (Sze, 1980), the proton gradient is dissipated, with the vesicle lumen pH rising and relative fluorescence of quinacrine returning to levels measured at the initial addition of ATP (Fig. 4.4). Thus, the vesicles isolated appear to be functional and capable of proton pumping across a proton impermeable membrane.
Figure 4.4. pH-dependent fluorescence quenching of quinacrine in inside-out vesicles from SY1-F, due to proton pumping. Relative fluorescence quenching of quinacrine commenced with the addition of ATP (2 mM final concentration) and was reversed by the addition of 10 μM nigericin.

4.3.1.2 Acid invertase is present in sec6-4 secretory vesicles

To test for any background sucrose movement or hydrolysis, isolated SY1-F yeast secretory vesicles were investigated for their responses to sucrose via stopped-flow fluorimetry. Note that these vesicles were from untransformed yeast, and so sucrose transporters are absent. In a system suitable for the study of transporter-mediated sucrose movement, an equimolar sucrose distribution across the vesicle membranes (where preloaded vesicles are injected against a test solution with identical sucrose and osmotic concentrations) should not result in any time dependent swelling or shrinkage of vesicles. This should hold true for energised (+ ATP) and non-energised conditions. Any vesicle shrinkage or swelling observed under these conditions would indicate sucrose metabolism, which is undesirable for this system.

The following data (contained in Fig. 4.5) was collected by Dr. Yuchan Zhou, with the assistance of Mr. Nathan Moon. In a non-energised state, the equimolar trans-
membrane sucrose distribution (0.3 M sucrose either side of the membrane) resulted in a negligible change in light scattering due to vesicle shrinking or swelling (Fig. 4.5, noting the slight shrinkage observed for HEPES trace). However, when ATP (in 0.3 M sucrose, 5 mM HEPES, 10 mM MgCl₂ to a 5mM final concentration) is added to the sec6-4 vesicles, vesicles underwent rapid swelling (Fig. 4.5, noting HEPES + ATP trace), which indicates that the intra-vesicular osmotic content exceeds that of the bath solution. Thus, the addition of ATP has driven an increase in the intravesicular osmotic content. One possible mechanism resulting in this increase is the cleavage of intravesicular sucrose by acid invertase. A glucose assay carried out on vesicles following incubation with ATP confirmed that sucrose hydrolysis had occurred (data not shown). So, despite being purportedly invertase-free, the data indicate that invertase is in fact still present and active in the intravesicular space.

Acid invertase, which is the secreted form of invertase, is trafficked to the plasma membrane via secretory vesicles. This enzyme has a reported pH optimum of 4.5-5.0 (Woodward and Wiseman, 1978). A likely mechanism driving sucrose cleavage upon ATP addition is mediated via the activation of endogenous, internally-directed ATP-activated proton pumping ATPases, acidifying the internal environment (see Fig. 4.4). As the vesicle lumen, which was buffered at pH 7.0, becomes acidified, the activity of residual acid invertase activity increases, driving sucrose cleavage into glucose and fructose. This cleavage of sucrose increases osmotic potential inside the vesicle, resulting in directional water movement and vesicle swelling (Fig. 4.5, noting HEPES + ATP trace).

Acid invertase of S. cerevisiae has a reported $K_m$ of 25 mM for sucrose (Andjelkovic et al., 2010), so the intravesicular concentration of 300 mM is sufficient to see cleavage of sucrose into hexoses, and a corresponding increase of osmotic potential inside the vesicle.

To confirm that the osmotic water movement results from invertase activity, the known acid invertase inhibitor Tris (Hatch et al., 1963) was substituted for HEPES both inside and outside of the vesicles. At 25 mM Tris, no swelling of vesicles occurred following the addition of ATP (Fig. 4.5, noting Tris + ATP trace), with the Tris alone and Tris + ATP traces being identical. The inhibition of vesicle swelling by Tris addition indicates that invertase-mediated sucrose cleavage is the likely cause.
Figure 4.5. Sucrose transport in energised and non-energised secretory vesicles isolated from SY1-F yeast. Typical fluorimetry traces of secretory vesicles isolated from SY1-F, preloaded with 0.3 M sucrose and 10 mM MgCl₂ in a zwitterionic buffer by equilibration. Vesicles were injected against an identical solution, with 5 mM ATP included in the +ATP treatments. For HEPES traces, 5mM HEPES buffer was used, while Tris traces used 25 mM Tris as the buffer.

Further, while the endogenous yeast maltose transporters are capable of energised sucrose transport (Stambuk et al., 1999), they would not contribute to the observed vesicle swelling. In vivo yeast maltose transporters mediate maltose and sucrose uptake, thus in this system they would mediate sucrose efflux, shrinking the vesicles (see Fig. 4.1C, noting the symporter response). So, although the SY1-F yeast strain of sec6-4 supplied was putatively invertase-deficient, due to invertase knock-out, it appeared that invertase was in fact present.

To enable unambiguous measurement of sucrose transport in the sec6-4 yeast vesicle system, endogenous sucrose metabolism by extracellular acid invertase must be inhibited. The inclusion of Tris base in the reaction solution provided dramatic reduction in invertase activity and prevented ATP-mediated vesicle swelling (Fig. 4.5).

Tris is a competitive inhibitor, along with sucrose, for the active site of several enzymes including invertase (Kolinská and Semenza, 1967) and trehalase (Chen et al.,
In addition, Tris competitively inhibits α-amylases (Aghajari et al., 1998; Ghalanbor et al., 2008), suggesting that it has the ability to compete with several glucosides for enzyme active sites (Ghalanbor et al., 2008). Thus, it seems likely that Tris may also compete with the binding of sucrose to the transporters, the system it is designed to investigate. Additionally, Tris has been shown to interact with cation binding sites (Gordon-Weeks et al., 1997). In light of the literature reporting Tris inhibition of enzymes, it appears likely that it may have an inhibitory effect on sucrose and possibly also proton transport. So, although it is an effective inhibitor of invertase (Fig. 4.5), the same properties make it a potential inhibitor of the sucrose transporters being characterised, leading to compromised data.

Other inhibitors of invertase have been reported, including sulphydryl modifiers, suggesting that the active site of invertase includes a sulphydrl group (Neumann and Lampen, 1967). This is a feature invertase has in common with members of the sucrose transporters, including the SUTs and SUFs cloned from pea and bean (and see Chapter 2). Thus, another approach, preferably one not utilising inhibitors due to the similarity of SUT and invertase active sites, is required to mitigate the effect of invertase activity. For example, eliminating invertase activity by avoiding its optimal pH range of 4.5-5.0. However, successful buffering the vesicle lumen to an alkaline pH would be technically very difficult, given the limited volume of the lumen and the activity of H⁺-ATPases (see Fig. 4.4, noting the pH-dependent quenching of intravesicular quinacrine following the addition of ATP). The effect of proton pumping mediated pH acidification is rapid, as shown by the vesicle swelling measured over the first five seconds (Fig. 4.5, noting the HEPES + ATP trace).

Removal of all invertase activity through genetic approaches (as was initially believed to be the status of SY1-F) eliminates the need for Tris or other invertase inhibitors during the assays, thus providing a superior system.

### 4.3.1.3 Engineering invertase-deficient sec6-4 yeast by gene disruption

In yeast, invertases (EC. 3.2.1.26) are encoded by the SUC (sucrose fermentation) genes. There have been multiple SUC genes identified in *S. cerevisiae*, with them being mapped to 9 different loci (Table 4.3).

All invertases except SUC2 are located in mobile telomeric regions of the chromosomes (Carlson et al., 1985). SUC2 or its non-functional allele (*SUC2*<sup>o</sup>) is present in all yeast strains; in addition some strains have one or more of the remaining
SUC genes (Carlson and Botstein, 1983). A high degree of homology exists between SUC2 and the other genes. For instance, SUC2 shares 94% and 93% similarity with SUC1 and SUC4 respectively.

Both the intracellular and extracellular forms of each invertase isoform are coded by a single gene, which produces transcripts for both forms (Perlman and Halvorson, 1981). Of these two forms, the extracellular form of invertase is of most relevance to our experimental system, as this invertase is contained within the secretory vesicles. The intracellular invertase is of lesser concern, as washing of the isolated secretory vesicles will remove any intracellular invertase remaining following cell lysis.

The significance of the chromosomal locations of SUC genes (Table 4.3) will be discussed further later.

The sec6-4 yeast mutant used in this study is SY1 (Nakamoto et al., 1991), and was generated from the crossing of the sec6-4 yeast strain NY503 with NY179, which was utilised to introduce a leu selectable marker into the progeny. The sec6-4 mutant, containing NY503, originated from an ethyl methanesulfonate-induced mutagenesis event using the laboratory yeast strain X2180-1A. Subsequent selection for temperature-dependent defects in extracellular invertase secretion was used to identify secretory pathway mutants (Novick et al., 1980).

Investigation into the identity of invertase genes present in the mother strain X2180-1A showed that SUC2 is the only invertase gene present in this strain (Naumov et al., 1996).

Stopped-flow fluorimetry with the invertase inhibitor Tris using the SY1-F cells demonstrated that invertase was still active in these cells (Fig. 4.5), despite claims that invertase has been knocked out. As X2180-1A is the mother strain of SY1, it was reasoned that SUC2 would most likely be the invertase present.
Table 4.3. Known invertase (SUC) genes in *S. cerevisiae*, and their chromosomal location.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Chromosomal Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUC1</td>
<td>VII</td>
<td>Telomeric</td>
<td>Carlson <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>SUC2 / SUC2”</td>
<td>IX</td>
<td>Non- telomeric</td>
<td>&quot;</td>
</tr>
<tr>
<td>SUC3</td>
<td>II</td>
<td>Telomeric</td>
<td>&quot;</td>
</tr>
<tr>
<td>SUC4</td>
<td>XIII</td>
<td>Telomeric</td>
<td>&quot;</td>
</tr>
<tr>
<td>SUC5</td>
<td>IV</td>
<td>Telomeric</td>
<td>&quot;</td>
</tr>
<tr>
<td>SUC7</td>
<td>XIII</td>
<td>Telomeric</td>
<td>&quot;</td>
</tr>
<tr>
<td>SUC8</td>
<td>X</td>
<td>Telomeric</td>
<td>Naumov and Naumova, 2010a</td>
</tr>
<tr>
<td>SUC9</td>
<td>XIV</td>
<td>TBC</td>
<td>Naumov and Naumova, 2010b</td>
</tr>
<tr>
<td>SUC10</td>
<td>XVI/XIII doublet</td>
<td>TBC</td>
<td>Naumov and Naumova, 2010b</td>
</tr>
</tbody>
</table>

A PCR-based knockout strategy was adopted, using a construct designed to target the kanamycin resistance to the SUC2 loci, knocking out SUC2 and creating kanamycin-selectable transformants. The invertase gene disruption fragment generated via PCR use the KanMX cassette (Fig. 4.6A) containing plasmid pUG6 (Table 4.1; Fig. 4.2; Güldener *et al.*, 1996) as template, with flanking regions designed to be homologous to the sequences immediately upstream and downstream of the yeast invertase gene. The disruption fragments generated were sequenced (Fig. 4.6B) to confirm successful amplification, then used in the transformation of *sec6-4* yeast cells using the approach described by Nakamoto *et al.*, (1991; also see Section 4.2.3).

The disruption fragment, which contains the KanMX cassette sequence and *SUC2* gene homologous regions, recombines into the genomic DNA displacing the coding region of the invertase gene (Fig. 4.6C). The supplied *sec6-4* yeast strain should show no kanamycin resistance (Kan^R; see Table 4.1) prior to transformation, allowing the Kan^R gene contained in the KanMX cassette to provide a selectable marker.
Figure 4.6. Kanamycin-mediated invertase gene disruption in yeast.

A. Schematic of the KanMX cassette, showing the LoxP sites flanking the marker gene. On transformation of yeast with the Cre recombinase plasmid pSH47, the KanR gene self-excises by recombining out at the LoxP sequences. P - promoter; T - terminator. Redrawn from Güldener et al., 1996.

B. Sequence of the kanamycin disruption fragment generated, using primers KandisF and KandisR (shaded grey) described in Table 4.2. The underlined sequence is homologous to the KanMX cassette contained in pUG6, while bolded sequence is homologous to the SUC2 regions. The LoxP sequences are shaded pink, while the coding region for KanR is shaded orange. The red A is from the ATG codon of the SUC2 sequence, showing the disruption of this gene by the KanMX cassette.

C. Gene disruption of SUC2, mediated by the homologous recombination of KanR into the coding region, with subsequent selection for kanamycin resistance in transformants.
A. 

B. 

C. 

Yeast nucleus 

Transformation with fragment 

KAN 

Yeast nucleus 

Homologous recombination 

Selection for transgene
Stopped-flow fluorimetry with the invertase inhibitor Tris using the SY1-F cells demonstrated that invertase was still active in these cells (Fig. 4.5). So, it was decided to attempt invertase knockout on this material, targeting SUC2, using the KanMX cassette strategy. Competent SY1-F were prepared, and used for transformation. Numerous putative transformants grew on media containing the selectable marker (in the form of G418 at 200 mg/L), and the KanR gene was shown to be present through colony PCR (Fig. 4.7A). However, during the PCR screening of transformants, it was discovered that the KanR gene also was present in untransformed SY1-F yeast (Fig. 4.7B). In addition, untransformed sec6-4 yeast cultured on selectable media grew at the same density as the successfully transformed strains. Taken together, these data suggest the sec6-4 yeast supplied for this work had been transformed with a KanR gene before receipt and therefore no selection was achieved through this re-transformation.

Upon further discussion with Prof. Frommer, it was revealed that there had been several attempts made at invertase disruption in this yeast prior to it being provided to our lab. The main strategy used had been the use of URA3 as a marker for homologous-recombination mediated SUC2 gene disruption. However, it could not be determined whether the KanR marker detected was a result of subsequent modification to this strain by the Frommer lab or contamination with another yeast strain. Although functional secretory vesicles were isolated from the SY1-F strain (Fig. 4.5), suggesting it is a sec6-4 mutant line, it was found to still contain a functional invertase gene (Fig. 4.6; 4.7A noting the positive control), in addition to the KanR marker (Fig. 4.7B). Given the uncertain identity of the SY1-F yeast, it was decided to source SY1 yeast from the originator lab for further development.
4.3.2 Phase II: sec6-4 yeast From the Slayman Lab

To circumvent the challenges introduced by using material already harbouring the kanamycin selection gene, sec6-4 yeast was sourced from Dr. C. W. Slayman (Department of Cell Biology, Yale University), and designated SY1-S. It was this lab that originally realised the potential for the sec6-4 mutant yeast to create vesicles for the characterisation of membrane transporters, in studying a yeast H⁺- ATPase (Nakamoto et al., 1991).

When plated on kanamycin, the SY1-S yeast was unable to grow (Fig 4.8A), and were confirmed to be KanR gene free by PCR. Thus, this yeast was used as the starting material for KanR-mediated gene knockout of invertase.

**Figure 4.7. KanR and invertase in SY1-F yeast.**

A. Colony PCR of putative transformants showing the presence of both invertase and KanR genes. Untransformed SY1-F yeast was used as a positive control (note the band for KanR) and H₂O as a negative control.

B. Colony PCR of untransformed SY1-F yeasts, showing presence of the KanR gene. The KanMX cassette-containing plasmid pUG6 was used as a positive control.
Transformation of competent SY1-S cells was carried out using the disruption fragment described previously (Fig. 4.6B), and putative transformants grew on kanamycin selection plates (Fig. 4.8B).

**Figure 4.8. Use of kanamycin as a selection agent for transformants.**

A. SY1-S sec6-4 yeast were unable to grow on kanamycin. Three separate sub-clones are shown.

B. Transformation of SY1-S yeast with the kanamycin-invertase gene disruption fragment yielded colonies able to grow on kanamycin, suggesting successful integration of the KanR gene.

Colony PCR was carried out on colonies growing on selection plates, and confirmed that the KanR gene was now present in these transformants (Fig. 4.9). Thus it appears that the kanamycin disruption fragment (with the KanR gene flanked by invertase gene sequences) had inserted into the yeast genome. Despite this however, the transformants tested positive for invertase gene expression by colony PCR (Fig. 4.9). Forty-three colonies were tested in total, and all were shown to have both KanR and invertase genes.
present. Note that the staining visible near the loading wells in the gel is non-specific staining of cellular debris carried over from the colony PCR template yeast cells.

![Image of gel electrophoresis result](image)

**Figure 4.9. Colony PCR of yeast transformants showing presence of both the Kan\textsuperscript{R} and Inv genes present in transformants.** The positive control (+) used for Kan\textsuperscript{R} was the KanMX containing plasmid pUG6, while for the invertase gene, untransformed SY1-S was used. For both negative controls (-), dH\textsubscript{2}O was used.

It appeared possible that the positive PCR for invertase resulted from residual contamination of colonies of Kan\textsuperscript{R} transformed yeast cells with traces of untransformed cells that were streaked out onto the selection plates following the transformation procedure. To determine whether this was the case, colonies of putative transformants were successively subcultured three times on selection media, and individual resulting colonies PCR tested. All of these subcultured colonies tested showed the presence of both Kan\textsuperscript{R} and invertase, thus ruling out the possibility of invertase gene contamination from untransformed cells.

To ensure that the failure to successfully knock out \textit{SUC2} was limited to the kanamycin-mediated disruption approach, invertase knockout using a different selectable marker, bleomycin resistance (Ble\textsuperscript{R}), was carried out on untransformed SY1-
S yeast. A second disruption fragment, using the Ble\textsuperscript{R} gene flanked with invertase gene sequences, was constructed, confirmed via sequencing and transformed into sec6-4 yeast. The structure of this fragment is identical to that for the Kan\textsuperscript{R} fragment (Fig. 4.6), except the bleomycin resistance gene replaced the kanamycin gene sequence. A similar result to that seen with the Kan\textsuperscript{R} gene strategy was encountered, with the invertase gene still present alongside the Ble\textsuperscript{R} gene in all 48 transformant colonies tested, a subset of which are shown in Fig. 4.10.

Fig. 4.10. Colony PCR of yeast transformants, showing both the Ble\textsuperscript{R} and Inv genes as present in transformants. The positive controls (+) used for Ble\textsuperscript{R} was the Ble\textsuperscript{R} containing plasmid pUG66, while for the invertase gene, untransformed SY1-S was used. For both negative controls (-), dH\textsubscript{2}O was used.

4.3.2.1 The kanamycin gene inserted correctly into an invertase locus

The failure of both the Kan\textsuperscript{R} and Ble\textsuperscript{R} strategies to remove invertase raised the possibility that the invertase disruption fragment was being mis-targeted, and homologously re-combining somewhere into the yeast genome other than into the invertase locus. Rates of successful homologous recombination into the correct locus is reported to occur in about 70\% of transformants (Güldener \textit{et al.}, 1996) when using the method adopted in our study utilising 60 bp primers comprising 20 bp homologous to the KanMX sequence and 40 bp homologous to the region flanking the target gene (and see Fig. 4.3). Further, the primer sequences designed against \textit{SUC2} flanking sequences
used for disruption fragment homology were Blast searched against the whole yeast genome sequence, showing upstream and downstream homology with SUC genes only. Thus, the disruption fragments exclusively should have targeted invertase. Nevertheless, primers were designed to amplify a 1.8 kb region surrounding the invertase locus, to determine whether insertion of the Kan\textsuperscript{R} disruption fragment had occurred correctly. Colony PCR of transformants with these primers revealed that a feint 1.6 kb fragment (Fig. 4.11) corresponding with the size of the Kan\textsuperscript{R} gene, was present in the invertase gene locus of transformants but not wild-type, in addition to a 1.8 kb band. The size of this 1.8 kb band corresponds to that expected when the invertase gene is still present in its locus. Thus, it appears that both the kanamycin gene and invertase gene are present within the invertase locus. This provides support to the idea that more than one invertase locus is present in this line of yeast.

To confirm the identity of the gene within the locus, the 1.6 kb and 1.8 kb bands were excised and extracted from the gel, and used as PCR template (Fig. 4.12), using primers for internal regions of the Kan\textsuperscript{R} and invertase genes. All of the fragments from Fig. 4.11 tested positive to both Kan\textsuperscript{R} and invertase (Fig. 4.12). Due to the size similarities, the two bands were not separated distinctly on the gel in Fig. 4.11. As such, cross-contamination of the bands would account for each one testing positive to the two genes. It does, however, confirm that the Kan\textsuperscript{R} disruption fragment has integrated successfully into an invertase locus. Further, the existence of invertase within an invertase locus, in parallel with the kanamycin in a second invertase locus, suggests that more than one invertase locus is present in sec6-4 yeast.
Figure 4.11. Colony PCR showing insertion of the kanamycin resistance gene, as indicated by a 1.6 kb band (arrows), within the invertase gene locus in transformed \textit{sec6-4 yeast}. Note that the transformants still show the presence of an invertase gene (1.8 kb band) within the invertase locus, while untransformed (ut) \textit{sec6-4} yeast shows the invertase gene only.

Figure 4.12. Identity of gene present within invertase locus. PCR carried out on 1.6 kb (A) and 1.8 kb (B) fragments generated from two transformants in Fig. 4.7. The gel extracted fragments were used as PCR template using internal gene primers for Kan\textsuperscript{R} and invertase.
4.3.2.2 Transformants have a reduced capacity to metabolise sucrose

The KanR transformants generated were grown on sucrose as a sole carbon source, to assess whether the invertase gene disruption had any effect on their ability to hydrolyse sufficient quantities of sucrose to support colony growth. 17 of 43 (40%) of the KanR transformants screened were deficient in their ability to grow on sucrose (see Fig. 4.13 for representative strains), indicating that invertase activity had been compromised by the successful insertion of the kanamycin gene into the invertase locus. Although some transformants were able to grow after an extended culture, several were unable to grow at all.

![Figure 4.13. Growth of selected KanR transformants on glucose and sucrose as the sole carbon source after incubation for 4 days at 30 °C.](image)

Strains of yeast with compromised growth on sucrose (those that were slow growing or unable to grow on sucrose) were assayed for invertase activity (Fig. 4.14). These transformants assayed had between 9 and 80% of the invertase activity of untransformed SY1 yeast. This supports the theory of multiple copies of the invertase gene being present in the source yeast.

The sequences used for knockout primer design (see Table 4.2) are conserved between SUCx1-5 and SUC7 (Hohmann and Gozalbo, 1988). A BLAST search of the SUC2 sequences used show homology of about 98% with the SUC sequences in
GenBank (*SUCs*1-4 and *SUC7*; Fig. 4.15), so *SUC2*-targeted knockout would also be able to homologously recombine into these other loci as well (Fig. 4.15). The *sec6-4* strain used, SY1, is derived from the strain X2180-1A (P. Novick, pers. com.), which is reported to have only one invertase gene - *SUC2* (Naumov et al., 1996). However, the pathway from strain X2180-1A is somewhat complex. As mentioned earlier, the original *sec6-4* yeast strains were derived from X2180-1A by ethyl methanesulfonate-induced mutagenesis, followed by an invertase secretion assay (Novick et al., 1980).

![Figure 4.14. Relative invertase activity of yeast transformants generated by kanamycin- and bleomycin-mediated gene disruption.](image)

Yeast transformants were selected as those with a severely compromised ability to grow on sucrose as a sole carbon source. Cells were suspended and incubated in 50 mM sucrose for 1 h, with an enzymatic assay carried out to determine free glucose in the culture medium at the end of incubation. Values are expressed as a percentage of the invertase activity calculated for untransformed *sec6-4* yeast. The invertase-null yeast strain SUSY7 was included as a negative control. Data presented are means ± SEs of 4 replicates per strain.

Thus, a mutagenesis event, followed by selection for invertase overproduction at a permissive temperature had occurred. Following this, the strain SY1 was derived from a crossing of two of the original *sec6-4* yeasts, to allow for the transfer of a *URA3* auxotrophic marker into the progeny. From the evidence collected, it appears that multiple invertase genes are present in SY1-S. It is possible that this event has arisen...
through duplication of a SUC2 gene, which has been recently observed as occurring as a chromosomal translocation (Naumov and Naumova, 2011). The other possibility is that an invertase allele other than \textit{SUC2} has been included through the complex genetic history of SY1. Multiple invertase genes have an additive effect on invertase activity (Hohmann, 1987). As the genes are located at different loci under separate promoters, as opposed to being within a single operon, the transcription of genes can occur independently of one another. This phenomenon may account for the observed different levels of invertase inhibition in Fig. 4.14.

<table>
<thead>
<tr>
<th>Primer 1</th>
<th>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUC1</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC2</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC3</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC4</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC5</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC7</td>
<td>CAAGCAAAACAAAAGCTTTTCTTTTCTACATATAGA</td>
</tr>
<tr>
<td>SUC8</td>
<td>not available</td>
</tr>
<tr>
<td>SUC9</td>
<td>not available</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer 2</th>
<th>GAGGTTATTTATTTTTTCATTGCTTTTTTTATTTTTTCAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUC1</td>
<td>GAGGTTATTTATTTTTTCATTGCTTTTTTTATTTTTTCAG</td>
</tr>
<tr>
<td>SUC2</td>
<td>GAGGTTATTTATTTTTTCATTGCTTTTTTTATTTTTTCAG</td>
</tr>
<tr>
<td>SUC3</td>
<td>not available</td>
</tr>
<tr>
<td>SUC4</td>
<td>GAGGTTATTTATTTTTTCATTGCTTTTTTTATTTTTTCAG</td>
</tr>
<tr>
<td>SUC5</td>
<td>not available</td>
</tr>
<tr>
<td>SUC7</td>
<td>GAGGTTATTTATTTTTTCATTGCTTTTTTTATTTTTTCAG</td>
</tr>
<tr>
<td>SUC8</td>
<td>not available</td>
</tr>
<tr>
<td>SUC9</td>
<td>not available</td>
</tr>
</tbody>
</table>

**Figure 4.15. Homology of primer sequences used to target \textit{SUC2} sequence to other SUCs.** Both primers have a high degree of similarity to corresponding sequences (where available) of other \textit{SUCs}.

So, antibiotic resistance-mediated gene disruption had resulted in invertase activity inhibition of up to 91\% (Fig. 4.11). However, as invertase levels in vesicles are the critical factor for the stopped-flow fluorimetry experimental system, it was decided that this was insufficient for our purposes, given the sensitivity of fluorimetry to small osmotic changes.

At this point in the yeast engineering process, it was discovered that a plasmid designed to transfer the \textit{sec6-4} mutation into a yeast strain of interest had become
available. It was determined that adopting this strategy was preferable to continue engineering the sec6-4 yeast strain via invertase knockout for several reasons. Firstly, it had become apparent that there were the multiple alleles of invertase present in the sec6-4 yeast. Although the Kan$^\text{R}$ marker was recoverable, and so could be reused for subsequent invertase knockout events, there was an uncertainty surrounding how many invertase alleles remained. The invertase activity assay however (Fig. 4.14), suggested that there may have been several present in total, given the three distinct levels of invertase activity observed. Secondly, the issue of endogenous maltose transporters still exists, and may have required similar gene disruption to remove the endogenous transporter(s), as they have the capacity to transport sucrose (Stambuk et al., 1999). Although no discernible impact of maltose transport was observed in initial stopped-flow fluorimetry experiments (Fig 4.5), this would have been difficult to detect due to the invertase interference encountered. In any case, a genetic background free of both sucrose metabolism and transport in vesicles is the ideal situation.

4.3.3 Phase III- Engineering of sec6-4 Mutation into SUSY7 Yeast

The sec6-4 mutant allele arises due to a single T to C point mutation in the functional SEC6 allele, resulting in a L633P amino acid substitution (Lamping et al., 2005). The sec6-4 mutant allele was incorporated into a vector (pSK-sec6-4-URA3 – Fig. 4.2) designed to introduce the mutant into yeast (Lamping et al., 2005). The vector relies on homologous recombination (following vector linearization) of the sec6-4 mutant, along with a selectable marker (URA3) into the yeast genome to generate stably transformed sec6-4 yeast (Fig. 4.14). This useful tool provided the opportunity to engineer the sec6-4 mutation into a yeast genetic background suitable for studying sucrose transporters. SUSY7 yeast (Riesmeier et al., 1992) was selected as an ideal candidate, being deficient in both invertase (due to the mutant invertase allele suc20) and maltose transport (Riesmeier et al., 1992). Additionally, the URA3 auxotrophy introduced into SUSY7 (Barker et al., 2000) enables the utilisation of the URA3 gene of pSK-sec6-4-URA3 as a selectable marker.

Transformation of SUSY7 yeast was carried out, and selection carried out on uracil-free medium. To facilitate screening, the pSK-sec6-4-URA3 plasmid contains the mutant sec6-4 gene, with a silent point mutation removing an EcoRV restriction site that
is present in the wild type SEC6 gene (Fig. 4.16). This point mutation allows for screening of putative transformants, using a PCR step followed by an EcoRV restriction digestion.

**Figure 4.16. Strategy for transforming the sec6-4 mutation into SUSY7 yeast, using the pSK-sec6-4-URA3 plasmid (Lamping et al., 2005).** A. The plasmid, following PstI-linearization, combines into the target gene site via homology. The inclusion of a silent point mutation, destroying the EcoRV restriction site, is designed to facilitate screening of putative transformants using restriction digestion. B. The functional sec6-4 mutant gene inserted into the SEC6 locus in the yeast genome, followed by the plasmid backbone and the promoter-less disrupted SEC6 gene.

4.3.3.1 The sec6-4 mutation was successfully introduced into SUSY7 yeast

Putative transformants were selected using the URA3 auxotrophic marker on dropout medium, and screened via colony PCR. Primers (SEC6f1927 and SEC6r2348, Table 4.2) amplifying a 421 bp section of the SEC6 and sec6-4 genes covering the EcoRV restriction site (which has been deleted in the sec6-4 mutation carried by the plasmid pSK-sec6-4-URA3) were used for a colony PCR of each putative transformant. A digestion of the resulting fragment was carried out using an excess of EcoRV, and the
digestion product run on a gel (Fig. 4.17A). Where successful integration of the sec6-4 gene (along with the deleted EcoRV) restriction site has been successful, only partial digestion of the PCR product is seen (for example, transformant G in Fig. 4.17A). The fragments that undergo digestion by EcoRV are either from the non-mutant SEC6 present in untransformed SUSY7 yeast, or from the disrupted SEC6 gene that is present downstream of the pSK-sec6-4-URA3 backbone integrated into the invertase locus following successful transformation (as illustrated in Fig. 4.16B).

**Figure 4.17. Insertion of the sec6-4 mutation into an invertase deficient yeast strain.** A. Restriction digestion showing SUSY7 transformed with the sec6-4 mutant. Uncut PCR product indicates the presence of the sec6-4 mutant allele, with destroyed EcoRV digestion site. B. Sequence data from the sec6-4 gene, showing the successful integration of the sec6-4 point mutation (red box) in the s6s7 engineered yeast strain, plus the deleted EcoRV site, confirming integration of the pSK-sec6-4-URA3 plasmid.

Sequencing was carried out on transformants harbouring the undigested fragment (Fig. 4.17B), and confirmed the insertion of the sec6-4 mutant gene into SUSY7. A
representative sec6-4 mutant-containing clone was designated s6s7 (sec 6-4-SUSY7) and used for vesicle functional studies.

### 4.3.3.2 s6s7 yeast yield functional vesicles

Secretory vesicles were isolated from s6s7 yeast, and a proton-pumping assay carried (Fig. 4.17) out to confirm that the vesicles are functional, sealed and in an inside-out orientation. The ability of ATP to induce pH-dependent quinacrine quenching is due to the action of endogenous H+- ATPases, which drives vesicle lumen acidification (see Fig. 4.1 C). The addition of the protonophore nigericin dissipates the intravesicular proton concentration, reversing the observed quenching (Fig. 4.18).

![Figure 4.18. pH-dependent fluorescence quenching of quinacrine in inside-out s6s7 vesicles.](image)

Relative fluorescence quenching of quinacrine commenced with the addition of ATP (2 mM final concentration) and was reversed by the addition of 10 μM nigericin.

### 4.3.3.3 s6s7 vesicles shrink and swell subject to osmotic changes

The success of the stopped-flow fluorimetry relies on the ability of the secretory vesicles to act as osmometers, shrinking or swelling in response to osmotic water...
movement. Thus, the ability of the isolated s6s7 vesicles to respond to osmotic gradients was investigated. The initial slope of vesicle shrinkage (over the first second) was measured using stopped-flow fluorimetry for a series of imposed osmotic gradients (Fig. 4.19). The response was linear over a range of osmotic gradients from 10 to 300 mosmol kg\(^{-1}\), indicating that the vesicles were acting as osmometers.

Thus, the s6s7 strain of yeast engineered in this Chapter provides an invertase and maltose transporter free background required to study the kinetics of sucrose transporters at the intracellular face, using the stopped-flow fluorimetry system.
Figure 4.19. Stopped-Flow Fluorimetry using s6s7 vesicles.

A. Initial rates of s6s7 vesicle shrinking under a range of imposed osmotic gradients, generated by the addition of sorbitol to the injection medium. Rates were calculated over the first second following injection, using light scattering to measure vesicle shrinkage. Traces (three per osmotic gradient) were fitted to a single exponential curve (where $y = a \exp(-bt)$) to obtain the initial rate constant $b$.

B. An example trace of vesicle shrinkage over the first second of vesicle shrinkage, detected by an increase in light scattering, for an imposed osmotic gradient of 200 mosmol kg$^{-1}$. 

$y = 0.0493x$

$R^2 = 0.9629$
4.3.4 Vector requirements for sucrose transporter characterisation in s6s7 Yeast

4.3.4.1 Vector requirements for s6s7

The newly developed yeast strain s6s7 has auxotrophies for tryptophan and uracil, which are present in the mother strain SUSY7. As such, these can be used as selectable markers for plasmids containing the \textit{URA3} or \textit{TRP1} gene, when used in conjunction with amino acid dropout growth medium. Thus, any vector used for the expression of sucrose transporters in s6s7 yeast will need one of these genes.

4.3.4.2 Heat-shock element sequencing

\textit{pRN90} (Table 4.4) is a plasmid designed to work in conjunction with the \textit{sec6-4} vesicle production system. Cloning the transporter gene of interest into the \textit{pRN90} behind a heat shock element (HSE) in the plasmid results in the production of transporter-enriched vesicles, as the yeast is subjected to a heat shock (37°C for >2 h) to drive vesicle accumulation. Protein of the transporter of interest can constitute 5-10 \% of the total vesicle protein (Nakamoto \textit{et al.}, 1991), demonstrating the ability of the heat shock promoter to drive transporter expression. Although not carried out in this study, this could be tested by measuring relative protein levels in vesicles containing the transporter gene of interest, under the control of the HSE in a plasmid, versus the vesicle protein levels resulting from cells harbouring an empty HSE plasmid. Any detectable differences in total protein should thus be due to vesicle enrichment with transporter protein.

\textbf{Table 4.4.} Yeast plasmids for the generation of a sucrose transporter expression plasmid suitable for use with s6s7 yeast.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{pRN90}</td>
<td>\textit{LEU2 CEN4 ARS1} centromeric plasmid containing 2 copies of heat shock element (HSE2) from yeast strain SSAl flanked by SalI and SacI restriction sites</td>
<td>Nakamoto \textit{et al.}, 1991</td>
</tr>
<tr>
<td>\textit{yCplac33}</td>
<td>\textit{URA2 CEN4 ARS1} centromeric plasmid</td>
<td>Gietz and Sugino, 1988</td>
</tr>
<tr>
<td>\textit{yCHSE}</td>
<td>\textit{URA3 CEN4 ARS1} centromeric plasmid containing 2 copies of heat shock element (HSE2) from yeast strain SSAl flanked by SalI and SacI restriction sites. MCS: BamHI; KpnI; SacI; EcoRI.</td>
<td>This study</td>
</tr>
</tbody>
</table>
Whole plasmid sequencing of pRN90(F) sourced from the Frommer lab showed mutations in several key sites, including within the heat shock element. However, plasmid sourced from the Slayman lab was mutation free, and suitable for inclusion into an s6s7 yeast- specific plasmid.

4.3.4.3 Approach for generating a suitable sucrose transporter expression plasmid

A plasmid, pCHSE, can be generated to incorporate both the HSEs of pRH90 and the URA3 (yeast) and AmpR (E.coli) selectable markers. To create pCHSE, the 931 bp SalI – BamHI digested fragment from pRN90 (containing the tandem heat shock elements) would be directionally ligated into the BamHI/SalI cut plasmid yCplac33, which harbours the URA3 and AmpR selectable markers (Fig. 4.20). In the resulting plasmid, several unique cutting sites from the multiple cloning sites, including BamHI, KpnI, SacI and EcoRI remain intact directly downstream of the heat shock promoter, facilitating the cloning of transporter genes of interest.

In conclusion, this study outlines the successful generation of a sec6-4 secretory vesicle system in an invertase and maltose- transporter free genetic background, making it suitable for the study of sucrose transport. The inside-out configuration of the transporter within the vesicle membrane, combined with the ability of stopped-flow fluorimetry to measure real-time vesicle shrinking or swelling due to osmotic water movement, and the expression vector pCHSE, make the system ideal for studying the novel sucrose effluxers presented in this thesis. Additionally, the s6s7 yeast developed also presents a system for testing and identifying the energisation of other sucrose transporters, including symporters and antiporters. The yeast engineering approach taken, using the sec6 gene knock-in plasmid pSK-sec6-4-URA3 (Lamping et al., 2005) may also be used to develop similar systems for studying other sugar transporters, such as hexose transporters, when used in conjunction with yeasts of suitable genetic background, particularly where endogenous transporters and secreted enzymes for the sugar of interest are absent.
Figure 4.20. Generation of *pCHSE*, a sucrose transporter expression plasmid for use with *s6s7* yeast. *pNR90* harbours a promoter consisting of two tandem copies of the heat shock element, HSE, located upstream of a multiple cloning site. This plasmid contains the yeast leucine *LEU2* selectable marker. *yCplac33* is similar to *pRN90*, but lacking the HSEs and containing a *URA3* selectable marker rather than *LEU2*. The resulting plasmid, *pCHSE*, is a yeast centromeric plasmid with two tandem copies of the HSE upstream of a multiple cloning site, with *URA3* as a yeast selectable marker, and Amp$^R$ for *E. coli*. 
CHAPTER 5

General Discussion
5.1 Summary of Principal Findings

The aim of this study was to further understanding of the molecular basis of sucrose release from plant cells. The approach taken was to characterise and localise transporters cloned by homology to known sucrose transporters from tissue functionally committed to sucrose efflux.

The characterisation of the five genes cloned by homology to SUT sequences from developing legume seed coats revealed that all are functional sucrose transporters (Chapter 2). Two of these, PsSUT1 and PvSUT1, were typical SUT Clade I family proteins, in that they are H⁺-sucrose symporters. Additionally, with apparent $K_m$ values for sucrose of 1.5 and 8.5 mM respectively, they have a similar (although slightly lower for PvSUT1) affinity for sucrose to other eudicotyledenous SUT1 transporters, which typically have a $K_m$ range of 0.07 to 2 mM (Kühn and Grof, 2010). The remaining three transporters, however, were found to be energy-independent sucrose facilitators. This is the first report of non-energised transport by sucrose transporter family members. To distinguish these facilitators from sucrose symporter SUTs, they were termed SUFs (sucrose facilitators). The three SUFs characterised had apparent $K_m$ values ranging from 28 to 100 mM, which are approximately 20 times greater than those reported for SUT1 transporters. It is interesting to note that these apparent $K_m$ values correspond to those reported for excised seed coats from developing Vicia seeds (Ritchie et al., 2003), which were about 500 mM for sucrose.

Studies using yeast cells pre-loaded with sucrose demonstrated the ability of the SUFs to function as effluxers, providing the most direct evidence of function as facilitators. Further, studies using yeast cells loaded with a high concentration of unlabelled sucrose and subsequently incubated in a low concentration of $[^{14}\text{C}]$ sucrose showed that a transient influx of sucrose occurred against the concentration gradient. This is characteristic of carrier, rather than channel mediated transport, where efflux initially drives a corresponding influx of substrate by what is termed counter-transport (Stein, 1986).

Localisation studies were carried out on a sub-set of these transporters, PsSUT1, PsSUF1 and PsSUF4 (Chapter 3). These three transporters were chosen as they were all cloned from pea, and were reasoned to be a more complete set of SUTs than those cloned from French bean, given that no SUT4 was cloned from that species.
Examination of the intracellular localisation of the transporter proteins was carried out. Transient expression of transporter-GFP fusion proteins in tobacco leaves confirmed that all three are plasma membrane-localised, thus mediate sucrose exchange between the cytoplasmic and apoplastic compartments.

Cellular localisation using transporter-specific antibodies were carried out in regions involved in sucrose efflux - the coats of developing seeds, and the minor veins of source leaves in which sucrose is loaded apoplastically into the collection phloem. For seed coats, the symporter, PsSUT1, was restricted to sieve elements of the chalazal vein, where it probably plays a role in retrieval of sucrose prematurely leaked from the phloem. The facilitators, PsSUF1 and PsSUF4, localised to the seed coat inner layers comprised of thin walled parenchyma cells, which are the putative site of sucrose efflux (Wang et al., 1995). Data from western blotting suggested that the SUFs are more abundant in developing seed coats in pre-storage phase seeds.

In the minor veins of source leaves, the facilitators did not localise to putative sucrose efflux sites (bundle sheath and/or phloem parenchyma; Giaquinta, 1983). Instead, they co-localised with the symporter in sieve elements. Given the outward-directed sucrose concentration gradient from sieve elements, it is unlikely that they function as sucrose facilitators in this location. To do so would result in sucrose leakage from the phloem and futile cycling with the co-localised symporter. Instead, it is proposed that they form part of a larger protein complex, which is a hetero-oligomer of sucrose transporters (see for example Reinders et al., 2002a) and perhaps other sucrose transport-related proteins.

To further understand the mechanisms of sucrose efflux, the need to access and study the cytoplasmic face of the transporter was recognised. A system was developed to enable this (Chapter 4), utilising the sec6 secretory vesicle mutation in yeast. The resulting s6s7 yeast mutant generated allows the accumulation of inside-out membrane vesicles (harbouring the sucrose transporter of interest) when cultured under heat shock conditions. Under heat shock conditions (yeast culture growth at 37 °C for at least 2 h), the heat shock promoter drives expression of the transporter of interest; in the resulting inside-out membrane vesicles the transporter of interest can constitute 5-10 % of the total vesicle protein (Nakamoto et al., 1991). These vesicles are produced in an invertase and otherwise sucrose-transport free background. When the transporter of interest is introduced into the s6s7 strain on an appropriate plasmid, the transporter
becomes embedded within the inside out vesicles, with its cytoplasmic face directed outward. This allows the functional characterisation of this face of the transporter, by exposure to metabolic inhibitors, varying sucrose concentrations, pH conditions and competing sugar species. Proof of concept studies using these vesicles show that, when coupled with stopped-flow fluorimetry, the changes in light scattering by vesicles due to osmotic shrinking and swelling enable rapid, real time evaluation of sucrose transporter activity in response to various treatments.

Due to the protracted development period of the s6s7 sucrose transporter characterisation system, the actual functional characterisation of the cytoplasmic face of these transporters fell outside the scope of this study. However, this system now provides the opportunity to explore this area. Of particular interest is to explore whether energy can be utilised by the cytoplasmic face of SUFs to transport sucrose against a concentration gradient.

**5.2 Implications of Findings**

The role of SUFs within source leaf minor veins can be best described as equivocal at this point, given the cellular localisation of transporter protein to the sieve elements. Further experimental work is needed to better identify their role in these cells. Given what is known about their in vitro function as facilitators, and a growing body of evidence of the association of SUT family members in hetero-oligomers (Reinders et al., 2002b; Schulze et al., 2003) and within lipid micro-domains in planta (Krugel et al., 2008; Liesche et al., 2011) it appears to be a promising area of investigation.

In the case of SUF function in seed coats, the situation appears more straightforward, with these putative effluxers localising to putative efflux sites during periods of high sucrose flux. Thus, it is possible to consider more fully the potential contribution of SUFs to sucrose efflux throughout seed development.

**5.2.1 Sucrose Efflux Across Seed Development- A Model**

Two distinct sucrose efflux mechanisms have been proposed for coats of developing legume seeds: sucrose release via an energy-independent transporter protein (Fieuw and Patrick, 1993; Walker et al., 1995; de Jong et al., 1996; van Dongen et al., 2001), and active sucrose release mediated by sucrose/H⁺ antiporters (Fieuw and Patrick, 1993; Walker et al., 1995). By mapping the conclusions drawn by these studies, and
incorporating the findings relating to SUFs gleaned in this study, it becomes possible to construct a preliminary model of sucrose efflux during seed development (Figure 5.1.) The seed model considers two distinct stages of development: the first is the pre-storage phase in which cell division and cell expansion drive seed growth. The subsequent stage, storage phase, is characterised by storage product accumulation (Weber et al., 2005).

In terms of sugar transport from maternal to filial tissues, the two phases differ in the species of sugar predominant in the intervening seed apoplasmic space. At both stages, sucrose is the predominant form of sugar released from the seed coats. However, in the pre-storage phase, high levels of cell wall invertase activity (Weber et al., 1995) result in sucrose hydrolysis. Hexoses resulting from sucrose cleavage may reach 100 mM in the apoplasmic space of developing *Vicia faba* seeds (Offler et al., 1997). Similarly, in developing seeds of other species, including barley (Weschke et al., 2003) and rice (Hirose et al., 2002) caryopses, cell wall invertase activity is highest in maternal tissues early in grain filling. A decline in invertase activity coincides with the transition from pre-storage to storage phases, resulting from the crushing of inner seed coat layers by cotyledon expansion in which the extracellular invertase is located (Weber et al., 1995).
Figure 5.1. Sucrose transport at the maternal/filial interface across seed development.  
A. Absolute seed growth rate (solid line) and apoplastic invertase activity (dashed line) across seed development. The relative ages (in DAF) are given for pea and Broad bean, along with the Briarty (1969) developmental phases and key publications investigating sucrose movement within seeds of these species. B. Localisation of sucrose and hexose transport-related transporters at the maternal/filial interface in I) pre-storage phase and II) storage phase seeds.
A. Pre-storage Phase → Storage Phase → Storage product accumulation → Desiccation

- Seed Set
- Cell division
- Cell expansion

RWC

90 80 70 60 50 40 30 20

B. I) Pre-storage Phase
- Phase I
- Phase II
- Phase III
- Phase IV

- This study
- Patrick 1994
- Weber et al 1997a
- Harrington et al 1997
- Fieuw & Patrick 2003
- Ritchie et al 2003
- Offler et al 1989
- Offler & Patrick 1993
- de Jong et al 1996
- van Dongen et al 2001

II) Storage Phase

- Relative Sucrose: Hexose Levels
- High
- Low

Cell Types
- twp - thin-walled parenchyma
- twp-tc - thin-walled parenchyma transfer cells
- ctc - crushed thin-walled parenchyma transfer cells
- sec - sub-epidermal cells
- sp - storage parenchyma
- etc - cell wall matrix

Transports and Enzymes - predominant site of activity
- Sucrose facilitator
- Glucose/H+ symporter
- Sucrose antipporter
- Fructose/H+ symporter
- Sucrose/H+ symporter (SUT1)
- H+-ATPase
- Invertase
- Sucrose binding protein
In the pre-storage phase, invertase activity within the cell wall space of thin-walled parenchyma cells (Weber et al., 1995) ensures an outward-directed sucrose concentration gradient. Thus, cells mediating sucrose release (the thin-walled parenchyma transfer cells; Wang et al., 1995) should be able to do so without energisation. The localisation of PsSUF1 and PsSUF4 to the thin-walled parenchyma cells (Fig. 3.17C, D) are support a role for these transporters in mediating this sucrose release. Although the immunolocalisation studies were carried out using seed coats entering early storage phase, the data from Western blots (Fig. 3.10) suggest that these proteins may be even more abundant in younger seed coats.

In pre-storage phase seeds, the glucose and fructose from sucrose resulting cleavage in the apoplasm are taken up by both the epidermal cells and storage parenchyma of the cotyledons, in symport with protons (Harrington et al., 2005). As the cotyledons continue to expand, and crush the innermost layers of seed coat cells, the amount of invertase in the system declines (Weber et al., 1995). By the time seeds are in the storage phase of development (Fig. 5.1), the sucrose gradient between efflux cells and the apoplasm has collapsed (Patrick, 1994). By this stage, the expression of SUFs in seed coats has declined (see Fig. 3.10). The physiological evidence in whole seed coats indicates that a sucrose proton antiporter is in operation at this stage (see Fieuw and Patrick, 1993; Walker et al., 1995). In the cell localisation study presented in Chapter 3, the seed coats examined were at the transition between pre-storage and storage phase, and so SUFs were still present, although probably declining, within the system. The relative contributions of energised to non-energised sucrose efflux in pre-storage seed coats have not been studied in detail and published. The protonophore CCCP, ATPase inhibitor EB and sulphydryl reagents pCMBS and NEM had an impact on sucrose efflux (with up to 50 % reduction) during the storage phase of development, suggesting that energised transport is of significance at this point in development (Fieuw and Patrick, 1993). This coincides with the collapse in sucrose concentration gradient that is required to drive facilitated efflux.

During the storage phase in developing Broad bean seeds, the outward directed transmembrane sucrose concentration difference between the seed coat and the apoplastic space surrounding the embryo is estimated to be approximately 40 mM, while in French
bean, it is 10 mM, thus a modest outward-directed sucrose gradient is present (Patrick, 1994). So, the effect of sucrose cleavage in maintaining an outward-directed sucrose gradient has been diminished in these seeds. The apparent $K_m$ value of the outer face of PsSUF1 (which localised to the inner layers of the seed coat) is 100 mM under the experimental conditions imposed in Chapter 2. In contrast, the apparent $K_m$ value for sucrose uptake by PsSUT1 in the pea cotyledons is 7.7 mM (Tegeder et al., 1999). As such, the limiting factor for sucrose movement, based on transporter capacity, from the seed coat to the cotyledon is the capacity of the uptake system at the cotyledons. This then provides the opportunity for cotyledons to regulate the delivery of sucrose through this system, by increasing the activity and abundance of PsSUT1 proteins. Indeed, it has been shown that $PsSUT1$ expression is enhanced at low intracellular sucrose concentrations (Zhou et al., 2009).

5.2.2 Facilitated Sucrose Efflux: Same Functionality, Different Locations?

Sucrose facilitators may possibly be of physiological significance in the plant at other locations where an outward-directed trans-membrane sucrose concentration difference is present. This is notwithstanding the localisation reported in this study in leaf minor veins, where the facilitators PsSUF1 and PsSUF4 were localised to SEs (Fig. 3.9), which are putative sites of sucrose uptake rather than efflux. If they were functioning as effluxers in planta, it would be expected that they would be present on the plasma membrane of the bundle sheath cells or phloem parenchyma (as predicted by plasmodesmatal frequencies in Fig 3.6B). However, semi-quantitative transcript detection has made it apparent that SUFs are not restricted to the developing seed coat and source leaf minor veins. PsSUF4 transcript was located throughout the plant, suggesting a generalist role (Zhou et al., 2007). In situ transcript localisation in developing cotyledons, however, shows that PsSUF4 is restricted to the abaxial epidermal transfer cells and sub-epidermal cells, and is absent from storage parenchyma cells (Zhou et al., 2009). This, together with the immunolocalisation studies in source leaves (Fig. 3.9) and developing seed coats (Fig. 3.15) show that it is probably not ubiquitous throughout all plant cells. PsSUF1, despite being present in (and cloned from) seed coats, had the highest transcript levels in cotyledons, and also in roots. In cotyledons, PsSUF1 transcript localised to abaxial epidermal layers, and to a lesser
extent in storage parenchyma (Zhou et al., 2009). In bean, a high level of PvSUF1 expression was detected in source leaves and stems. Transcript was also abundant in sink leaves, flowers and roots (Zhou et al., 2007).

Given their function, SUF family proteins could potentially be present in any cells where facilitated movement of sucrose across a plasma membrane is likely, in accordance with the prevailing concentration gradient. Other sites within the plant where this may occur include the anthers, where symplasmic discontinuity occurs between the tapetum and pollen grains (Clément and Audran, 1995).

In roots, the movement of sucrose and other small molecules from protophloem to root tips is generally considered to be symplasmic, with companion-cell expressed free GFP able to move throughout the cell layers unimpeded (Stadler et al., 2005). Beyond this, root tips and elongation zones are able to exude sucrose and other soluble carbohydrates to the rhizosphere to stimulate proliferation of soil microbes (Marschner, 2011). Additionally, this zone is able to uptake sucrose (and other carbohydrates) when provided artificially in growing media. Chaudhuri et al. (2008) monitored sucrose flux into roots (using a fluorescent sucrose sensor), noting that flux was pH and ionophore insensitive. They suggested that SUFs are a possible candidate for mediating this observed flux.

Facilitated delivery of sucrose is most likely active in other terminal sinks. For instance, in potato stolons (which are derived from shoot apical meristems), sucrose delivery is apoplastic during the elongation phase (Viola et al., 2001). Thus, there is a potential role for facilitators in these situations. Also, facilitators may be located along the transport phloem, effluxing sucrose to meet the metabolic needs of surrounding tissues (Ayre, 2011). The high level of transcript observed for PvSUF1 in stems may reflect this role.

Otherwise, if SUFs are found to function in concert with other membrane proteins (such as other SUT family members), as proposed in Chapter 3, their distribution may be extended to other regions where symplasmic discontinuity occurs, but without the thermodynamically favourable sucrose gradient. For instance, although a facilitator when expressed in yeast, they might participate in energised transport in concert with other proteins.
Although the SUFs described are functional in yeast and expressed in seed coats, it appears that they may not be the only transporters responsible for sucrose efflux to filial tissues of developing seeds. In experimental conditions favouring facilitated transport (with a zero external sucrose concentration), at most only 50% of sucrose efflux from seed coats (Fieuw and Patrick, 1993; Walker et al., 1995) was energy-independent. The remaining 50% of sucrose efflux is driven by sucrose antiporters (Fieuw and Patrick, 1993; Walker et al., 1995). It is worth noting that the yeast heterologous system used in Chapter 2 is not conducive to the characterisation of antiport. Hence the need for the sec6-4 inside-out vesicle system developed in Chapter 4. So, the possibility that SUFs are also able to support H⁺/sucrose antiport needs to be explored using the sec6-4 system before it can be excluded. However, it appears more likely that, rather than being, the SUFs are unable to support, when other cases of a change of transporter function from symporter to facilitator are taken into account. For example, for the E.coli H⁺/lactose symporter LacY, a single amino acid mutation leads to the loss of proton coupling, resulting in a lactose facilitator. This was reported to occur for two separate amino acid point mutations, suggesting that particular residues are involved in energy coupling (for a review, see Varela and Wilson, 1996). No corresponding change of function mutants- from symporter to antiporter, nor of facilitator to antiporter have been reported to date.

At the commencement of this project, the only plant transporters that were unequivocally shown to be sucrose transporters were SUT family members. Although the sucrose binding proteins (SBP) were also reported to mediate sucrose transport in yeast (Overvoorde et al., 1996), there were other conflicting reports, and doubts cast on their role as putative transporters. This is due to SBP being a member of the seed storage protein family, and the lack of multiple membrane spanning domains typically seen with transporter proteins (Contim et al., 2003). However, the concept of SBP functioning as a sucrose facilitator has now been supported by evidence showing that SBP overproduced in, and isolated from E. coli, binds sucrose after the protein has undergone re-naturing (Rocha et al., 2007). Additionally, SBP appears to be membrane-localised, with VrSBP1 from cowpea being localised to the tonoplast (Wang et al., 2009).
As SUTs were the only sucrose transporter confirmed to have multiple, functional members across several plant species when this study was designed, it was reasoned that a homology-based cloning approach focusing on efflux tissue was appropriate for this study. This strategy was successful, given the cloning of novel sucrose efflux (facilitator) proteins from both pea and bean. Thus, the non-energised sucrose efflux observed in whole tissue studies has at least been partially accounted for.

5.2.3 Sucrose Transporters in Pea and Bean – A Snapshot

Three full-length SUT/SUF genes cloned from pea have been reported in this study. However, immunolocalisation revealed an additional uncloned SUT1 family member in the companion cells of leaf minor veins (Fig. 3.8D). This was indicated by the labelling of a protein by the generic SUT antibody PEP2, but no corresponding labelling by the specific antibodies for PsSUT1, PsSUF1 nor PsSUF4. So, it appears that at least four genes from this family are present in pea. In the case of French bean, two full length SUT/SUFs were sequenced. In addition, a partial length SUT1 homolog ( provisionally named PvSUT3) was cloned, with transcript studies showing that it is restricted to flowers (data not shown; Zhou et al., 2007). No SUT4 family homologs have been cloned from bean to date; however, plant species that have been sequenced and have sequence data readily available (including Arabidopsis, Ricinus, Medicago, cassava, poplar and lotus) have a SUT4 homolog, so it is reasonable to expect one in bean also. A similar case may apply to pea and bean in the case of SUT2 clade transporters. By this reasoning there should be at least five SUT/SUF family members in each of pea and bean.

In species and cells that have strong outward-directed sucrose concentration gradients that are in excess of the inward-directed potential energy of the proton motive force, sucrose efflux may occur via SUT (symporter) proteins. In these situations, the physiological conditions make symporter reversal likely. The ability of ZmSUT1 to act in reversal mode, to efflux sucrose in symport with protons, has been demonstrated (Carpaneto et al., 2005) where the potential energy in the outward-directed sucrose gradient is sufficient to overcome the inward-directed pmf. Although induced under experimental conditions, there are situations in planta that may support such function. For instance, in sugarcane, the symporter ShSUT1 localises to the plasma membrane of the mestome sheath and the vascular parenchyma cells of leaves, which are the putative
sites of release from the mesophyll cell symplasm (Rae et al., 2005). In this case, the high sucrose content within the mesophyll-vascular parenchyma symplasm (of up to 670 mM) may be sufficient to drive proton reversal.

5.2.4 Sucrose Efflux Beyond SUFs

The physiological evidence obtained from whole seed coat studies indicates that there is a second, energy dependent sucrose transporting protein or proteins (Wolswinkel and Ammerlaan, 1983; Minchin and Thorpe, 1990) that exist alongside the SUT/SUFs mediating sucrose efflux in antiport with protons (Fieuw and Patrick, 1993; Walker et al., 1995). Thus, the exciting opportunity to clone and characterise this unidentified sucrose antiporter is presented. This will be facilitated by recent advances in bioinformatics, screening and functional characterisation systems. It is possible that the as-yet-uncloned antiporter lies outside the SUT family phylogeny. Several recent studies describing sucrose transport outside of this family in plants (as well as other organisms) give weight to this notion.

In one of these studies, a new family of sugar transport proteins, the SWEETs (Chen et al., 2010), were identified in plants and other organisms including humans and Caenorhabditis elegans. This family, of which there are 17 members in Arabidopsis, includes several sucrose uniporters (Chen et al., 2012). Interestingly, this family were formerly only known as proteins with homology to Medicago truncatula nodulins. Two of the SWEETs studied, AtSWEET11 and AtSWEET12, were highly expressed in leaves, appearing in what may be phloem parenchyma of both major and minor veins. This localisation however is only putative at this stage, and needs more rigorous confirmation. These findings, along with the impaired leaf sucrose export in SWEET mutant plants, suggest a role in sucrose efflux to the phloem apoplasm, for subsequent uptake (by SUT1 proteins) into the SE-CC complexes. For the cloning of these novel proteins, Chen et al., (2010; 2012) used human cell lines, reasoning that they should lack endogenous sucrose transport. The online plant membrane protein database Aramemnon (http://aramemnon.uni-koeln.de) was used to select transporters of unknown function with multiple membrane spanning domains from Arabidopsis for screening. The authors then screened the selected genes by co-expressing them with a sucrose FRET sensor in human cell lines, identifying AtSWEET1 as a glucose
transporter (Chen et al., 2010). Further screening of SWEET family members revealed the sucrose uniporters, AtSWEET11 and AtSWEET12 (Chen et al., 2012).

Another group of transporters capable of sucrose transport have been recently described. The Tonoplast Monosaccharide Transporter (TMT) proteins (Wormit et al., 2006) were identified through proteomic approaches using highly purified mesophyll tonoplast (Carter et al., 2004; Endler et al., 2006). Originally described as glucose/H⁺ and fructose/H⁺ antiporters (Wormit et al., 2006), subsequent analysis also showed the ability to mediate sucrose/H⁺ antiport (Schultz et al., 2011). As a tonoplast-localised transporter, it is unlikely that it accounts for the plasma membrane antiport detected in developing legume seed coats by Fieuw and Patrick (1993) and Walker et al. (1995). However, a plasma membrane localised TMT family member may mediate the observed transport. The case both of SWEETs and TMTs, illustrate the potential of other sucrose transporters to reside within families of proteins for which function had not previously been elucidated.

There are several families of poorly-known transporter proteins in plants in addition to those containing the TMTs and SWEETs. One of these families alone, the multidrug and toxicity efflux (MATE) family, has 58 paralogs in Arabidopsis. The few MATEs characterised to date have been shown to transport various solutes, including alkaloids, flavonoids, citrate, malate and other metabolites in antiport with protons. In plants, MATE family members have been localised to the tonoplast and plasma membrane (Omote et al., 2006). Interestingly, one of these transporters, TT12 (a vacuolar flavonoid/H⁺ antiporter), transports beta-glucosides when expressed in yeast (Marinova et al., 2007). This suggests that the active site may be similar to that of SUTs, which also transport beta-glucosides (see for example Chandran et al., 2003; Sivitz et al., 2007). It is also worth noting that TT12 was identified as a sucrose responsive gene by Sivitz et al. (2008). This evidence suggests that it is possible that a sucrose-proton antiporter may be contained within the MATE family. However, due to the large numbers of unknown transporter proteins outside the MATE family, a functional cloning approach to identifying sucrose antiport would be most useful (see section 5.3.3).

Recent studies comparing a tonoplast-localised inositol transporter (AtINT1) and closely related tonoplast-localised transporter (AtINT4) identified a targeting sequence
that, when incorporated into otherwise plasma membrane transporters (including AtINT4, AtSUC2 and AtSWEET1) re-routes them to the tonoplast (Wolfenstetter et al., 2012). The sequence identified in AtINT1 is responsible for targeting to the tonoplast, and accounts for the different subcellular localisations sometimes observed between closely related transporters. The possibility of similar such targeting sequences in other membrane transporters, including SUTs, SWEETs and MATEs, appears likely. Thus, there is a distinct possibility that a close relative of a vacuolar sucrose/H+ antiporter (such as a TMT) may lack such a vacuolar targeting sequence, and function as a plasma membrane sucrose/H+ antiporter.

5.3 Proposed Future Directions

5.3.1 Characterisation of SUF Proteins at the Intracellular Face

A major outcome of this study is the development of the s6s7 yeast, to enable the study of the cytoplasmic face of SUFs (and other sucrose transporters) using stopped flow fluorimetry. Fundamental questions asked about the extracellular face of the transporters in Chapter 2, including energisation, affinity for sucrose and substrate specificity can now be addressed for the cytoplasmic face. An interesting question that may be examined is whether the transporters have functional symmetry, or whether they are polarised, with faces of differing affinity for sucrose and/or other sugars. Functional asymmetry has been reported in other transporters, including the human Na+/glucose transporters SGLT1 (Quick et al., 2003) with more than an order of magnitude difference in affinity between the two faces.

5.3.2 Reverse Genetics and SUF-mediated Contributions to Sucrose Transport

A limitation of the series of studies presented in this thesis is the lack of reverse genetic studies in elucidating SUF gene function in planta. The principal reason for this is that there are no immediately apparent paralogs of SUFs in Arabidopsis, despite the presence of 9 SUT genes within this species. Of these 9, 7 fall within the SUT1 clade, one in the SUT2 clade, and one is a SUT4 clade member. AtSUT4 is reported to be a sucrose-proton symporter (Weise et al., 2000), as is SUT2 member AtSUC3 (Meyer et al., 2000). For SUT1 clade members, AtSUC1 and AtSUC2 (Sauer and Stolz, 1994), AtSUC5 (Ludwig et al., 2000), AtSUC8 and AtSUC9 (Sauer et al., 2004) all encode
functional symporters, whereas AtSUC6 and AtSUC7 appear to be pseudogenes, encoding aberrant proteins in most ecotypes (Sauer et al., 2004). So, the use of Arabidopsis insertional mutants to study the phenotypic effects for SUF knockdown is not possible. This absence of SUF paralogs in Arabidopsis may be due to their limitation to some plant species only (for example, legumes) as a result of a specialisation in function.

A possibility that remains is creating pea lines under and/or over-expressing SUF genes. This approach has already been taken with studying SUT1 over-expression (Rosche et al., 2002; 2005), using StSUT1 under control of the seed-specific vicilin promoter. Similarly, antisense PsSUT1 lines (using the PsSUT1 cloned in reverse orientation behind the lipid transfer protein (LTP) promoter have been generated (Patrick, unpublished). A similar approach could be taken with SUFs. In particular, it would be interesting to create an antisense PsSUF1 under the control of the companion-cell specific AtSUC2 promoter. The resulting knockdown of PsSUF1 in the SE-CC complexes of leaf minor veins could help reveal whether this protein is contributing to phloem loading (possibly by interaction with PsSUT1) despite its in vitro facilitated efflux function.

5.3.3 Possible Strategies for Cloning a Sucrose- proton Antiporter

A next apparent step in fully describing sucrose efflux from plant cells involves identifying the sucrose-proton antiporter previously detected in developing legume seeds using whole coat studies (Fieuw and Patrick, 1993; Walker et al., 1995). In particular, the aim is to identify the transporter(s) at a molecular level, and determine whether it belongs to a known class of sucrose transporter (SUT, SBP, SWEET, TMT) or another, perhaps undescribed, family. To this end, a novel strain of yeast has been generated (Y Zhou, unpublished) to facilitate the functional screening of cDNA libraries for additional sucrose effluxers. The yeast strain, Supy (sucrose producing yeast), has been engineered to express sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP), enabling the production of sucrose from hexoses or ethanol supplied in the growth media. In the presence of a sucrose antiporter (or other sucrose effluxer), sucrose is effluxed from the cell and detected in the medium. This detection may utilise an enzyme coupled sucrose assay. The success of the Supy yeast system has already
been demonstrated using *PsSUF4* cDNA, with significant efflux of sucrose detected via enzyme-linked sucrose assay when compared to empty vector transformed yeast (Y Zhou, unpublished). Thus it appears to be a promising screening system for identifying novel sucrose effluxers, whether SUT/SUF family members, or from other transporter families. Additionally, the Supy yeast has the potential to be combined with other emerging technologies, such as the FRET sucrose sensor (Lager *et al.*, 2006). This sensor used by Chen *et al.* (2012), could be utilised to facilitate rapid screening. In this situation, the sensor needs to be present in the extracellular environment to sense sucrose released from the cells. The sensor could be contained within a second yeast strain or bacteria, with its growth and sensing contingent on sucrose release from Supy. Alternatively, the FRET sucrose sensor could be expressed within Supy and targeted for secretion and binding into the cell wall compartment, thus enabling sensing of sucrose released to the apoplasm. There are now several sequence tags that have been characterised and utilised to target and bind proteins to the yeast cell wall (for a review see Pepper *et al.*, 2008), and placing the FRET sequences under control of such a promoter may make such targeting achievable. An advantage of a FRET-Supy yeast system is that it can exploit fluorescence-assisted cell sorting (FACS), rather than requiring the culturing and assaying of each individual yeast transformant harbouring a particular cDNA molecule separately.

The selection of tissue for sampling to create a cDNA library that is likely to contain the putative sucrose effluxer(s) can be informed by the model presented in Fig. 5.1B. This predicts that the antiporter would be most active and abundant in seed coats during the storage phase. Supy-cDNA yeast cells that return a positive result for FRET and/or enzyme linked assay based sucrose detection can have the insert sequenced, and moved across to the sec6-4 vesicle system developed in Chapter 4. The novel transporter isolated can then characterised, with the mode of energisation (if any), *Kₚ*, and substrate specificity identified.

Several approaches could be taken to examine what, if any, proteins SUFs complex within the plasma membrane. Protein complex immune-precipitation is one such approach, where a bead-bound SUF-specific antibody (generated for this project) is used to probe a solution of non-denatured, cross-linked (for complex stability) membrane protein complexes. The protein complexes bound to the antibody-coated beads are then eluted, individual proteins are separated by SDS-PAGE, and identified
by mass spectroscopy analysis. Antibodies raised against any co-precipitating proteins used to repeat the procedure should be able to isolate the starting target protein (in our case, the SUFs). To carry out this study, the plant plasma membrane used would ideally be derived from source leaves, to isolate SUFs (and any associated proteins, possibly including PsSUT1, which co-localised in Fig. 3.9) localised in minor vein sieve elements. The successful antibody labelling of SUFs in Chapter 3 indicates that the epitope is exposed for antibody binding, increasing the chance of success with this technique.

For studying potential homo-oligomerisation of SUFs, blue native PAGE, such as that carried out by Liesche et al. (2008) on SoSUT1, would be appropriate, while the split ubiquitin system presented by Reinders et al. (2002) would be useful to determine whether interactions occur between SUT/SUF proteins when expressed heterologously in yeast.

Isolating the detergent-resistant membrane fraction from leaves and probing with SUF antibodies could also prove informative. However, this technique has recently come into question (Tanner et al., 2011) so other approaches mentioned above should be preferred.

### 5.4 Conclusion

This work has demonstrated that members of the sucrose transporter family also include transporters that are facilitators, in addition to the sucrose proton symporters previously reported. These facilitators, shown to be plasma membrane transporters, were localised to regions supporting high sucrose flux within the plant, although their transcripts were not limited to these regions. *In planta*, the cellular localisation of these facilitators on sieve elements suggests that their function may be more complex than just simple sucrose facilitation. Further studies using reverse genetics, the s6s7 inside–out vesicle generating yeast mutant (developed in this study), and on association and interaction of SUFs with other proteins will provide a more complete picture of the contribution of SUFs to sucrose flux. This, together with the cloning of sucrose-proton antiporters, will advance our understanding of sucrose efflux, a most fundamental component of plant biology.


