UNIVERSITY OF SOUTHAMPTON

THE ROLE AND REGULATION OF SUGAR TRANSPORTERS IN ARABIDOPSIS

by Duncan James Legge

Doctor of Philosophy

FACULTY OF SCIENCE
SCHOOL OF BIOLOGICAL SCIENCES

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

Signature: 

Date: 26th May 2004

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
SCHOOL OF BIOLOGICAL SCIENCES

Doctor of Philosophy

THE ROLE AND REGULATION OF SUGAR TRANSPORTERS IN ARABIDOPSIS

by Duncan James Legge

Sugar transporters are essential for assimilate partitioning and cellular nutrition, and their activities must be carefully controlled to meet the demands of plant growth and development under changing environmental conditions. Analysis of the Arabidopsis genome, in this and other studies, has revealed that there are large, multi-gene families of sugar transporters. The next challenge is to determine their individual physiological function. This thesis describes a number of different approaches to understand the role of sugar transporters in Arabidopsis thaliana. Insertion mutants from the Sainsbury Laboratory Arabidopsis dSpm Transposant (SLAT) collection were used to investigate the function of the sucrose transporters AtSUC1 and AtSUC2. Phenotypic analyses of these mutants demonstrate that AtSUC1 and AtSUC2 have substantially different roles in growth and development of Arabidopsis. The suc1-1 mutant was similar to wild-type (WT) plants in terms of general growth and development. In contrast, knocking out AtSUC2 had severe effects at all developmental stages. The suc2 mutants were severely retarded in their growth and, at maturity, were significantly smaller in height with fewer and smaller leaves and a reduced number of flowers. Siliques developed in some cases, but these were generally shorter in length and produced significantly smaller seed than in WT plants. Importantly, although previously isolated suc2 mutants were reported as sterile, those isolated in this study could produce viable seed although viability varied between individual plants. Exogenously supplied sucrose did not fully rescue the suc2 mutant phenotype.

An alternative approach to understanding gene function is to investigate the regulation of gene expression. This was undertaken for representative genes from two important sugar transporter families, the disaccharide transporter, AtSUC2, and the monosaccharide transporter, AtSTP4. Gene expression was investigated in Arabidopsis seedlings using RT-PCR and reporter gene technology concentrating on two potential regulators of sugar transporter gene expression, sugar availability and light. Both activate signalling pathways in plants and sugar transporters may be targets for regulation by these stimuli. Experiments using transgenic Arabidopsis plants containing promoter: *GUS* fusions demonstrated that AtSUC2 and AtSTP4 were both expressed in all organs with AtSUC2 expression concentrated in the vascular tissue. Neither AtSUC2 nor AtSTP4 expression was dramatically different in light- and dark-grown seedlings. However, both genes were repressed by exogenous sucrose with AtSUC2 inhibited more than 50% by just 10 mM sucrose, but not markedly affected by glucose, fructose or mannitol. AtSTP4 was much less sensitive to sucrose than AtSUC2.

A more global approach to understanding light regulation of sugar transporter expression was taken by utilising microarray technology with a custom-made Arabidopsis membrane transporter array containing oligonucleotides representing all sugar transporter genes. This transcriptomics approach identified several transporters showing changes in gene expression in response to far-red light, which specifically activates phytochrome A. These results will provide a starting point for further targeted analysis of the role of sugar transporters in light-regulated seedling development.

Contents

Acknowledgements

Firstly, I would like to thank my supervisors Dr. M.J. Terry and Dr. L.E. Williams for their guidance throughout my project and in writing this thesis

Throughout my research project I have been fortunate to have worked with many post-doctoral researchers who have been very generous in giving me their invaluable advice and experimental expertise. I would like to thank Dr. Alex McCormac, Dr. Jon Pittman, Dr. Gerard Krijger, Dr. Ian Beech, Dr. Aram Buchanan. I have also found encouragement in the support and friendship of colleagues also studying for their PhDs, namely, Vassilis Fotopoulos, Russell Vaughan and Paul Baccarini.

Finally, I must recognize the seemingly endless support from my family.

Abbreviations

3-OMG	3-O-methyl glucose
ABA	Abscisic acid
ADP	Adenosine diphosphate
AMPL	*Arabidopsis* membrane protein library
AMT	*Arabidopsis* membrane transporter
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AtSTP	*Arabidopsis thaliana* sugar transporter
AtSUC/T	*Arabidopsis thaliana* sucrose carrier/transporter
AtTFDB	*Arabidopsis thaliana* Transcription Factor Database
BLAST®	Basic Local Alignment Search Tool
bp	Base pairs
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CRE	*Cis*-regulatory elements
CTAB	Cetyltrimethylammonium bromide
CTP	Cytosine triphosphate
DEPC	Diethyl pyrocarbonate
dATP	Deoxyadenosine triphosphate
ddNTP	Dideoxynucleotidetriphosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotidetriphosphate
EBI	European Bioinformatics Institute
EDTA	Ethylenediaminetetraacetate
EMBL	European Molecular Biology Laboratory
EST	Expressed sequence tag
EtBr	Ethidium Bromide
FR	Far-red light
FW	Fresh weight
gDNA	genomic DNA
GTP	Guanidine triphosphate
GUS	β-glucuronidase
HCl	Hydrogen chloride
HXK	Hexokinase
HPLC	High-performance liquid chromatography
I/B ratio	Intensity / background ratio
IAA	Isoamyl alcohol
IPTG	Isopropylthiogalactoside

kb	Kilobase pairs
K_d	Dissociation constant
kDa	Kilo daltons
K_m	Michaelis constant
L	Light
LB	Luria-Bertani media
LiCl	Lithium chloride
M*At*DB	MIPS Arabidopsis thaliana database
MFS	Major facilitator superfamily
MIPS	Munich Information Centre for Protein Sequences
mM	Millimolar
MRAT	Median background corrected single pixel ratio
mRNA	Messenger RNA
MS	Murashige and Skoog media
MU	4-methylumelliferyl
MUG	4-methylumelliferyl-β-D-glucuronide
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrophosphate
NaOH	Sodium hydroxide
nM	Nanomolar
NMR	Nuclear magnetic resonance spectroscopy
Oligo	Oligonucleotide
PCMBS	*p*-Chloromercuribenzene sulphonic acid
PCR	Polymerase chain reaction
PHY	Phytochrome
PLACE	Plant Cis-acting Regulatory DNA Elements Database
PPT	Phosphinothricin
R	Red light
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
SAS	Shade avoidance syndrome
SDS	Sodium dodecyl sulphate
SE	Standard error
SE/CC	Sieve element / companion cell
SLAT	Sainsbury Laboratory *Arabidopsis* Transposant Filter
STF	Sugar transporter family
T-DNA	Transposon DNA
TAE	Tris / acetic acid / EDTA
TAIR	The *Arabidopsis* Information Resource
TE	Tris / EDTA

TMD	Transmembrane domain
Tris	Tris(hydroxymethyl)aminomethane
µl	microlitre
UTP	Uridine triphosphate
UV	Ultraviolet light
WT	Wildtype
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-giucuronide

Chapter 1

Introduction

1.1. Sugar synthesis and transport in plants

Plants are photoautotrophic organisms capable of fixing carbon dioxide into sugars by photosynthesis. This occurs mainly in the mesophyll cells of mature leaves and as the latter are responsible for the net export of sugars they are classified as source tissues. Plants also have many heterotrophic tissues, such as roots, seeds and developing leaves, and these require a source of sugars to be supplied for growth and development. These are classified as sinks because they are net importers of sugars. The process of carbon partitioning from source to sinks is required to allow growth and development and it has to be precisely co-ordinated according to the demands of each particular tissue. A diagrammatic representation of long-distance sugar transport showing the contribution of sugar transporters and key enzymes important for carbon partitioning is shown in figure 1.1 (reviewed in Lalonde *et al.*, 1999; Williams *et al.*, 2000a).

The initial process of carbon dioxide fixation via the Calvin cycle occurs in the chloroplast. The products, triose phosphates, can be shuttled into several biosynthetic pathways within the chloroplast (e.g. for starch and lipid synthesis), or may be exported to the cytosol via the triose phosphate translocator (TPT) where they are converted to sucrose (figure 1.1). This is the major form of sugar that is translocated in higher plants. It is a stable, non-reducing disaccharide that is electroneutral, inert and soluble in water (Eschrich, 1994). Importantly, even at relatively high concentrations is shows low viscosity in solution. These properties make it well suited as a vehicle for energy transport. Some plant species also translocate sugar alcohols (mannitol and sorbitol) in addition to sucrose whereas others transport the raffinose series of oligosaccharides (raffinose, stachyose, and verbascose) in addition to sucrose (Zamski and Schnaffer, 1996). Sucrose appears to be the major translocated sugar in *Arabidopsis* although small amounts of raffinose are also transported (Haritatos *et al.*, 2000).

Export from source to sink tissues is facilitated by membrane bound transport proteins and a simplified model for this will be discussed (figure 1.1). Sucrose synthesised in the mesophyll may be loaded into the SE/CC complex either through plasmodesmata or via the apoplast. Apoplastic phloem loading requires sucrose export (1) from the mesophyll or the vascular parenchyma and uptake (2) into the SE/CC complex. Solute transport in the phloem is considered to occur by mass flow, driven

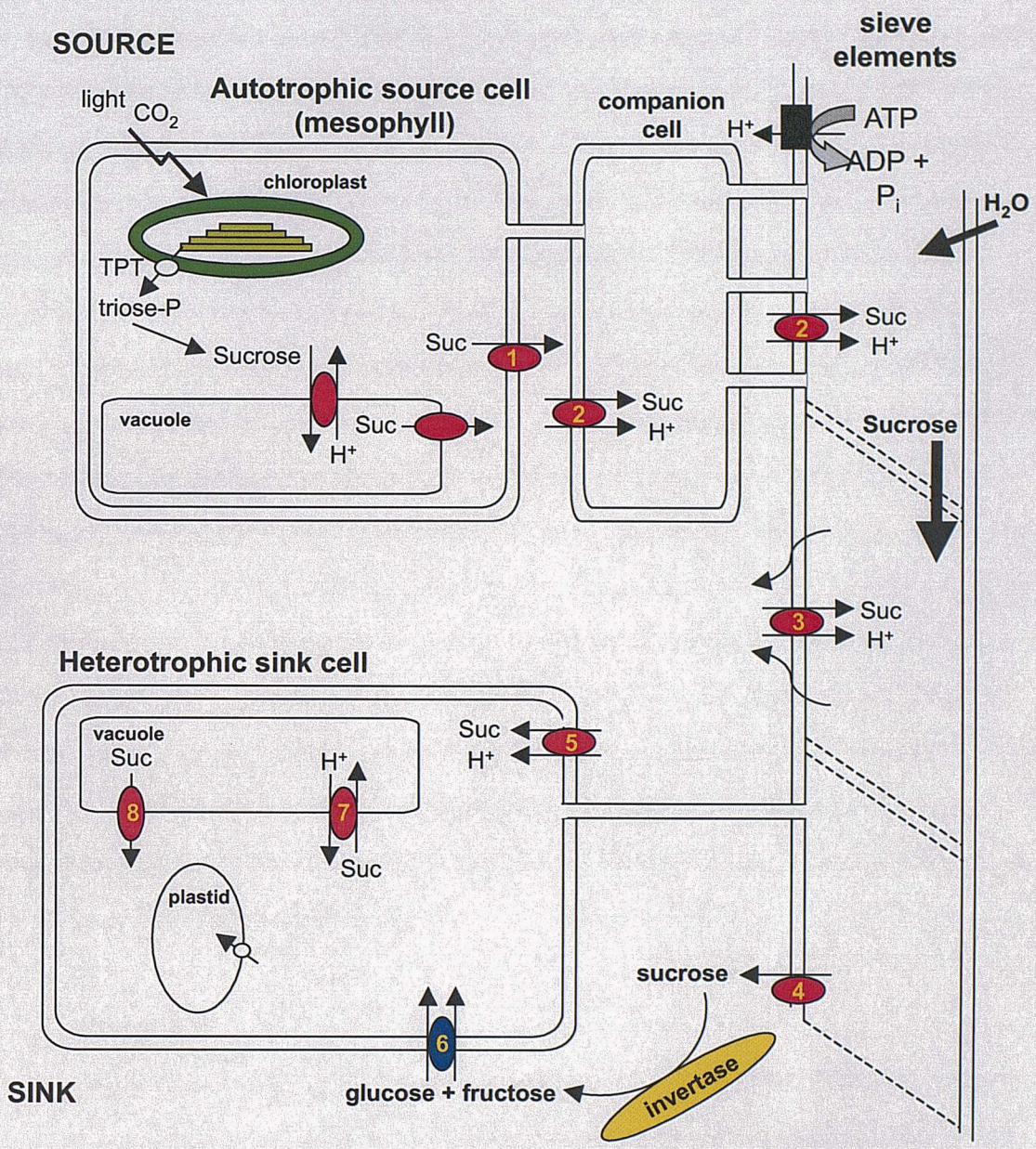


Figure 1.1 Simplified model of long-distance sugar transport between sources and sinks in higher plants illustrating the contribution of sugar transporters and some key enzymes. Sucrose is synthesised in the mesophyll and may be loaded into the SE/CC complex either through plasmodesmata (shown connecting the different cells) or via the apoplast. Apoplastic phloem loading requires sucrose export (1) from the mesophyll or the vascular parenchyma and uptake (2) into the SE/CC complex. Sucrose is transported in the phloem sap by bulk flow toward sink tissue. Passive leakage can occur along the path (indicated by arrows) and retrieval (3) can also takes place. Sucrose can enter sink tissues via plasmodesmata or, alternatively, a sucrose exporter may occur to unload sucrose into the apoplast in sink tissues (4). A sucrose carrier may be responsible for uptake into sink tissues (5) or cells may take up hexoses following the hydrolysis of sucrose by an apoplastic invertase (6). A H^+/sucrose antiporter may be responsible for sucrose uptake into the vacuole (7) whereas a uniporter may be responsible for release (8). Figure adapted from Lalonde *et al.* (1999). See text for further details.

by an osmotically generated pressure gradient between source and sink (according to the pressure-flow model first proposed by Münch in 1930). Passive leakage can occur along the path (indicated by arrows) and retrieval (3) can also take place. Sucrose can enter sink tissues via plasmodesmata or, alternatively, a sucrose exporter may occur to unload sucrose into the apoplast in sink tissues (4). A sucrose carrier may be responsible for uptake into sink tissues (5) or cells may take up hexoses following the hydrolysis of sucrose by an apoplastic invertase (6). A H^+/sucrose antiporter may be responsible for sucrose uptake into the vacuole (7) whereas a uniporter may be responsible for release (8) (reviewed in Lalonde *et al.*, 1999; Williams *et al.*, 2000a). As stated, this is a simplified model, and it is now clear that there are many sucrose and monosaccharide transporters (see next section) together with different isoforms of invertase and the H^+-ATPase are present to coordinate sugar transport in diverse tissues, at different developmental stages and under varying environmental conditions (reviewed in Williams *et al.*, 2000a). These must be carefully controlled so that the carbon partitioning is integrated with the metabolic activities of both the source and sink tissues. There is good evidence for the occurrence of sucrose transporters at the companion cell and sieve element and for various monosaccharide transporters in sink tissues as indicated in the model (figure 1.1) but strong molecular evidence for some of the other transporters shown is still weak or lacking (e.g. vacuolar sugar transporters). A more detailed discussion of phloem loading and unloading will be given after an introduction to sugar transporters.

1.2 Sugar Transporters

Studies using whole tissues, suspension cells and protoplasts indicated that sugar transport proteins were responsible for sugar uptake into cells (reviewed in Reinhold and Kaplan, 1984). The sugar/H^+ cotransport theory was put forward in the seventies to account for sugar uptake into plant cells (Komor *et al.*, 1977; Baker 1978) and this model stands today where it is proposed that the plasma membrane H^+-ATPase generates the proton- motive force to drive the uptake of sugars via a proton-coupled symport process. Evidence in support of this came from methods using isolated membrane vesicles, which had a number of advantages: ability to isolate specific membrane (plasma membrane, tonoplast); the elimination of compartmentation and metabolism; access to either surface of the membrane, and the ability to manipulate intra- and extravesicular solution composition (Bush, 1993). These techniques demonstrated that sucrose could be transported across the plasma membrane via proton-coupled transporters (Bush 1989, 1990; Lemoine *et al.*, 1989; Williams *et al.*, 1990). Although an improvement, the properties observed for sugar transport across plasma

membrane vesicles could include a contribution from all sugar transporters in that membrane. Molecular approaches were introduced which resulted in the isolation of individual sugar transporter cDNAs. This enabled researchers to assess the function and role of individual sugar transporters and use the initial cDNA sequences as probes with which to discover other sugar transporters (see below). Obtaining the cDNA sequences also enabled scientists to look at the amino acid components and investigate the structure and mechanism of membrane sugar transport. Such studies and whole genome analysis have shown that there are multi-gene families for sugar transporters (reviewed in Williams *et al.*, 2000a). Since publication of the genome sequence for the model plant *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000) many putative sugar transporters have been identified and by homology have been grouped into families. Forty-eight monosaccharide transporters were identified by Ward (2001; http://www.cbs.umn.edu/arabidopsis/, Organic Solute Cotransporters, Glucose Transporter Family), however, some are putative transporters and have yet to be characterised. The monosaccharide and disaccharide transporters have been classified as belonging to the sugar porter family (2.A.1.1) of the Major Facilitator Superfamily (MFS, 2.A.1) of families of transport proteins (Saier *et al,*. 1999; http://www-biology.ucsd.edu/~msaier/transport/titlepage2.htm, 2001). The sugar porter family also contains sugar transporters from organisms as diverse as protozoa, yeast, fungi bacteria and animals such as rat and humans (Saier *et al.*, 1999). It would seem that sugar transporters share inter species homology to some degree. Of the 48 monosaccharide transporters predicted from the *Arabidopsis* genome, 14 have been classified as *AtSTPs* (***A**rabidopsis **t**haliana* monosaccharide **s**ugar **t**rans**p**orters) due to their similar homology (Büttner *et al.*, 2000).

The first plant sugar transporter to be cloned was the H^+-monosaccharide transporter HUP1, from the algae *Chlorella kessleri* (Sauer and Tanner, 1993). This was isolated by differential screening of a cDNA library prepared from glucose-induced cells and radiolabelled cDNA from induced and non-induced cells (Sauer and Tanner, 1993). The *HUP1* sequence was used as a probe to obtain genomic and full-length cDNA clones of an *Arabidopsis thaliana* sugar transporter, *STP1* (Sauer *et al.*, 1990a). Hydrophobicity plots reveal that AtSTPs conform to the characteristic 12 transmembrane spanning domains that are common to the MFS (Marger and Saier, 1993). Homology between the first and second six domains suggests that the structure of the sugar transporters evolved from a duplication of the ancestral gene (Sauer *et al.*, 1990a). To demonstrate that *HUP1* and *STP1* encode sugar transporters, these clones were expressed in yeast (*Saccharomyces pombe* and *S.cerevisia*) and transport studies using radiolabelled substrates indicated that they were indeed

monosaccharide transporters (Sauer *et al.*, 1990b; Sauer and Stadler 1993; Caspari *et al.*, 1994). Monosaccharide transporters have since been isolated from a range of other species using various techniques including RT-PCR (Weig *et al.*, 1994). Most work has been carried out with the *Arabidopsis* monosaccharide transporters of the AtSTP family and these show different tissue localisation, kinetic properties and also regulation. Interestingly, some *AtSTPs* appear to be highly responsive to the conditions of the plant; this is supported by wound and pathogen induced expression of *AtSTP4* and *AtSTP3* (Truernit *et al.*, 1996; Büttner *et al.*, 2000, Fotopoulos *et al.*, 2003). Some monosaccharide transporters transport a range of monosaccharides whereas others are more specific (Büttner *et al.*, 2000). Substrate specificity has generally been studied following heterologous expression and therefore the substrates actually transported by these carriers in the plant is not necessarily the same and will depend on the local concentration of sugars and prevalence in the particular tissue.

The first higher plant sucrose carrier was isolated by complementation of an engineered yeast mutant lacking the native invertase and maltose carrier, and expressing a plant sucrose synthase enzyme (Riesmeier *et al.*, 1992). This yeast is unable to grow on sucrose as the sole carbon source but is capable of metabolising internal sucrose. Transformation of the yeast with a spinach cDNA library in a yeast expression vector followed by selection for growth on sucrose resulted in the isolation of the *SoSUT1* clone (Riesmeier *et al.*, 1992). This was characterised and displayed properties consistent with it being a proton-coupled sucrose transporter (Riesmeier *et al.*, 1992). Further homologues have now been isolated from a range of plant species including *Solanum, Arabidopsis, Plantago* and *Ricinus* (Riesmeier *et al.*, 1993; Sauer and Stolz, 1994; Gahrtz *et al.*, 1994; Weig *et al.*, 1996). The first sucrose transporters cDNAs isolated from *Arabidopsis* were *AtSUC1* and *AtSUC2* (***A**rabidopsis **t**haliana* **su**crose **c**arrier) (Sauer and Stolz 1994) and there appear to be a total of nine different SUCs in *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000). Five of these (*AtSUC1*, *AtSUC2*, *AtSUT2/AtSUC3*, *AtSUT4 and AtSUC5*) have been characterized to varying degrees: (Sauer and Stolz 1994; Barker *et al.*, 2000; Ludwig *et al.*, 2000; Weise *et al.*, 2000). The nomenclature in the literature for plant sugar transporters has become rather confusing with SUC and SUT being used for disaccharide transporters and STP, MST, HEX and ST being used for monosaccharide transporters. In some cases the same gene sequence has been published with different names e.g. *AtSUT2* (Barker *et al.*, 2000) and AtSUC3 (Meyer *et al.*, 2000) and in this case both names are used together in the thesis (AtSUT2/AtSUC3). Functional studies have indicated that AtSUC1, 2 and 3, transport sucrose with high affinity (K_m values of 0.5, 0.77 and 1.9 mM respectively;

Sauer & Stolz 1994; Meyer *et al.*, 2000), whereas AtSUT4 is a low affinity transporter (K_m 11.6 mM; Weise *et al.*, 2000). AtSUC5 can transport vitamin H (biotin) as well as sucrose (K_m 1 mM) but it is not yet known if this is a general property of these plant disaccharide transporters (Ludwig *et al.*, 2000).

Sucrose transporters are also part of the MFS as they too show a predictive structure of twelve membrane domains (see chapter 6). A structural analysis of PmSUC2 has been performed using monoclonal antibodies and bacteriophage lambda surface display (Stolz *et al.*, 1999). In spite of the apparent similarity to the MFS superfamily of transporters, sucrose transporters are not directly sequence-related to other carriers. For example, very few common elements exist with the monosaccharide transporter plant gene family (Büttner and Sauer, 2000; Williams *et al.*, 2000a), with an average of 20% of identical amino acids between sucrose and monosaccharide plant transporters (Sauer *et al.*, 1994). Unlike monosaccharide transporters which can sometimes transport a range of monosaccharides, sucrose transporters are relatively specific to sucrose; as high as ten fold excess of glucose, fructose, lactose, mannose, melibiose and raffinose does not inhibit sucrose transport (Bush, 1999; Lemoine, 2000). Only maltose has been shown to inhibit sucrose uptake (Gahrtz, 1994; Shakya and Sturm, 1998).

Plant sugar transporters have specific tissue locations (shown by mRNA *in situ* hybridisation, reporter genes and antibodies (reviewed in Williams *et al.,* 2000a and Truernit, 2001). Some transporters appear to play a purely nutritional role, supplying sugars to cells for growth and development, whereas others are involved in generating osmotic gradients required to drive mass flow or water movement (Stadler *et al.*, 1999; Meyer *et al.*, 2000; Williams *et al.*, 2000a). It has also been suggested that some of these transporters may function as sucrose sensors (Barker *et al.*, 2000) although this has yet to be shown (Meyer *et al.*, 2000; Barth *et al.*, 2003). One of the sugar transporters investigated in this thesis study (AtSUC2) is suggested to play an important role in phloem loading and possibly unloading and therefore these processes will be discussed briefly in the next sections.

1.3. Phloem Translocation of Sugars

Plants employ an intricate vascular network that covers all of the organs to efficiently keep sink tissues supplied with sugars. This vascular network is composed of two main types of tissues; the xylem that carries water and mineral salts in the transpiration stream and the phloem that contains a sap rich in both organic and inorganic substances (Taiz and Zeiger 1998). The phloem is a tissue of tubular structure that consists of inter-connected sieve elements, themselves of a cylindrical shape.

Pores in the adjoining walls of the sieve elements (sieve plates) allow a continuous passage along the tubular phloem. Sugars loaded into the phloem flow between the sieve elements as phloem sap. This is a thick mixture consisting mainly of sugars but also contains amino acids, ureides, organic acids, protein and inorganic elements (Riens *et al.*, 1991). Sucrose concentration in the phloem sap ranges from 200 to 1600 mM, depending on the plant species (Kallarackal *et al.*, 1989; Winzer *et al.*, 1996).

The sieve elements of the phloem are unique amongst plant cells, as at maturity they do not contain the many organelles essential for cell maintenance. Instead, they are supported by companion cells. These companion cells are connected to the sieve elements via specialized plasmodesmata. The size exclusion limits for plasmodesmata are reported to show a similar range to that for the gap junctions of animal cells, i.e. approximately 800-1000 Da (Spray and Bennet, 1985). However, some studies have reported the movement, through the phloem of molecules with much larger molecular mass, for example the 30 kDa tobacco mosaic virus (TMV-MP) in tobacco plants (Lucas and Wolf, 1999). The close ontogenic and functional relationship between the two cell types is reflected in the term sieve element/companion cell complex (se/cc complex). Exporting leaves can also contain two specialized types of companion cells: transfer cells and intermediary cells. Transfer cells are characterized by cell wall invaginations, which increase the surface area 3- to 10-fold (Pate and Gunning, 1972; Wimmers and Turgeon, 1991). It has been suggested that this increase in plasma membrane surface area may be a response to an increase in solute transport and, therefore, the presence of transfer cells may indicate intense localised apoplastic transport activity (Ward *et al.*, 1998). Intermediary cells are an indication of a functioning symplastic pathway. Intermediary cells have many plasmodesmata connections to parenchyma and bundle sheath cells (Turgeon and Beebe, 1991). They can also be characterized by small vacuoles and a lack of starch grains in the chloroplasts.

Sugars are translocated in the phloem by mass flow driven by a pressure gradient between source and sink tissues (based on the hypothesis by Münch, 1930). This is created by the active loading of sugars in the SE/CC of the source leaves, increasing the solute potential, resulting in the influx of water from the xylem and thus increasing the turgor pressure. Phloem unloading of solutes at sink sites causes water to exit and the pressure gradient is maintained. The relatively high turgor pressure in SE/CC of source leaves causes the movement of solutes along the phloem. This turgor pressure gradient along the SE/CC between source and sink sites is aided by the varying proportional volumes of SE and CC in these different zones (van Bel, 1996). Collection phloem (in source leaves) consists predominately of CC whereas the release phloem (in sink tissues) are mostly if not entirely

SE. This increase in SE diameter encourages bulk flow as release phloem imposes a lower turgor pressure than collection phloem enabling a gradient for bulk flow. Additionally, unloading from release phloem either symplastically down a concentration gradient or apoplastically induces the movement of water out of the phloem further decreasing the turgor pressure and therefore encouraging bulk flow (Fisher and Cash-Clark, 2000).

1.3.1. Phloem loading

Two main pathways for the loading of solutes into the phloem have been proposed, namely the apoplastic and symplastic pathways. The symplastic pathway involves the movement of solutes through the cytosol continuum including the sieve tubes, via connecting plasmodesmata (see below). Apoplastic phloem loading involves the release of solutes into the cell wall apoplast before uptake across the plasma membrane of the sieve tube and/or companion cell via membrane carriers. This is illustrated in figure 1.1. In this model, the H^+-ATPases hydrolyses ATP to pump H^+ into the apoplast. This creates a proton-motive force which sugar transporters use to transport sugars into the phloem sieve cells and/or companion cells. In support of this duo, AtSUC2 and AHA3 (*Arabidopsis* plasma membrane H^+-ATPases isoform) have both been localized to the companion cells of the phloem in *Arabidopsis* (Stadler and Sauer, 1996; DeWitt *et al.*, 1996). There is now good evidence for a range of sucrose transporters located in the phloem, which may be involved in phloem loading. (Riesmeier *et al.*, 1993; Gahrtz *et al.*, 1994; Truenit and Sauer, 1995; Kuhn *et al.*, 1997; Bick *et al.*, 1998; Burkle *et al.*, 1998; Shakya and Sturm, 1998; Gottwald *et al.*, 2000; Weise *et al.*, 2000).

There are two main models for apoplastic phloem loading (Ward *et al.*, 1998). In the first, sucrose is exported into the apoplast only at the sieve elements and then it is taken up by the latter cells; this is the predicted method of loading in the Solanaceae (e.g. tomato, tobacco). The second model depicts sugar transport into the apoplast at the parenchyma cells and in this case sugars are loaded into the companion cells that, in turn, then load the sieve element via a symplastic route. This is the predicted method of phloem loading in *Arabidopsis* and *Plantago*. Thus, different plant species translocate assimilates by different methods and, hence, it is plausible that the various sugar transporters have different roles in each group of plants. This sometimes complicates attempts to assign specific functions to individual sugar transporters. RNA *in situ* experiments have shown that the sucrose transporter *StSUT1*, is expressed in the sieve elements (Riesmeier *et al.*, 1993) of potato. As plants in which the expression had been drastically reduced by antisense technology show reduced tuber yield and the accumulation of sugars in mature leaves, which presumably was a result

of impaired export (Riesmeier *et al.*, 1994). Microscopy of leaves from these antisense *StSUT1* plants showed dramatic ultrastructural effects with increased starch accumulation especially in the chloroplasts and lipid deposits in the form of oleosomes (Schulz *et al.*, 1998). Knockout mutants for *AtSUC2* (localised to companion cells, Stadler and Sauer, 1996) were severely retarded in their growth and development and these showed a reduction in the transfer of labelled sugars from source to sink tissues (Gottwald *et al.*, 2000). This phenotype is consistent with a plant that cannot efficiently move sucrose from its source leaves to sink tissues.

For purely symplastic routes, unlike apoplastic loading and unloading, it is harder to imagine how specificity is achieved and also how the concentration gradient during sucrose loading is overcome. Evidence in support of sugar transport being symplastic has been produced for some plants by the use of radio-labelled sugars (van Bel *et al.*, 1994). Radioactive sugars were traced through the loading translocation pathway and this transport was found not to be prevented by PCMBS, an inhibitor of the activity of sucrose transporters proteins. The use of cytoplasmic fluorescent dyes has been used to gather evidence of symplastic pathways functioning in roots (Lucas *et al.*, 1993). A 27kD GFP protein expressed under the control of the *AtSUC2* promoter has shown macromolecular trafficking from companion cells into the sieve elements, through the phloem, and into many sink tissues (Imlau *et al.*, 1999). Some plants that have intermediary cells show no requirement for an apoplastic pathway but, instead, provide phloem loading against a concentration gradient by means of chemical modification steps and the arrangement of different diameter plasmodesmata to prevent back-flow. The polymer-trapping model of Turgeon and Beebe, (1991) proposes that sucrose is translocated from the bundle sheath cell to the intermediary cell, where the sucrose is converted to raffinose by the addition of galactose. This serves to sustain the sucrose gradient in the direction of the sieve elements. Raffinose is unable to diffuse back into the bundle sheath cells due the diameter of the plasmodesmata between the two cells being selective. This, thereby, increases the concentration of sugars in the intermediary cells and sieve elements. In support of this theory, raffinose has been found to be present in the phloem sap of type I plants eg. *Cucurbita pepo, Cucumis melo, Coleus blumei* (Gamalei, 1996) and these plant types are also seen to have high plasmodesmatal frequencies (Turgeon and Gowan, 1990).

The maize mutant, *sed1*, is unable to translocate symplastically and, hence, has been used to study the role of the symplast in phloem loading of this species (Russin *et al.*, 1996). It was shown that these mutants, with their blocked plasmodesmata transport, have drastically impaired growth and development. Furthermore, large starch granules and excessive amounts of anthocyanins were

present in the leaves, so supporting the importance of symplastic pathways. These phenotypes are also present in mutant plants with attenuated StSUT1 expression and AtSUC2 insertional mutants (Riesmeier *et al.,* 1994; Gottwald, *et al.,* 2000).

1.3.2. Phloem unloading

The efficiency of phloem unloading depends largely on the sink strength of the corresponding tissue. The sink strength is determined by the activity of enzymes involved in sucrose catabolism and/or starch synthesis (Kühn *et al.*, 1999). Several reviews discuss the different models of phloem unloading (Patrick, 1997; Lalonde *et al.*, 1999). In general, unloading into sink tissues may occur either symplastically down a concentration gradient via plasmodemata, or apoplastically with sugar transporters responsible for uptake from the apoplast. Both pathways might operate in the same organism (Williams *et al.*, 2000a). As shown in figure 1.1, sucrose released into the apoplast at sink sites could be taken up as sucrose into the sink cells directly via sucrose transporters or, alternatively, it can be hydrolyzed to glucose and fructose by cell-wall invertases and imported via monosaccharide transporters.

Assuming that sucrose concentrations are lower in the sink tissues and enough plasmodesmata connections are available, it seems conceivable that exit from sieve elements could be entirely symplastic. One advantage of symplastic transport is the greater photoassmilate flux that may be in the range 194-700 $X10^{-8}$ mol m^{-2} cell wall interface s^{-1} as compared to that of apoplastic transport that is in the range 2.2-16 $X10^{-8}$ mol m^{-2} cell wall interface s^{-1} (Patrick and Offler, 1995). In developing seeds there are no plasmodesmata connections between storage tissues and sink tissues and, therefore, an apoplastic pathway must be present.

Sieve elements have been shown to leak sucrose and thus there is a need for the reloading of sugars along the length of the phloem. However, some sucrose must leave the phloem to satisfy the sink requirements of the stem tissues and, therefore, not all unloaded sucrose needs to be reloaded. To prevent this reloading, the sucrose may be broken down to monosaccharides providing a pool of energy available to the cell and regulated by the turnover of invertase enzymes. Compartmentalization of sugars into the vacuole of the cell decreases the osmotic pressure in the cytoplasm, increasing the sucrose concentration gradient and, consequently the import of sucrose into the cell. By contrast, starch is osmotically ineffective and so this polysaccharide form can be used to store sucrose produced throughout the day in order to supply the cell with energy during the night and also to increase the sucrose concentration gradient. *AtSUC2* is expressed throughout the phloem

of all organs and as well as its role in phloem loading (Gottwald *et al.,* 2000*)* it has been suggested that it may have a role in retrieval and in the unloading process, although this has yet to be confirmed (Truernit and Sauer 1995). Numerous other sugar transporters have been localised in sink tissues where they may have a role in the unloading processes (reviewed in Williams *et al.*, 2000a). In summary, the precise mechanism of phloem unloading varies between plant species and between different sink tissue, which have varying frequencies of plasmodesmatal connections and also different cellular composition at the site of unloading (Patrick, 1997).

1.4. Regulation of Sugar Transporters

Sugar transporters play a key role in source/sink interactions and thus it is likely that they are tightly controlled. Research is currently underway investigating the regulation of specific sugar transporter genes. There is evidence that regulation occurs at the stage of transcription, translation and also at post-translational stages (reviewed in Delrot *et al.*, 2000, Williams *et al.*, 2000a).

The expression of sugar transporter genes correlates to various stages of plant development. Both *StSUT1* (potato) and *AtSUC2* (*Arabidopsis)* show up-regulation during the sink to source transition of developing leaves (Riesmeier *et al.*, 1993; Truernit and Sauer, 1995). Whether the signal is that of increased sugar concentration upon initiation of photosynthesis or part of the changes in response to light it is not known. *AtSTP2* expression in the early stages of gametophyte expression suggest that it has a role in packing hexose sugars into maturing pollen. *AtSUC1,* found in the connective tissue of the anthers is thought to have role of the movement of sucrose in and out of the anthers to provide osmotic gradients for anther growth and dehiscence (Stadler *et al.*, 1999).

Reports have noted a number of other factors that affect the expression of sugar transporters. The putative tonoplast hexose transporter *AtERD6* has been shown to be up-regulated when exposed to either dehydration or cold treatment (Kiyosue *et al.*, 1998). Wounding and pathogen infection increases transcription of *AtSTP4, AtSTP3, AtSUC1*and *AtSUC3* (Truernit *et al.*, 1996; Fotopoulos *et al.*, 2003; Sakr *et al.,* 1997; Büttner *et al.,* 2001; Meyer *et al.,* 2004) presumably to fuel repair and defence responses to invading pathogens. Harms *et al.*, (1994) have reported induction of a sucrose transporter by auxin and cytokinin but not by sucrose. It could be that changes in the expression of sugar transporters by influences from the environment are signalled by plant hormones; these would initiate transcription factors and/or other signalling cascades which co-ordinate gene expression.

Light signals the diurnal rhythm to plants and can produce dramatic effects on the way plants grow (this is discussed in more detail in subsequent sections). Therefore it is reasonable to assume

that this brings with it changes in the allocation of sugars to fuel growth. As yet only relatively basic photobiology experiments have investigated the effect of light on expression of sugar transporters. Several studies have indicated that expression of sucrose transporters in the phloem is diurnally regulated at the mRNA level (Shakya and Sturm 1998, Kühn *et al.*, 1997; Aoki *et al.*, 1999). Highest expression levels were observed during the daylight hours in tomato (*LeSUT1*, Kühn *et al.*, 1997) and carrot (*DcSUT1*, Shakya and Sturm, 1998) and transcript levels were reduced during darkness hours. However diurnal regulation in Zea *mays* (ZmSUT1) in the leaf was slightly offset to that reported for tomato and carrot, with highest expression occurring during the dark hours; expression levels were positively correlated to soluble sugar levels and therefore these could be the regulatory factor (Aoki *et al.*, 1999).

In germinating embryos of rice, *OsSUT1* was expressed in the light (as early as 12h post germination) but not in the dark, in the absence of exogenously supplied sugars (Matsukura *et al.*, 2000). They suggested that this transporter might be regulated by the cellular sugar status. Hirose *et al.*, (1997) had previously shown that transcripts of *OsSUT1* were higher in the shoots of etiolated seedlings (11 days after sowing) than in the light-grown seedlings but it was not clear whether seedlings were supplied with a sugar source. Hirose *et al.*, (1997) also examined *OsSUT1* transcript levels in seedlings grown in the dark for 11 days and then subjected to illumination. Transcripts started to decrease between 12 and 24 h after illumination and at 72h they were very low. The results of these two studies appear contradictory and it is therefore not clear if light does have an effect on *OsSUT1* expression and whether this is due to a change in sugar status of the cells.

Evidence for sugar regulation of expression of sugar transporters is limited and does appear to be dependent on the particular sugar transporter under investigation (Williams *et al.*, 2000a, Atanassova *et al.*, 2003). This will be discussed in detail in chapter 4 but relevant to this is the topic of sugar sensing, which will be discussed briefly in the next section.

1.5. Sugar Sensing

The role of sugars as simple sources of chemical energy is rapidly being expanded to incorporate further roles in signalling (Sheen *et al.*, 1999), metabolic regulation (Roitsch, 1999; Pego *et al.*, 2000), cell cycle (Riou-Khamlichi *et al.*, 2000), light perception (Dijkwel *et al.*, 1997), germination (Pego, *et al.,* 1999) and even protecting cells when frozen (Uemura and Steponkus, 2003). In plants, sugars are synthesized endogenously and this process must be regulated with regard to the utilization of sugar in the plant. Therefore mechanisms must be in place to monitor sugar

levels and communicate this to the gene promoters of photosynthetic genes. Koch (1996) wrote a review compiling various reports of gene expression affected by carbohydrates in plants. The genes were categorized into those that were enhanced upon sugar depletion, which were called famine genes, and those that were enhanced upon sugar abundance and thus were named the feast genes. The feast genes were those involved in responses to abundant sugar levels and included those involved in, metabolism of sugars, storage of excess sugars, transport of sugars, plant growth and relative sink activity whereas the famine genes were concerned with responses to low sugar levels and included those involved in photosynthesis to produce more sugar, remobilisation of stored sugars, export of sugars to sink tissues and relative source activity.

Sugar sensing is a fast developing area of plant research and several studies have investigated this process. Sheen (1990) showed using greening maize protoplasts that either 300 mM sucrose or 300 mM glucose produced a 15-fold decrease in the promoter activity of pyruvate phosphodikinase, a key enzyme in the pathway of C4 photosynthesis. The sucrose repression of pyruvate phosphodikinase was shown to be concentration dependant, thus when the sucrose concentration was more akin with the physiological levels (30 mM) found in photosynthetically active cells, the repression was no longer significant in comparison to the negative control. Later Jang and Sheen (1994) showed that another gene involved in the regulation of photosynthesis, chlorophyll a/b binding protein (*CAB*), is even more sensitive but only to glucose and not sucrose. Transfected green and greening maize protoplasts incubated with as little as 1 mM glucose caused cab promoter activity to be decreased to ~33% that of the water control. Sucrose also showed a repression in the CAB promoter activity but the reduced effect suggested to the author that glucose was more likely to be the direct signal. These studies demonstrate that these sugars can influence the expression of the genes that produce proteins involved in sugar synthesis. More relevant to the work here involving the effect the sugar regulation of sucrose transporters is a study by Chiou and Bush (1998) that showed sucrose transporter activity to decrease to 35-50% in plasma membrane vesicles isolated from leaves which had been fed 100 mM sucrose (compared to water controls). Since a similar response was not observed by feeding glucose, their results imply a relationship between sucrose, transport and regulation with a possible role for sugar sensing. From studies on the regulation of the phloem sucrose transporter, Vaughn *et al.* (2002) postulated that a sucrose sensor in the phloem responds to increased sucrose in the phloem by down-regulating transcription of the sucrose transporter gene; due to the rapid transporter protein turnover and mRNA degradation, the phloem loading capacity would be reduced.

Investigations regarding the mechanism of sugar sensing have developed from three main approaches: Comparison to sugar signalling in yeast (Johnston, 1999) which is further developed than that in plants; forward genetics, investigating the role of specific genes of mutants that display either an insensitive or hyper-sensitive response to sugars; reverse genetics, studying the affect of knocking out orthologues in yeast glucose sensing and the use of sugar analogues that are non-metabolizable.

Yeast hexokinases have been show to possess a bifunctional role; the protein contains a catalytic domain for the phosphorylation of glucose and a seperate domain that produces the glucose signalling Entian and Fröhlich (1984). Jang and Sheen (1994) investigated the role of hexokinase in plants and found that neither addition of phosphate or ATP relieved the down-regulation of the *CAB* promoter suggesting that glucose repression was instigated prior to the full processing through glycolysis. Furthermore, since non-metabolizable sugars 6-deoxyglucose and 3-O-methyl-glucose are transported into the cell but do not repress *CAB* it would suggest that transport is either very specific in sensing sugars or an intra-cellular sensor monitors sugar levels. Narrowing down the point of glucose sensing further,it was shown that while glucose, the substrate for HXK, produces a decrease in *CAB* promoter activity when incubated with maize greening protoplasts, incubation with glucose-6-phosphate, the product of the reaction between glucose and HXK, did not. This implied that HXK was responsible for glucose sensing, a theory strengthened by another experiment by Jang and Sheen (1994) in which inhibition of HXK with mannoheptulose, a competitive inhibitor of HXK, resulted in the loss of glucose repression.

However HXK is not the only glucose sensing system to operate in yeast. Two genes from the yeast *Saccharomyces cerevisiae, RGT2* and *SNF3,* which both show a high degree of similarity to mammalian and yeast glucose transporters (Laing and Gaber, 1996), have also been shown to be responsible for altering the expression of hexose transporter genes in response to glucose levels, thus implicating them as glucose sensors (Özcan *et al.,* 1996). Further analysis using yeast complementation revealed that although they show a high degree of similarity to glucose transporters, neither restored glucose transport in yeast defunct in hexose transporter genes even when over-expressed, indicating that these glucose sensors do not transport glucose (Özcan *et al.*, 1998). Unlike yeast glucose transporters, RGT2p and SNF3p have extended cytoplasmic C-terminal extensions (Özcan *et al.*, 1996). The C-terminal extensions are dissimilar except for a stretch of 25 amino acids, 16 of which are identical among repeats (Özcan *et al.*, 1998). RGT2 has two of these sequences whereas SNF3 has only one (Özcan *et al.*, 1998). Deletion of the RGT2 C-termini tail prevents its

ability to sense high levels of glucose and induce HXT1 expression; similarly, deletions of the SNF3 terminal tail prevents its ability to sense low levels of glucose and induce HXT2 expression (Özcan *et al.*, 1998). The transfer of the SNF3 tail to HXT1 and HXT2 converts these glucose transporters into glucose sensors, suggesting that the requirements for the sensing function are present entirely in the C-termini (Özcan *et al.*, 1998). Interestingly, two *Arabidopsis* sugar transporters *F23E12.140.* (At4g35300, from here in named AtPHS1 ***A**rabidopsis* **t**haliana **p**utative **h**exose **s**ensor **1**) and *AtSUT2/AtSUC3* (A2g05860, *Arabidopsis* sucrose transporter/sensor) also have extended cytosolic extensions. However they are not at the C-terminal regions but between the putative transmembrane domains 6 and 7.

To identify genes that have roles in sugar signalling mutants that show hypersensitive or insensitive responses to sugars in mutant screen have been characterized. There now a range of mutants that have been isolated and analysed (Rolland *et al.,* 2000; table1.1) which collectively have identified genes from a broad range of functions that contribute to sugar signalling. Along with confirming the participation of genes such as *HXK1* these investigations have also introduced some unexpected interactions with hormones such as ABA, auxin, cytokinin, and ethylene and also salt resistance and low temperatures (Nemeth *et al.,* 1998; Zhou *et al.,* 1998; Rook and Bevan, 2003; Rolland *et al.,* 2002). Add to these factors to the interplay of sugar sensing with nitrogen sensing (Coruzzi and Zhou, 2001), oxygen levels (Koch *et al.,* 2000) and light (Smeeken, 2000) and it seems that such a complicated system will take some time to truly understand.

1.6. Light Perception and Regulation of Plant Growth and Development

As autotrophic, sedentary organisms, plants must monitor and respond to the prevailing light conditions by changing their patterns of growth and development (such responses are termed photomorphogenesis). The main objective of such plastic responses is to maximise photosynthetic capacity and hence photoassimilate yield. The end-point therefore is to produce more sugars in photoautotrophic tissues, which will then be destined for transport around the plant. Thus, light-signalling and photomorphogenesis are intimately and co-operatively involved with the processes of sugar transport. This is highlighted by experiments showing that redistribution of sugars accompanies light-dependent changes in growth habits (Kasperbauer and Kaul, 1986). Sugar is also known to affect the expression of genes encoding photosynthetic components (Smeekens, 1998). It is the interaction of light-signalling pathways with the sugar transport pathways that this project specifically

Table 1.1. Arabidopsis Sugar Signaling Mutants. Taken from Rolland *et al.,* (2002).

Name	*Phenotype*
Sugar insensitive	
cai (carbohydrate insensitive)	Growth on low nitrogen
gin (Glc insensitive)	Growth on 330 mM Glc
isi (impaired sugar induction)	Reduced ApL3::P450 expression
l*ba* (low-level -amylase)	Low -amylase activity on 175 mM Suc
mig (Man-insensitive germination)	Germination on 7.5 mM Man
ram (reduced -amylase)	Reduced β-amylase in pgm mutant
rsr (reduced sugar response)	Pat(B33)-GUS expression on 90 mM Suc
sis (sugar insensitive)	Growth on 300 mM Suc or Glc
sun (Suc uncoupled)	Expression of PC-LUC on 88 mM Suc
sig (Suc insensitive growth)	Growth on 350 mM Suc
Sugar hypersensitive	
core (conditional root expansion)	Short, expanded roots on 4.5% Suc
fus (fusca)	Growth arrest on 3% Suc
glo (Glc oversensitive)	Growth arrest on 4% Glc
gss (Glc supersensitive)	Growth arrest on 56 mM Glc
hba (high-level -amylase)	High-level -amylase on 175 mM Suc
prl1 (pleiotropic regulatory locus)	Growth arrest on 175 mM Suc
sss (Suc supersensitive)	Growth arrest on 350 mM Suc

ApL3, ADP-Glc pyrophosphorylase; GUS, -glucuronidase; Luc, luciferase; PC, plastocyanin; pgm, phosphoglucomutase.

aims to address by examining the role of sugar transporters in light regulation of seedling development in *Arabidopsis*.

Figure 1.2 shows *Arabidopsis* seedlings grown in the dark or under white light. The dark-grown or etiolated seedlings display an elongated hypocotyl with closed and folded cotyledons. This is a skotomorphogenic growth strategy. Once transferred to the light, seedlings de-etiolate and follow a photomorphogenic growth strategy, which results in cotyledon expansion and chloroplast development, inhibition of hypocotyls elongation and root growth (Osterlund *et al.*, 1999; Neff *et al.*, 2000; Sullivan and Deng, 2003). These changes are mediated by specific photoreceptor proteins. To date nine photoreceptors have been identified in *Arabidopsis*; five red/far-red light-absorbing phytochromes, two each of the blue-light absorbing cryptochromes and phototropins (Briggs and Olney, 2001; Sullivan and Deng, 2003). These photoreceptors influence overlapping physiological responses and together regulate all aspects of plant development.

The most specialised group are the phototropins that are primarily involved in phototropic responses, chloroplast movement and stomatal opening (Briggs and Christie, 2002). The other class of blue-light photoreceptors, the cryptochromes are involved in regulation of flowering time, circadian entrainment and de-etiolation responses where cryptochrome 1 is the primary cryptochrome involved (Lin, 2002). The most extensively studied photoreceptor family are the phytochromes that are involved at all stages of plant development and play key roles in two responses that are likely to involve regulation of sugar transporter activity; de-etiolation and the shade avoidance response (Whitelam *et al.*, 1998; Møller *et al.*, 2002). As mentioned above there are five distinct phytochrome species in *Arabidopsis* encoded by a family of five genes, namely *PHYA*, *B, C, D* and *E* (Sharrock and Quail, 1989; Clack *et al.*, 1994). Each subunit consists of an apoprotein (the polypeptide component) and a chromophore moiety that is the light absorbing pigment. The chromophore is a linear tetrapyrrole called phytochromobilin that is attached to the apoprotein via a thioether bond that is formed autocatalytically (i.e. no cofactors or energy are required) to give a holoprotein (Elich *et al.*, 1989). Each apoprotein has two domains: a 74 kDa N-terminal domain to which the chromophore is covalently linked and a 55 kDa C-terminal domain that contains the site for dimerization (Cherry *et al.*, 1993). Thus each apoprotein has a molecular mass of 129 kDa. Each phytochrome holoprotein may exist in either of two, photo-interconvertible forms. The proportion of total molecules in each form depends on the ratio of red (R; 650-680nm) to far–red (FR; 710-740nm) light wavelengths. Phytochrome in the Pr form absorbs R light and is transformed to the Pfr form whereas Pfr absorbs maximally at FR wavelengths and, so, is converted back to Pr (Vierstra and Quail, 1986; Fururya,

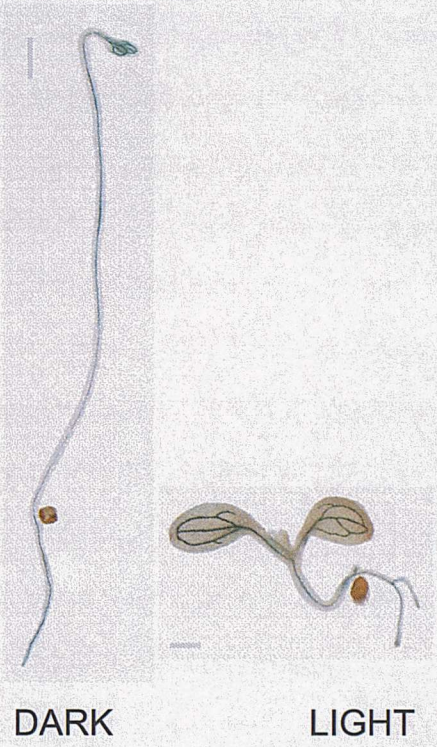


Figure 1.2. Seedling development in *Arabidopsis*. The dramatic phenotypic differences are a consequence of the light environment in which they are grown. Both seedlings were stratified for 48hrs then spread onto MS plates (1X Murashigue and Skoog media, 0.8% w/v bactoagar, 2% w/v sucrose) and placed under WL for 1 h to initiate germination. They were then placed in their respective light conditions and grown at 22 °C for 7 days. These seedlings showing GUS staining in the vascular tissue directed by the promoter of the sucrose symporter *AtSUC2*. Bar = 1 mm.

1993). This photoreversibility is the result of an isomerisation at the C15 bridge between rings C and D of the chromophore (Rüdiger *et al.*, 1983). The bond is in the *cis* formation in the Pr state, R light converts this to a *trans* bond in the Pfr state.

A number of distinct modes of phytochrome responses have been characterised. These are defined according to the qualitative and quantitative nature of the irradiance treatment to elicit the response and are termed the very low fluence response (VLFR), low fluence response (LFR) and high irradiance response (HIR) (Whitelam *et al.*, 1998). The classical form of phytochrome response is displayed in the LFR mode. In this mode, Pr/Pfr reversibility is demonstrated and conforms to the law of reciprocity which states that response magnitude is related to the total fluence ($\mu mol.m^{-2}$) and not the rate at which the fluence is delivered (Casal *et al.*, 1998). The VLFR mode is characterised by being saturated at Pfr/Pr ratios of <3% and hence a FR pulse alone produces a maximal response (Casal *et al.*, 1998). Consequently, R/FR reversibility is not displayed. The third response mode, the HIR, has been mostly studied under FR wavelengths. The FR-HIR is characterised by a requirement for continuous irradiation (or very frequent pulses), as opposed to being elicited by a single pulse (Casal *et al.*, 1998). Typically in the FR-HIR, the response amplitude is related to the fluence rate ($\mu mol.m^{-2}\ s^{-1}$) of delivery. The different response modes have been characterised to date with respect to the two dominant phytochromes, PHYA and PHYB. The distinctive photokinetic properties of these two phytochromes are summarized in table 1.2 (Casal, 1998).

Table 1.2. The Photo-kinetic Properties of phyA and phyB.

Phytochrome A	Phytochrome B
Most abundant in etiolated seedlings	Constitutively expressed
Labile as Pfr	Stable as Pfr
Mediates VLFR and FR-HIR	Mediates LFR
Response to cFR	Response to cR

Adapted from Casal, 1998.

1.7. Photomorphogenesis in mature plants and Implications for Altered Sugar Allocation by Sugar Transporters

The adaptation of plants to changing light conditions requires changes in morphology that must be accompanied by alterations in the allocation of resources such as sugars. One cause of reduced light availability is through competitive shading by other plants. The photoreceptors that are responsible for sensing shading are the phytochromes, which mediate a complex series of photomorphogenetic responses called the shade avoidance syndrome (SAS) (see table 1.2). In this

response, a plant's growth habit is altered in response to vegetative proximity so as to increase elongation growth and, thereby, place the photosynthetic organs (i.e. the leaves) in an advantageous position in the canopy. For reviews on shade avoidance see, Aphalo *et al.*, 1999; Ballare, 1999; Schmitt *et al.*, 1999; Morelli and Ruberti, 2002; Smith, 1995; Neff *et al.*, 2000). Leaves preferentially absorb R light and so transmitted light is characterised by having low R:FR ratio. Under these conditions there is less of the active Pfr form of phytochrome, which results in the reduced inhibition of stem elongation and the other responses characteristic of a shaded plant (table 1.3).

Table 1.3 The Shade Avoidance Syndrome

Physiological Process	Response to shade (i.e. reduced R:FR response)
Germination	**Retarded**
Extension Growth Internode extension Petiole extension Leaf extension	**Accelerated** Rapidly increased (lag. ~5 mins) Rapidly increased Increased in cereals
Leaf Development Leaf area growth Leaf thickness	**Retarded** Marginally reduced Reduced
Chloroplast Development Chlorophyll synthesis Chlorophyll *a:b* ratio	**Retarded** Reduced Balance changed
Apical dominance Leaf thickness Tillering (in cereals and grasses)	**Strengthened** Inhibited Inhibited
Flowering Rate of flowering Seed set Fruit development	**Accelerated** Markedly increased Severe reduction Truncated Truncated
Assimilate distribution Storage organ deposition	**Marked change** Severe reduction

Adapted from Smith, 1995

1.8. The Functions of Phytochromes as indicated by Mutant Studies

The individual physiological roles of each phytochrome species are becoming increasingly well characterised. One direct approach to investigating the biological function of a protein is to remove it from the system and observe the changes. To this end, mutants specifically deficient in one or myltiple phytochromes have been isolated. These specific mutants have lesions in the appropriate *PHY* apoprotein gene. Mutants that impair the tetrapyrrole pathway lead to chromophore deficiency and, hence, reduced total phytochrome activity. A number of mutants that fail to perceive light and grow like etiolated seedlings both in the light and dark have been isolated (Koornneef *et al.*, 1980). Koornneef *et al.* (1980) named these mutants *hy* mutants due to their phenotype of constitutive long hypocotyls.

In etiolated wild-type (WT) *Arabidopsis* seedlings, hypocotyl extension growth is dramatically inhibited under continuous irradiation by FR. Such responsiveness is typical of a HIR, mediated by phytochrome A (PHYA). The *hy8, fhy2* and *fre1* mutants deficient in PHYA (and now termed *phyA*) were selected by their failure to show this light response (Parks and Quail, 1993; Nagatani *et al.*, 1993; Whitelam *et al.*, 1993). This was pre-empted by experiments in which transgenic overexpression of a PHYA species was able to enhance the sensitivity of this response mode and also cause its persistence in the light-grown plant where, normally, responses mediated by more stable phytochrome pools become predominant (Whitelam *et al.*, 1992; Nagatani *et al.*, 1993). Under continuous FR light, PHYA is the only active phytochrome species and mediates partial cotyledon expansion, apical hook opening and changes in genes expression in addition to a strong inhibition of hypocotyl elongation mentioned above. However, under FR very little chlorophyll synthesis can take place and full de-etiolation cannot proceed. In the light, mature *phyA* mutants display few gross morphological differences from WT plants, but they have been shown to be deficient in detecting increases in photoperiod that initiate flowering (Johnson *et al.*, 1994). Thus although PHYA is light labile it still functions in light-grown plants. However, in WT plants the phytochrome-mediated photomorphogenic responses are predominantly a function of the light-stable phytochrome species and, in particular, PHYB (Smith, 1995).

Mutants deficient in PHYB (formally known as *hy3*) exposed to end-of-day pulses of FR (EOD-FR) fail to show elongation of hypocotyls, stems, petioles and root hairs in the light, have lower chlorophyll content, reduced number of rosette leaves and are earlier flowering than WT (Koornneef *et al.*, 1980; Goto *et al.*, 1991; Nagatani *et al.*, 1991; Reed *et al.*, 1993). The WT response to an EOD-FR treatment also includes changes in sugar allocation to stem tissues (Kasperbauer and Kaul, 1986; Kasperbauer *et al.*, 1970; Keiller and Smith, 1989; Casal *et al.*, 1995) and thus these effects may be under the direct control of PHYB. The LFR modes, including the SAS, are severely impaired in PHYB-deficient mutants and it is clear that, in light-grown plants, PHYB is the dominant photoreceptor (Viestra, 1993). However, removing PHYB does not prevent all of the shade avoidance characteristics, revealing that other phytochromes may also contribute.

The discovery of a naturally occurring mutation in the *PHYD* gene in the *Arabidopsis* ecotype Wassilewskija was utilized to study its function. When this *PHYD* mutant was introduced into the ecotype Landsberg *erecta* and grown under high R/FR light the plant showed increased hypocotyl extension, decreased cotyledon expansion and decreased anthocyanin levels compared to the WT (Aukerman *et al.*, 1997). Devlin *et al.* (1999) compared the difference between *PHYB* and *phyBphyD*

mutants and *phyAphyB* and *phyAphyBphyD* triple mutants. It was found that PHYD was directly involved in mediating flowering time, leaf area, R/FR responses, elongation of petioles and elongation of internodes, all characteristics of the shade avoidance response. However, mutants of *PHYD* only show a response when accompanied by a *PHYB* mutation, when PHYB is present it seems that the role of PHYD is redundant. Similar studies have demonstrated that PHYE also has an important role in shade avoidance (Devlin *et al.*, 1998). More recently a *PHYC* mutant has been isolated and this has revealed a role of PHYC as a modulator of other photoreceptors interacting with other phytochromes and the cryptochromes (Franklin *et al.*, 2003a). A summary of phytochrome-mediated photomorphogenetic responses is shown is table 1.4.

1.9. Plant Genomics

For biologists researching the molecular biology of plants the *Arabidopsis* genome represents an efficient genome to study (Wilson *et al.*, 1991). Compared to most plants *Arabidopsis* has an exceptionally small genome. While the haploid genome sizes of tobacco and pea are 1.6×10^9 base pairs (bp) and 4.5×10^9 bp respectively. the *Arabidopsis* haploid genome has a size of 7×10^7 bp. The reason for this smaller size is not due to a reduced number of genes, but due to a decrease in the proportion of non-coding DNA (Wilson *et al.*, 1991). Thus investigations should prove fruitful due to the higher content of coding DNA. It was this property that prompted scientists to use this plant genome as the first to be fully sequenced and which is now completed (The *Arabidopsis* Genome Initiative, 2000). This represents a milestone in plant biology as such information has enabled new areas of plant molecular biology and new approaches in the study of plant genes. One obstacle to overcome is the handling of this enormous amount of information and how we can best use this information for scientific discovery. Aided by the ability of the world-wide-web to share large amounts of information almost instantly across the globe, the area of bioinformatics has the task of maximising the potential of this "gold-mine" of information. John Ward has pioneered the discovery of novel families of membrane proteins from the *Arabidopsis* genome (Ward, 2001). An automated search was used to identify families of related putative membrane proteins from the 25,470 proteins predicted from sequencing. Of those predicted proteins, 4589 (18%) were found to have 2 or more putative trans-membrane domains. Sequencing initiatives for the organisms *D.melanogaster* and *S.cerevisiae* also found that 18% of the predicted proteins contain 2 or more putative trans-membrane domains. However, this statistic for *C.elegans* was 27%, quite an increase compared to the others. In all species a high number of 12 trans-membrane domains proteins were identified that correspond to

Table 1.4. A summary of phytochrome-mediated photomorphogenesis responses

Adapted from Smith, 1995.

PROCESS	PERCEPTION	RESPONSE	FUNCTION
Germination	PHYA	VLFR	Promotes Germination
	PHYA/PHYB	FR-HIR	Antagonistic promotion/ inhibition
	PHYB	LFR	Graded Response
Etiolation/ De-etiolation	PHYA	VLFR	Inhibits Extension Growth
		FR-HIR	Inhibits Extension Growth
	PHYB	LFR	PfrB Inhibits Extension growth of Etiolated Seedling.
Vegetative Development	PHYB	LFR	Low PrB/PfrB Promotes: Hypocotyl Extension; Stem Extension; Petiole Extension Flowering; Apical Dominance
	PHYC-E?	LFR	Radial Expansion Leaf Area Growth Flowering
Photo-periodism	PHYA	FR-HIR	Day-Extension in LDP
	PHYB	LFR	SD Perception

members of the Major Facilitator Superfamily (Saier *et al.*, 1999), which includes various transporters. A comprehensive website is now active featuring the sequences, hydropathy plots and MIPS codes (Munich Information Centre for Protein Sequences, http://mips.gsf.de/proj/thal/) of the gene families devised by Ward (http://www.cbs.umn.edu/*Arabidopsis*/). An invaluable part of the MIPS site is the MATDB (Munich *Arabidopsis thaliana* Database, Schoof, *et al.*, 2002), which contains all sequences derived from the *Arabidopsis* Genome Initiative sequencing project. Analysis of these families of sugar transporters is featured in chapter 5 including a phylogentic tree of all the sugar transporters both putative and characterized plus microarray analysis of their expression during FR de-etiolation.

Another reason why plant biologists have chosen this weed to be the model plant is its physiology and development. It is a relatively small plant at maturity and the dimensions of a healthy plant range up to 15 cm wide and 40 cm high. The lifespan from seed to senescence is only approximately 8-12 weeks for a WT plant depending on growth conditions (Wilson *et al.,* 1991; Boyes *et al.,* 2001). In addition, each plant can produce several thousand of seeds, which can be germinated on a wide range of media in petri dishes. Furthermore *Arabidopsis* can be transformed with *Agrobacterium*, which enables foreign genes to be inserted into the genome. Thus the study of transgenic plants is a possibility, which allows more techniques to be used to study its genetics and specific genes.

1.10. Aims

The sequencing of the *Arabidopsis* genome has revealed that there are large, multi-gene families of sugar transporters (The *Arabidopsis* Genome Initiative, 2000). The next challenge is to determine their individual physiological function. Sugar transporters are essential for assimilate partitioning and cellular nutrition, and their activities must be carefully controlled to meet the demands of plant growth and development. The aim of this study is to use a number of different approaches to understand the role of sugar transporters in *Arabidopsis thaliana*. Firstly, a reverse genetics approach will be used to investigate the function of the sucrose transporters *AtSUC1* and *AtSUC2*. The second approach to understanding the function of sugar transporters is to investigate their regulation using reporter gene analysis and microarray analysis. This thesis will concentrate on two potential regulators of sugar transporter gene expression, sugar availability and light. Both activate signalling pathways in plants and light, in particular, has a dramatic effect on plant architecture. Therefore, it is hypothesized that sugar transporters will be targets for regulation by these stimuli. This will be tested

by determining their effects on sugar transporter expression levels and tissue specificity. The regulation of a disaccharide transporter, *AtSUC2,* and a monosaccharide transporter, *AtSTP4,* will be investigated using transgenic *Arabidopsis* plants expressing promoter:*GUS* reporter fusions and RT-PCR. Studies investigating the role of two putative sugar sensors will be undertaken which may reveal novel mechanisms of sugar sensing in plants. Finally a more global approach will be taken to examine the regulation of all sugar transporter genes using transcriptomics. In this case, the experiments will focus on the role of sugar transporters in response to de-etiolation by far-red light, which specifically activates phytochrome A responses.

Chapter 2.

Materials and Methods

2.1 Plant Material

2.1.1 Growth of *Arabidopsis thaliana* on media plates

Arabidopsis thaliana L. cv. Columbia seeds were surface sterilised in 10% (v/v) Domestos™ bleach for 10 min and then washed in two volumes of distilled water (dH_2O) four times. Under sterile conditions, seeds were spread onto Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) (44.3 g/10 L, Sigma recommendation), containing 1% (w/v) agarose. In some cases media was supplemented with 2% (w/v) sucrose. Kanamycin (50 μg/ml) was added when growing GUS-transformed plants, and phosphinothricin (PPT, 10 μg/ml) for plants with transposon insertions. After the water had evaporated, the plates were covered, sealed, and then seeds were stratified at 4°C for 24 hr. For plants that were to be grown to maturity on soil following selection, the plates were first transferred to a controlled environment growth room (180 μmol m^{-2} s^{-1}, 22°C) for 2 weeks and then transferred to soil (see components in next section) in individual pots; plastic sleeves were placed around the pots to guide growth, avoid cross pollination and reduce seed loss/dispersal.

For experiments involving the growth of seedlings transformed with GUS constructs under different light treatments, the three different light conditions were as follows: continuous white light (180 μmol m^{-2} s^{-1}) for 7 days (L), dark for 7 days (D) and finally dark for 6 days followed by light (fluence) for 1 day (D-L). In all cases the temperature was constant at 22°C.

2.1.2. Growth of *Arabidopsis thaliana* on soil.

Seeds of *Arabidopsis thaliana* L. cv. Columbia mixed with sand were sown in trays with soil composed of 1pt. Minster John Innes No. 2, 1pt. Shamrock™ Irish moss peat and 1pt. Vermiperl® graded horticultural vermiculite (medium grade). The soil mixture was sterilized by autoclaving at 120°C for 15 min at 1 bar pressure. The trays were covered with Clingfilm™ (Netto, in-house stores) and seeds were stratified at 4°C for 48 hr. The trays were than transferred to a controlled-environment growth room 16 hr light / 8 hr dark cycle (22°C in light and 20°C dark). Relative humidity was kept constant at 75% while irradiance was set at 120 μmol m^{-2} s^{-1}. Upon germination, the Clingfilm™ was removed and the trays were watered daily with tap water.

2.1.3. Phenotypic analysis of mutants

The mutants obtained from the SLAT (Sainsbury Laboratory *Arabidopsis* Transposant) collection of insertional mutants were isolated previously in the lab (Marvier, Pittman and Williams, unpublished) and provided for this study. For phenotypic analysis of soil-grown plants, these were grown as described above under glasshouse conditions or in a controlled environment growth room. Various parameters (e.g. plant height, number of leaves, number of siliques etc) were recorded throughout the growth period). To determine seed viability, seeds were plated as above on 1X MS plates plus or minus 2% (w/v) sucrose, stratified for 3 days at 4°C, then grown at 23°C in a 16 hr light/8h dark cycle.

To monitor seedling growth under different light treatments, seeds were plated as above without sucrose, stratified as above and then transferred to 23°C. All seeds were given a 1 hr white light treatment to initiate germination and then grown in the dark for 48 hr. Seedlings were then exposed to various light conditions (white light, dark, far red or red) for 48 hr. The light sources for the supply of white light, red, and far-red wavebands were the same as described by McCormac *et al.*, (2001). White light was provided at a fluence rate of 100 mmol m^{-2} s^{-1} by 70 W type 84 fluorescent tubes (Philips, Eindhoven, Netherlands) with a 16 hr photoperiod. Red (R), far-red (FR) and blue (B) light was provided with a 16 hr photoperiod in temperature-controlled LED growth chambers (Percival Scientific Inc., Boone, IA, USA). Red light (620-700 nm, peak 666 nm) was provided at a fluence rate of 70 mmol m^{-2} s^{-1}, far-red light (700-780 nm, peak 740 nm) at a fluence rate of 10 mmol m^{-2} s^{-1}. Irradiance was recorded using a LI-COR LI-250 meter (LI-COR Inc., Lincoln, Nebraska, USA). Two additional neutral density filters (#116 and #172; Lee filters, Andover, UK) were used in the far-red light cabinet to attenuate wavelengths > 700 nm.

2.2. Isolation of nucleic acids from *Arabidopsis thaliana* tissues.

2.2.1. Isolation of total RNA

The preparation of pure, intact RNA is complicated by the presence of contaminating RNA-degrading enzymes (RNases) that are present not only within the cells from which the RNA is being extracted but also possibly present on all equipment that comes into contact with the RNA preparation. It is important therefore that precautions are taken to prevent the likelihood of contamination by RNases. Therefore, all glassware, eppendorfs and tips used in the isolation and handling of RNA were baked at 140°C for at least 8 hr, and sterile disposable centrifuge tubes were

used. Spatulas and magnetic stirrers were also baked at 140°C for at least 8 hr. Additional precautions included the autoclaving of all buffers and solutions (except those containing Tris or SDS), which inhibits several types of RNases. Tris-containing buffers and SDS solutions were prepared using autoclaved DEPC-treated water. Plastic-ware was soaked in 0.1 M sodium hydroxide, again to prevent RNase contamination. Finally gloves were worn at all times and changed at regular intervals.

Total RNA was isolated using a method based on Verwoerd *et al.*, (1989). Plant material was harvested into liquid nitrogen and ground in a pestle and mortar with liquid nitrogen to a light green powder. This was transferred to a 50 ml Falcon tube and 8 ml LiCl extraction buffer: phenol (1:1) was added The LiCl extraction buffer contained 0.1 M LiCl, 0.1 M Tris/HCl (pH 8.0), 10 mM EDTA and 1% SDS (w/v). The material was mixed by vigorous inversion for about 1 min and 1 vol chloroform: IAA (24:1) was added and mixed as before. After standing for 15-min on ice, the solution was centrifuged at 5,000 *g* for 30 min at 4°C. The aqueous phase was transferred to a fresh Falcon tube and an equal volume of 4 M LiCl was added and the solution was stored at 4°C overnight. The solution was centrifuged 12,000g for 20 minutes. The pellet was washed in 70% ethanol and resuspended in DEPC-treated water, typically 100-500 µl depending on size of pellet. The suspended pellet was then transferred to a sterile eppendorf for ethanol precipitation. For this 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) were added. This was mixed thoroughly by repeated inversion and stored at -20°C overnight. To collect the nucleic acid, the solution was centrifuged at 13,000X *g* for 20 min at ~4°C in a Sorvall RMC 14 centrifuge (Sorvall, Stevenage, UK). The pellet was rinsed in 70% ethanol prior to suspension in ~30 µl DEPC-treated H_2O. Further cleaning was carried by the phenol:chloroform:IAA method. One volume of phenol:chlorofom:IAA (25:24:1) was added, mixed by inversion and centrifuged at 13,000X *g* for 10 min at ~4°C. The top phase was transferred to a microfuge tube and ethanol precipitation was repeated as before. The RNA was stored at -70°C, to be thawed on ice when required.

2.2.2. Isolation of genomic DNA

Genomic DNA was extracted from tissue frozen in liquid N_2 and ground to a powder as described by Carroll *et al.* (1995) in 200 mM Tris (pH 7.5), 50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 0.38% (w/v) sodium bisulphite, 15 µg RNase A and 1% (w/v) lauroylsarcosine at 65°C. DNA was further purified by extraction in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in an

equal volume of isopropanol at room temperature. DNA pellets were resuspended using 50 µl pre-warmed (50°C) dH_2O. All centrifugation steps were at 13000 x *g* in a Sorvall MC12V microfuge (Sorvall, Stevenage, UK).

2.2.3. DNA purification and precipitation

Certain experiments required DNA to be further purified and/or precipitated to increase its concentration. For small-scale preparations (1-5 samples), the QIAquick PCR Purification Kit (QIAgen, UK) was used following the manufacturers instructions. For larger scale preparations (>6 samples), DNA was cleaned and concentrated following a slightly modified version of the method described by Sambrook *et al.* (1989). One volume of phenol:chloroform:IAA (25:24:1) was added to the sample, and the solution was centrifuged at 13000 x *g* in a Sorvall MC12V microfuge (Sorvall, Stevenage, UK) for 5 min. The supernatant was transferred in a new tube, and its volume was made up to 200µl with sterile dH_2O. Two volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) were added and the solution was precipitated in –20°C for 30 min. This was centrifuged at 13,000 *x g* for 20 min to pellet the precipitate. The supernatant was discarded and the pellet washed with 70% ethanol, before resuspending in a small volume (10-20 µl) of dH_2O.

2.2.4. Determining the concentration and purity of nucleic acid samples by spectrophotemetry

The purity and concentration of DNA or RNA in a given sample was determined by spectrophotometry. Optical density (OD) readings were taken at 260 nm and 280 nm. A sample of DNA or RNA was diluted 1:250 in either TE (DNA samples) or DEPC-treated water (RNA samples) in a quartz cuvette and measured in a UV absorbance spectrophotometer. Since the optical density at 260 nm of a 1 mg/ml solution of double stranded DNA is 20, the concentration of a diluted aliquot (µg/µl) = $^1/_{20}$ x OD_{260nm} x dilution factor x 2. The optical density at 260 nm of a 1 mg/ml solution of single stranded RNA is 25, therefore the concentration of a diluted aliquot of RNA (in µg/µl) = $^1/_{25}$ x OD_{260nm} x dilution factor x 2. The measurement at OD_{280nm} is used to determine the level of contaminating protein. The ratio of OD_{260nm}/OD_{280nm} can be used to give an indication of the sample purity, an ideal ratio is between 1.8 - 2.0. A higher ratio for RNA can indicate considerable degradation of the sample and/or polysaccharide contamination, while a low ratio for DNA may indicate protein or phenol contamination. All nucleic acid samples used were within the 1.8 - 2.0 ratio.

2.3. Polymerase Chain Reaction (RT-PCR) amplification of cDNA

PCR was used to amplify various cDNAs and gDNA from *Arabidopsis thaliana.* Thermocyclers used were either Biometra Uno II (Thistle Scientific Ltd, Glasgow, UK) or Perkin Elmer 9700 (Perkin Elmer, Buckinghamshire, UK).

2.3.1. Reverse Transcriptase-(RT) reaction

First-strand complementary DNA (cDNA) was prepared from total RNA isolated from different tissues of *Arabidopsis thaliana.* 5 μg of RNA was added to 10 μl of DEPC-treated dH_2O and denatured at 70°C for 20 min 4 μl of 5 x Superscript buffer (Gibco BRL, UK), 2 μl of 10 mM DTT (1.5 mM final concentration), 100 μM of each dNTP, 1 μl (10 ng) of oligo- dT primer and 1 μl (30 units) of RNase inhibitor ('RNAGuard'; Gibco BRL, UK) were added before heating at 37°C for 2 min. 2 units of 'Superscript' reverse transcriptase (Gibco BRL, UK) were added and the reaction was incubated at 37°C for 1 hr. The reaction was stopped by heating to 94°C for 10 min before use.

2.3.2. Oligonucleotide Primers

Specific primers were designed based on the sequences released in various databases (EMBL at EBI, http://srs.ebi.ac.uk; MATDB at MIPS, http://mips.gsf.de/proj/thal/db/index.html, Schoof, *et al.*, 2002) (table 2.1). Most primers were designed to anneal to regions just outside the coding region, and many were designed to contain restriction sites to facilitate cloning. To check for any chances of binding to other sequences, the primers were analysed using the TAIR BLAST search facility (http://www.*Arabidopsis*.org/Blast, Altschul *et al.*, 1990).

2.3.3. PCR Reactions

PCR reactions were routinely performed using the TAKARA Ex Taq system (Takara, Japan, distributed in the UK by Biowhittaker) as per manufacturers' instructions in either 10 or 20 μl reactions volumes. The thermocycle program was as follows.

1. an initial 10°C for 10 min while the samples were being transferred from ice to the PCR block
2. an initial melt comprising 95°C for 1 min
3. another melt as in step 2
4. annealing temperature see table 2.1

Table 2.1. List of Oligonucleotide Primers. Table includes recommended conditions such as annealing temperature (T^A), elongation time (Et) and expected product size (EPS) for cDNA and gDNA (given in italics).

Primer *Eco*R1 sites	T^A(°C)	Et (min)	EPS (Kbp)
Actin F 5' GGT AAC ATT GTG CTC AGT GGT GG 3' R 5' CTC GGC CTT GGA GAT CCA CAT C 3'	60	1	0.33 *0.36*
AtPHS1 (F23E12.140) F 5' CTC CCC ACA TGA AGA AGA CGC TTA AGG A 3' R 5' AGG AAT TCG GTC ATG CTT TTT GGT GTT ATC TC 3'	60	3	2.2 *2.4*
AtSUC1 F 5' CGG GAA TTC TTC CTC TAA AGT TTC TAT TTT G 3' R 5' CGG GAA TTC AGA CGA CCC AAG GTT TAT TAG ATT T 3'	58	2	1.8 *1.9*
AtSUC2 F 5' CGG GAA TTC CAA CCA CCA AAA ACC CTC TCA AA 3' R 3' CGG GAA TTC GGT AAA ATA CAA ACC AAC CCA ATG AG 3'	60	2	1.7 *2.2*
AtSUC3/SUT2 F 5' CGG AAT TCC TAG ATC GAC GAG ATC AAA A 3' R 5' AAG AAT TCA TAT ACG CCG AGA GGA GAA C 3'	50	2	1.9 *3.8*
AtSUT4 F 5' GAC TCC GCG GCG AGA AAT GGC TAC TTC CG 3' R 5' TAA CCC GCG GGA GAA TCT CAT GGG AGA GG 3'	62	2	1.6 *1.8*
AtSUC5 F 5' CGG GAA TTC CTT TTC ATA ATG GGA GCC TTG G 3' R 5' CGG GAA TTC AAC TAA TGG CCT CCC ATA GCC C 3' (Taken from Juergensen *et al.*, 2003)	60	2	1.5 *2.0*
AtSTP4 F 5' TGC TAT GTT TGT CTG AAA CAG ACA G 3' R 5' ATG AAA GCT AAG TCT CTG AAG C 3'	53	2	1.7 *1.9*
AtSTP35 F 5' ATT GGG ATA ATG CTG GCG TA 3' R 5' TGA GTA GAA GCC GAC GAC CT 3'	58	1	0.5 *0.8*

5 elongation consisting of 72 °C, 1 min per 1 KBP, then back to step 3 ~35 times

6 a final elongation period of 72 °C for 10 min

7 a hold temperature of 10°C.

When maximum fidelity was required, proofreading polymerases systems were used. These are composed mainly of Taq. but also include a proof-reading polymerase. KlenTaq LA (Sigma-Aldrich Ltd, Gillingham, UK) is an enzyme mix containing KlenTaq-1 DNA polymerase (a 5'-exo-minus, N-terminal deletion of Taq DNA polymerase) and a small amount of a proofreading polymerase (not stated by manufacturer). Each system had its own composition of reaction mix and was used in conjunction with the manufacture's recommendations. The only adjustment when using this system is a decrease in the elongation temperature to 68 °C as suggested by the manufacturer.

2.4. DNA gel electrophoresis

Gel electrophoresis of DNA was performed as described by Sambrook *et al.* (1989). DNA was separated on a 1.2% (w/v) agarose gel containing 1X TAE (40 mM Tris/acetate (pH 8.0), 1 mM EDTA). The running buffer also consisted of 1 x TAE (40mM Tris/acetate (pH 8.0), 1mM EDTA). The gel and running buffer contained 1mM ethidium bromide. The DNA was loaded in buffer containing 0.25% (w/v) bromophenol blue and 15% (w/v) Ficoll 400 in distilled water (1 µl loading buffer per 5 µl DNA sample). Electrophoresis was carried out at 80 V for 1-2 hr depending on the size of the gel. DNA was visualised under UV light. Specific products were identified by comparison to a 1 kbp Ladder (Gibco BRL, UK). Quantification of products could be estimated by comparison to the 1.6 kbp band of the 1 kbp Ladder that was known to be 50 mg of DNA.

2.4.1 Purification of DNA from agarose gels

After separation on an agarose gels, DNA fragments were physically cut from the gel using a sterile scalpel blade. The DNA was purified using the Qiagen PCR Purification Kit, following the manufacturer's instructions (Qiagen, UK). The pellet was resuspended in an appropriate volume of dH_2O depending on the size of the pellet.

2.5 RNA gel electrophoresis

Gel electrophoresis of RNA was carried out as described by Sambrook *et al.* (1989). RNA was separated on a 1.2% (w/v) agarose gel containing 0.9 M formaldehyde and 20 mM MOPS buffer (containing 200mM MOPS, 50mM sodium acetate and 1mM EDTA (pH 8.0)). Immediately before

loading, the RNA was denatured at 65°C for 15-20 min in 0.8 vol formaldehyde, 2 vol formamide, and 0.4 vol 10X MOPS buffer, and snap cooled on ice. RNA was then loaded in sample buffer containing 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue, and 2μg of ethidium bromide per 15 μl of sample. Electrophoresis was carried out at 80 V for 1-2 hr, depending on the size of the gel. The RNA was then visualised under UV light.

2.6. Cloning of PCR-amplified products

2.6.1. Ligation of DNA into plasmid vector

DNA fragments amplified by PCR from cDNA templates were cloned into plasmid vectors to allow sequencing, labelling, and to provide a means to amplify the DNA fragment when necessary. The pGEM®-T Easy Vector System (Promega, UK) was routinely used as it facilitates cloning of DNA products, since it eliminates the need to digest, dephosphorylate and purify the vector DNA, as well as the need to digest the insert DNA. It also allows blue/white colony screening, and the release of the insert by a choice of three different single-enzyme digestions. The standard reaction as suggested in the Technical Manual (Promega, UK) was used for ligation. 5 μl of 2X Rapid Ligation Buffer, 1 μl pGEM®-T Easy Vector (50 ng), 1 μl of T4 DNA Ligase (3 Weiss units/μl) and also the appropriate volume of PCR product were incubated overnight at 4°C.

2.6.2. Preparation of competent *Escherichia coli* cells

Cultures of *E. coli* DH5α bacteria cells were grown overnight at 37°C in Luria-Bertani (LB) broth. 100 ml of LB was inoculated with 1 ml of overnight culture containing *E.coli* DH-5α. This was maintained on a shaker at 37°C until the $O.D_{550}$ was between 0.4 and 0.5 (approximately 2.5 to 3 hr). The cells were placed on ice for 5 minutes and then centrifuged at 3000 X g for 5 minutes. The supernatant was discarded and the cells resuspended in 20 ml of ice-cold 100 mM $CaCl_2$. Cells could be stored at –70°C in 15% (v/v) glycerol (final concentration) for later use but as far as possible fresh competent cells were made up for each transformation. An LB agar plate was also inoculated for more immediate use.

2.6.3. Transformation of *E.coli* with plasmid DNA (containing ampicillin resistance gene)

10 ng of recovered plasmid were added to 200 µl of competent cells (as described by Promega, JM109, as part of pGEM®-T Easy Vector System II Kit) and left on ice for 10 min. The mixture was heat shocked at 42°C for 90 seconds then left on ice for another 5 min. 200 µL of LB at 42°C was added and samples of 50 µl, 100 µl and 200 µl were plated on LB agar media containing ampicillin (40 mg/ml). The plates were incubated at 37°C overnight. Any colonies growing on the plate could represent transformants and, routinely, between 4 and 6 were picked off and grown up overnight in liquid LB containing ampicillin (40 mg/ml).

If the plasmid contained the *LacZ* gene, X-Gal (40 ng/ml should this be 40 µg) and 500 µM IPTG were included in the plate media. Recombinant colonies could be selected by the blue/white colour screening method. Recombinant white colonies were removed and grown overnight at 37°C in 1 ml of LB. Plasmid DNA was isolated from each set of colonies to check that they contained the vector and insert DNA.

2.6.4. Isolation of Plasmid DNA from *E. coli*

Clear white colonies were picked from the plate and placed into 6 ml of LB media containing ampicillin (40 mg/ml) and incubated at 37°C overnight. The cells were centrifuged at 13,000 *g* for 10 min. The pellets were suspended in 100 µl glucose/ EDTA /Tris medium (1% (w/v) glucose, 50 mM EDTA, 25 mM Tris). To lyse the bacterial cell walls, 20 µl 10% (w/v) SDS and 4 µl 10M NaOH was added, mixed by inversion and left to stand for 10 minutes. Plasmid DNA was separated from genomic DNA, protein and other nucleic acids by precipitation with the addition of 150 µl 3 M potassium acetate; this was gently mixed by inversion for 10 min and centrifuged at 13,000 *g* for 20 min. The supernatant, which contained the plasmid, was transferred to a separate microcentrifuge tube and concentrated to 20 µl in TE buffer by ethanol precipitation as described previously. This could be further purified using the Qiagen PCR Purification Kit (Qiagen, UK).

2.6.5. Restriction digestion of plasmid clones

For confirmation that an insert was present in the plasmid, restriction digests with the appropriate restriction enzymes were used followed by gel electrophoresis to determine the size. The restriction enzyme, *Eco*R1, was routinely used to isolate the insert from the plasmid. For a 10 µl digest, 0.5 ng of plasmid DNA, 1 µl of 10X *Eco*RI restriction buffer, 1 µl BSA and 1 µl (2-10 units)

*Eco*R1 enzyme were added and made up to 10 μl with DEPC-treated dH_2O. The reaction was mixed and incubated at 37°C for 4 hrs. To isolate the insert, the digestion was run on a gel and then purified using the Qiagen PCR Purification Kit (Qiagen, UK).

2.7. Long-term storage of the transformed bacteria

For long-term storage of the transformed bacteria, glycerol stocks were prepared. To 500 μl of culture (transformed bacteria in LB media grown overnight), 160 μl of 60% (v/v) glycerol was added, giving a final glycerol content of 15% (v/v). For shorter-term storage, colonies were grown on LB plates containing ampicillin (50 μg/ml), overnight at 37°C; these were stored in a cold room at 5°C. Colonies from these plates were used to inoculate LB media containing ampicillin to grow fresh stocks for isolation of the plasmid or insert.

2.8. DNA Sequencing

2.8.1. Big Dye Sequencing

The DNA sequence of the PCR amplified products was deduced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, UK processed on the ABI PRISM® 377 DNA sequencer (PE Applied Biosystems, UK). This automated DNA sequencing method is based on a combination of the cycle sequencing method (Murray, 1989) and the dideoxy sequencing method (Sanger *et al.*, 1977). Initially, PCR was performed that incorporates a dideoxynucleotide base with a frequency-specific fluorescent dye attached. The dideoxy structure of the nucleotide terminate elongation and the specific dye enables identification with the sequencer. For a PCR product of 1-2 kbp, 10–100 ng of DNA was required. Alternatively, the plasmid of the cloned product could be sequenced and in this case 200-500 ng of DNA was required. To the template, 4 μl of the Terminator Ready Reaction Mix and 1.6 pmol of one of the primers were added. This was made up to 10 μl with dH_2O and then run on the BigDye Thermocycle program. Reactions were cycled 30 times at 96°C for 30 sec, 50°C for 5 sec, and 60°C for 4 min. The PCR products were precipitated to remove the unincorporated dyes. 10 μl of dH_2O, 50 μi 95% ethanol and 2 μl 3 M sodium acetate was added and mixed. The mixtures were centrifuged at 13,000 g for 20 min and the supernatant was discarded. The pellet was washed three times with 70% ethanol (v/v) and left to air dry. The samples were processed and run on the sequencing gel in the ABI377 Prism Sequencer by

the School Sequencing Facility. The sequences obtained were visualized graphically using the Chromas Version 1.45 computer program.

2.9. DNA sequence analysis

Sequence analysis was performed using software from the GCG package (part of the Wisconsin Package, Version 10 (1999), Genetics Computer Group, Madison, Wisconsin, USA) on the Human Genome Mapping Project facility (telnet address: hgmp.mrc.ac.uk), MRC Laboratory, Cambridge, UK. EMBL/GenBank and EST sequence databases were searched using the TAIR BLAST program (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al.*, 1990). *Arabidopsis*-specific sequence databases were searched using the BLAST program via the *Arabidopsis thaliana* Database (AtDB) at http://www.*Arabidopsis*.org/blast/index.html (Altschul *et al.*, 1997). Identities between sequences were found using the GAP and/or BESTFIT program (GCG package), while sequence alignments were carried out using the PILEUP program from the same package, or CLUSTALW v1.8.2 (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the DRAWTREE and FITCH programs of the PHYLIP package 3.6a3 (http://evolution.genetics.washington.edu/phylip.html; Felsenstein, 1989). Bootstrap analyses were conducted with the SEQBOOT program from the same package.

2.9.1 Prediction of transmembrane regions

Prediction of transmembrane regions and orientation was performed using the TMpred computer program (www.ch.embnet.org/software/TMPRED_form.html; Hofmann and Stoffel, 1993). TMpred makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring.

2.10. β-glucoronidase (GUS) reporter gene Analysis

2.10.1. Histochemical assay:

This method was derived from technical advice by Jefferson (1987, 1989). Seedlings were placed in the staining solution, X-Gluc (1 mM X-Gluc (Melford Laboratories, UK), 1 mM Na-EDTA, 0.5% (v/v) Triton-X100, 0.5% (v/v) Dimethylformamide, 50 mM $NaHPO_4$) and incubated overnight at 37°C. The X-Gluc was replaced by the decolouration/fixation solution (D/F) (50% (v/v) ethanol, 10%

(v/v) formaldehyde, 5% (v/v) glacial acetic acid) and was further incubated at 37°C for 2 hr to fix the stain and remove the green pigment, chlorophyll, to increase staining contrast. The D/F was then discarded and the seedlings were stored in 70% ethanol (v/v) for >4 days to further clear the seedlings of chlorophyll. Seedlings were observed in 70% (v/v) ethanol within a small petri dish.

2.10.2. *Fluorometric quantification assay*

β -Glucuronidase activity was determined using 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate based on the method by Jefferson (1987, 1989).

Ten whole seedlings, pairs of cotyledons, hypocotyls or roots were placed in 100 µl GUS Extraction Buffer (GUS-EB: 50 mM $NaHPO$ (pH 7.0)$_4$, 10 mM EDTA, 0.1% (v/v) Triton-X100, 0.15% (v/v) β -mercaptoethanol, 0.1% (w/v) N-lauryl sarkosyl) and stored at -70°C. GUS has been shown to be most active in the presence of thiol reducing agents such as β -mercaptoethanol or DTT, but can be inhibited by some divalent heavy metal ions, hence the inclusion of EDTA in the GUS-EB. Tissue samples were thawed, and then ground thoroughly to release the GUS enzyme.

The assay was started with the addition of 200 µl substrate buffer (SB:4-MUG) (Melford Laboratories, UK), 1 mM final concentration to the crude extract. For assays with a number of time points, the reaction volume was increased to allow for the increased number of aliquots required. The reaction was stopped at appropriate time points by allocating samples into 0.2 M Na_2CO_3. Routinely 90 µl of the reaction was added to 1410 µl of the stop buffer, but this was adjusted for some experiments. The final volume was always 1500 µl. The alkaline pH of the stop buffer also serves to develop the fluorescence of the 4-methyl umbelliferyl (4-MU) product (Jefferson, 1987). The samples were assayed with a spectrofluorometer using an excitation wavelength of 365 nm and emission wavelength of 455 nm with an appropriate blank (100 µl GUS EB and 200 µl GUS). As standards, known concentrations of 4-MU were prepared and the fluorescence measured. This method is similar to that utilized by Goosey *et al.* (1997).

2.11 DNA Microarray analysis

2.11.1 RNA Isolation and Reverse Transcription Cy-dye Labelling

DNA microarray was carried out following a protocol described by Dr. A. Amtmann (University of Glasgow, UK) and Maathuis *et al.* (2003). 100 µg of total RNA was prepared per array using the

Qiagen Plant RNeasy Miniprep Kit (Qiagen, UK) according to the manufacturers instructions, and concentrated to a volume of 10 μl using Microcon MC 30 spin columns (Amicon, UK) by centrifugation at 10,000rpm for 20 min at 4°C in a Sorvall MC12V microfuge (Sorvall, Stevenage, UK). Sample RNA was mixed with spiking RNA in RNase-free water to a final volume of 20 μl, and 0.5 μl of oligo $(dT)_{20}$ primer (2 μg/μl; MWG Biotech, Germany) was added to the reaction mix. This was incubated at 65°C for 10 min, followed by an incubation at room temperature for 10 min, and chilled on ice for 2 min. 4 μl of dNTP master mix (5 μl of 100 mM dATP, dGTP, dTTP, 2 μl of 100 mM dCTP and 83 μl RNAse-free water), 4 μl of 0.1M DTT, 8 μl of 5X 'Superscript II' 1st strand buffer (Gibco BRL, UK), 2 μl Cy3 (or Cy5)-dCTP (Amersham, UK) and 200 units of 'Superscript II' reverse transcriptase (Gibco BRL, UK) were added before mixing the solution and incubating at 39-42°C for 2 hr while covered to protect from light. The reaction was stopped by adding 10 μl 1M NaOH and incubating at 65°C for 10 min, and neutralized by adding 10 μl 1 M HCl and 200 μl 1 M TE buffer (pH 7.5). Unincorporated dNTPs, fluorescent dyes and primers were removed using the Qiaquick PCR Purification Kit (Qiagen, UK) according to the manufacturers' instructions.

2.11.2 Blocking, Hybridisation and Washing

The surface of the microarray was blocked directly before use by pre-heating blocking solution (25 ml 20 x SSC, 1 ml 10% SDS and 1 g BSA in 100 ml sterile dH_2O) to 42°C and immersing array in solution for 45 min at 42°C. The array was rinsed five times in deionised dH_2O at room temperature, and air-dried by centrifuging in a 50 ml conical tube at 3,000rpm for 15 min in an IEC Micromax RF centrifuge.

The labelled cDNA was dried prior to hybridisation by vacuum centrifugation, and re-dissolved in 12 μl hybridisation buffer provided by MWG (formamide based). Cy3 and Cy5- labelled samples were mixed by adding 12μl hybridisation buffer to Cy3-cDNA, pipetted up and down with a Gilson pipette, then the mixture was transferred to tube with Cy5-cDNA, mixed by pipetting, and heated in a heating block at 95°C for 3 min. The hybridisation mixture was cooled on ice for 30 sec, centrifuged briefly, and applied to the blocked microarray. The array was covered with a cover slip (Hybri-Slip 22 x 22 cm, Sigma), squeezing any air bubbles out by gently pressing the cover slip with a pipette tip, and placed in a hybridisation chamber after pipetting 20 μl 2xSSC into the corners of the chamber. The chamber was then transferred into a water bath and incubated at 42°C for 16-24 hr. Following the overnight incubation, the microarray was immersed vertically in a 50ml conical tube

containing washing buffer 1 (2 x SSC, 0.1% SDS) that was pre-warmed at 30°C, the cover slip removed, and washed for 5min with gentle agitation on an orbital shaker at room temperature. The procedure was repeated with washing buffer 2 (1x SSC, 0.1% SDS) and washing buffer 3 (0.5x SSC, 0.1% SDS). Excess buffer was drained from the array by placing it vertically on a dust free tissue, and air dried by centrifugation. Finally, hybridized slides were stored in a dark, cool, dry place (slide box).

2.11.3 Microarray scanning and data analysis

Hybridized arrays were scanned with a Packard Lite 4000 (Packard BioChip Technologies, UK). The intensities of the spots were measured using Scanalyze v4.24 (http://genome-www5.stanford.edu/microarray/smd/restech.html). Fluorescence intensities were measured and averaged over a defined spot area. Median background signals were determined over a defined area in the immediate spot environment and spot signal/background ratios were calculated. Spots with background-corrected intensity values smaller than 2.0 for at least one channel were discarded because of low signal. In addition to data exclusion on the basis of signal/background ratios, all spots were visually inspected and flagged as bad in the event of aberrations in fluorescence or background signals. For the final analysis, data points were averaged from two replicates using Microsoft Excel. It is probable that due to the stringent quality controls, which were applied to define the working data set, results may underestimate the extent of altered gene expression.

2.12. Statistical Tests

The spreadsheet program Microsoft Excel was used to analyse data. The unpaired parametric t-test was used to test the significance of results. Microsoft Excel has this incorporated and allows the unequal variance of the samples to be compared. This is important with the relatively small replicate sizes variance might fluctuate. A larger number of figures per data set might have produced a data set with a normal distribution with equal variance between the two data sets. However, in practise the experiment could not allow for so many replicates within each data set without tens of replicates. The 95% confidence level of $P = \geq 0.05$ was chosen as the cut-off point for significance.

2.13. Sources of reagents and materials

Chemicals not indicated in the text were obtained from Sigma Chemicals, Dorset, UK; Fisher Scientific, Leicestershire, UK; and GibcoBRL Life Technologies Ltd., Paisley, UK; Molecular biology

enzymes were from Promega, Hampshire, UK; Hybaid Ltd., Middlesex, UK; and Stratagene Ltd., Cambridge, UK. All primers were obtained from MWG Biotech, Germany.

AtSTP4-GUS and *AtSUC2*-GUS seeds were kindly donated by Norbert Sauer (Erlangen, Germany). The transposon intentional mutants were identified from the Sainsbury Laboratory *Arabidopsis* d*Spm* Transposant (SLAT) library (Tissier *et al.*, 1999) and obtained from Sainsbury Laboratory (Norwich, UK).

Chapter 3.

A reverse genetics approach to investigating the physiological role of *AtSUC1* and *AtSUC2*.

3.1. Introduction

A number of techniques are now available for studying the physiological role of individual transporters and for investigating their regulation. One approach to investigating the function of a particular gene product is to use a gene knockout or null mutation (Krysan *et al.*, 1999). Rendering the gene non-functional may have a marked effect on plant growth and development and this can be monitored by phenotypic analysis of the resulting mutants. This reverse genetics approach, where one begins with a mutant gene sequence and then investigates the resulting change in the phenotype, is fundamentally different from forward genetics where one starts with a mutant phenotype and then determines the genotype (Krysan *et al.*, 1996).

The easiest way to inactivate a specific gene is to disrupt it with an unrelated segment of DNA. In yeast, *E. coli* and mouse, homologous recombination is used for targeted mutagenesis. The basis of this is to allow the recombination of a wild-type copy of the target gene with a disrupted version of the gene carried in a cloning vector. Homologous recombination with intact *Arabidopsis* plants has been reported (Kempin *et al.*, 1997), although the frequency is thought to be too low to be used as a viable method for generating knockout mutations in a large number of genes (Krysan *et al.*, 1996). Therefore, an alternative strategy is to use insertional mutagenesis.

In *Arabidopsis*, transposable elements and T-DNA (transfer-DNA) have been used to generate large populations of insertion mutants. The insertion disrupts and hence inactivates individual genes. Individual plants with an insertion in a gene of interest can be identified by PCR using a pair of primers, one that anneals to the insertion sequence and the other to the gene of interest (McKinney *et al.*, 1995). Pooling strategies are implemented so that individuals containing the insert in the gene of interest can be directly identified from large populations (Krysan *et al.*, 1996). Several PCR-based strategies to isolate insertional mutants have been described for *Arabidopsis* using either T-DNA or transposon lines (Krysan *et al.*, 1996; McKinney *et al.*, 1995; Speulman *et al.*, 1999; Tissier *et al.*, 1999; Young *et al.*, 2001; Sessions *et al.*, 2002). With the sequencing of the *Arabidopsis* genome, we now have a great opportunity to use reverse genetics strategies and many

groups are taking advantage of the availability of insertional mutants to learn more about gene function. Knockout mutants for *Arabidopsis* transporter proteins, including H^+-ATPases, a K^+ channel and a Ca^{2+}-ATPase, have been previously identified in a collection of T-DNA-transformed lines (Krysan *et al.*, 1996). More recently using such mutant lines, genetic evidence for a role of AtSUC2 in phloem loading has been reported (Gottwald *et al.*, 2000). The *suc2* insertional mutants displayed retarded growth and development and sterility, with an excess of sugars present in the leaves and an inadequate supply to the rest of the plant (Gottwald *et al.*, 2000). A variety of mutant collections is available from various stock centres such as the Nottingham *Arabidopsis* Stock Centre (NASC) and *Arabidopsis* Biological Resource Center (ABRC).

The aim of the work in this chapter was to examine the physiological function of AtSUC1 and AtSUC2 using transposon-insertion mutants. The original insertion mutants used in this study were isolated prior to the start of this project in the Southampton Membrane Transport Laboratory (Pittman, Marvier and Williams, unpublished) from the Sainsbury Laboratory *Arabidopsis* Transposant (SLAT) library (Tissier *et al.*, 1999). In the following section, a description of the SLAT library and its construction is given with an overview of the procedures carried out to isolate the original sugar-transporter insertional mutants.

3.1.1. Sainsbury Laboratory *Arabidopsis* Transposant Collection

Tissier *et al.* (1999) have produced a library of transposants called the SLAT collection. The SLAT collection was produced by insertional mutagenesis using non-autonomous derivatives of the suppressor-mutator element (d*Spm*) and represents approximately 48,000 independent transposants (Tissier *et al.*, 1999). In their procedure, a single T-DNA construct (figure 3.1) was used to transform *Arabidopsis* via *Agrobacterium*-vacuum infiltration.

The important constituents of the construct and the processes in obtaining the final SLAT library are discussed in detail by Tissier *et al.* (1999) and will be described below. The selectable genes (described below) were chosen for their ability to be screened on soil, thus avoiding the need to prepare sterile media and seeds and allowing for a more efficient screening process (Tissier *et al.*, 1999).

The defective *Spm* (d*Spm*) element (transposon) carries the BAR gene which confers resistance to the herbicide phosphinothricin (PPT). PPT is used to select for T-DNA integration and for transposon reinsertion. Thus, plants that are resistant to PPT carry the transposon. A terminator region of the nopaline synthase (nos) gene at the 3' end of the BAR gene occurs in the opposite

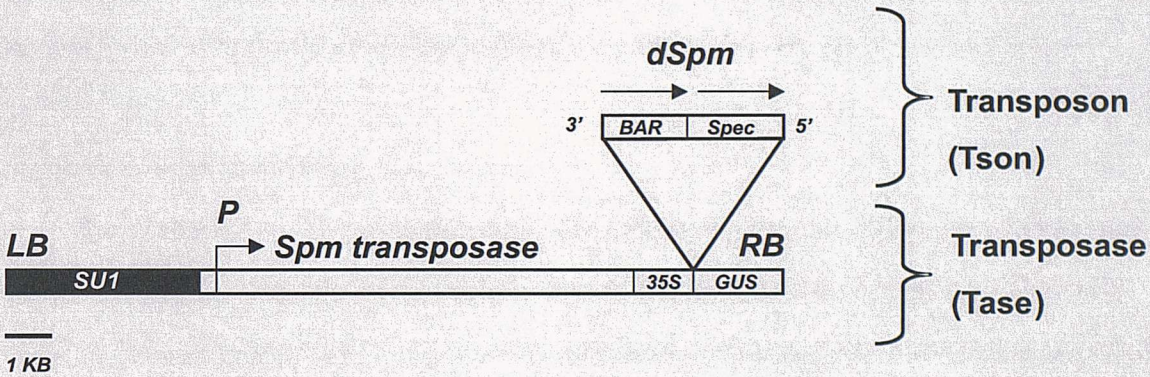


Figure 3.1. Diagram of the construct used to insert transposons into the genome of *Arabidopsis*. LB and RB, left and right borders respectively; P, promoter driving the expression of the transposase (*Spm, 35S or AtDMC1* promoters); Spec, spectinomycin resistance gene for selection in bacteria; Su1, counter-selectable marker (Adapted from Tissier *et al.*, 1999)

orientation to the termination region of the *Spm* element. Thus, insertions of this mutagenized gene into an intron of a gene should abolish gene function (Tissier *et al.*, 1999). d*Spm* lies between the cauliflower mosaic virus 35S promoter and the start codon of a β-glucuronidase (GUS) gene. This acts as an excision marker to monitor the activity of the element. If plants stain positive for GUS this means that the transposon has been excised from the T-DNA construct. This was used to check for germinal excision of the initial transformants (see below). The transposase gene fragment coding for both the TnpA and TnpD proteins, was cloned under the control of a 35S promoter, the *Spm* promoter, or a meiosis-specific promoter from the *AtDMC1* gene. So essentially there were three T-DNA constructs used in the study differing in the promoter controlling the transposase. The *SU1* gene from *Streptomyces griseolus* encodes a cytochrome P450 that confers sensitivity to R7402 (a sulfonylurea proherbicide; O'Keefe *et al.*, 1994). The counter-selection cassette containing the *SU1* gene was cloned between the left border and the transposase gene in the same orientation.

3.1.2. Selection of active lines

The initial stage in the production of the SLAT library involved the selection of "active lines" (Tissier *et al.*, 1999). *Arabidopsis* was transformed with the three different T-DNA constructs and multiple transformants were recovered. Since the presence of multiple unlinked T-DNA inserts would greatly reduce the probability of recovering double resistant plants, it was important at this stage to isolate transformants with T-DNA inserts at a single locus. This was achieved by plating 100 seeds from each transformant on PPT or R7402 (to select for *BAR* and *SU1* respectively). Only the transformants segregating as a single locus for both markers were retained for the production of transposition events (Tissier *et al.*, 1999).

As a further selection, the T2 progeny from each of these primary transformants were sown on soil and subjected to double selection by PPT and R7402. The frequency of double resistant plants was recorded for each line. It was also important at this stage to stain the double resistant plants for GUS to eliminate lines in which the *SU1* gene had been inactivated by insertion of the *dSpm*. From these results, Tissier *et al.* (1999) selected active lines that were double-resistant and used these for large-scale production of transposition events.

The principle for recovery of unlinked transposition events has been explained in detail by Tissier *et al.* (1999) and will be summarised here. Seeds from a plant heterozygous for the T-DNA insert were collected, allowed to germinate on soil and treated with PPT and R7402. If a transposition of the *dSpm* element to an unlinked site, on either the same or a different chromosome, occurs before

or at meiosis then the T-DNA and the newly transposed element may segregate in the progeny (see figure 3.2). In this case, plants carrying the transposon and lacking the T-DNA will be resistant to both PPT and R4702. However, if the transposon transposes to a linked site, double-resistant (DR) plants can only be recovered if there is recombination between the T-DNA and the transposon (i.e. there is a rearrangement of genes in combinations that differ from those in either parent). Double-resistant plants could also result from inactivation of the *SU1* gene, either by insertion of the *dSpm* or by silencing.

Unlinked transposition events can be recovered only in the progeny (called F_1) arising from the self-pollination of plants that are heterozygous for the T-DNA locus (Tissier *et al.*, 1999). The strategy devised relied on the fact that the progeny of homozygous plants should not contain individuals resistant to the double selection of R7402 and PPT. The seeds that were subjected to this double selection could then be harvested not from heterozygous plants (F_1 plants) but from their PPT-resistant progeny (F_2). The ratio of heterozygotes to homozygotes from the F_2 population will thus be one to three. Although one out of three F_3 seeds therefore should not contribute any transposition events, this strategy allows rapid production of massive numbers of selectable seeds. One heterozygous plant can produce up to 20,000 seeds, of which 10,000 are heterozygous for the T-DNA. When these are grown with PPT-resistant homozygous individuals, they can produce 1000 to 10,000 seeds each (depending on the density of the plants). Three hundred plants per tray (21 x 35 cm^2) will produce ~300,000 seeds, that is, 1000 per plant, which are harvested from a sector of the tray (Tissier *et al.*, 1999). Under those conditions, within two generations, 107 selectable seeds can be generated from a single heterozygous plant. Transposition events that occurred in the F_1 generation will be transmitted and may give rise to large numbers of DR plants in the F_3 progeny. Therefore, only one or two plants are collected from each half tray (20,000 seeds in which >50 DR F3 plants are present. In addition, plants expressing the *SU1* gene in the absence of selection exhibit a slightly different growth habit than do untransformed plants (they are shorter, bushier, and darker green; Tissier *et al.*, 1999). The wild-type plants, which may carry unlinked transposition events, in PPT-resistant F_2 population, were removed to prevent contamination of the F_3 seeds (Tissier *et al.* 1999).

3.1.3. Organisation of the transposant library

Seeds and DNA from plants that survived the double selection were harvested in pools of 50 individuals and assigned to a superpool of 48 pools (Tissier *et al.*, 1999). To date there are 20

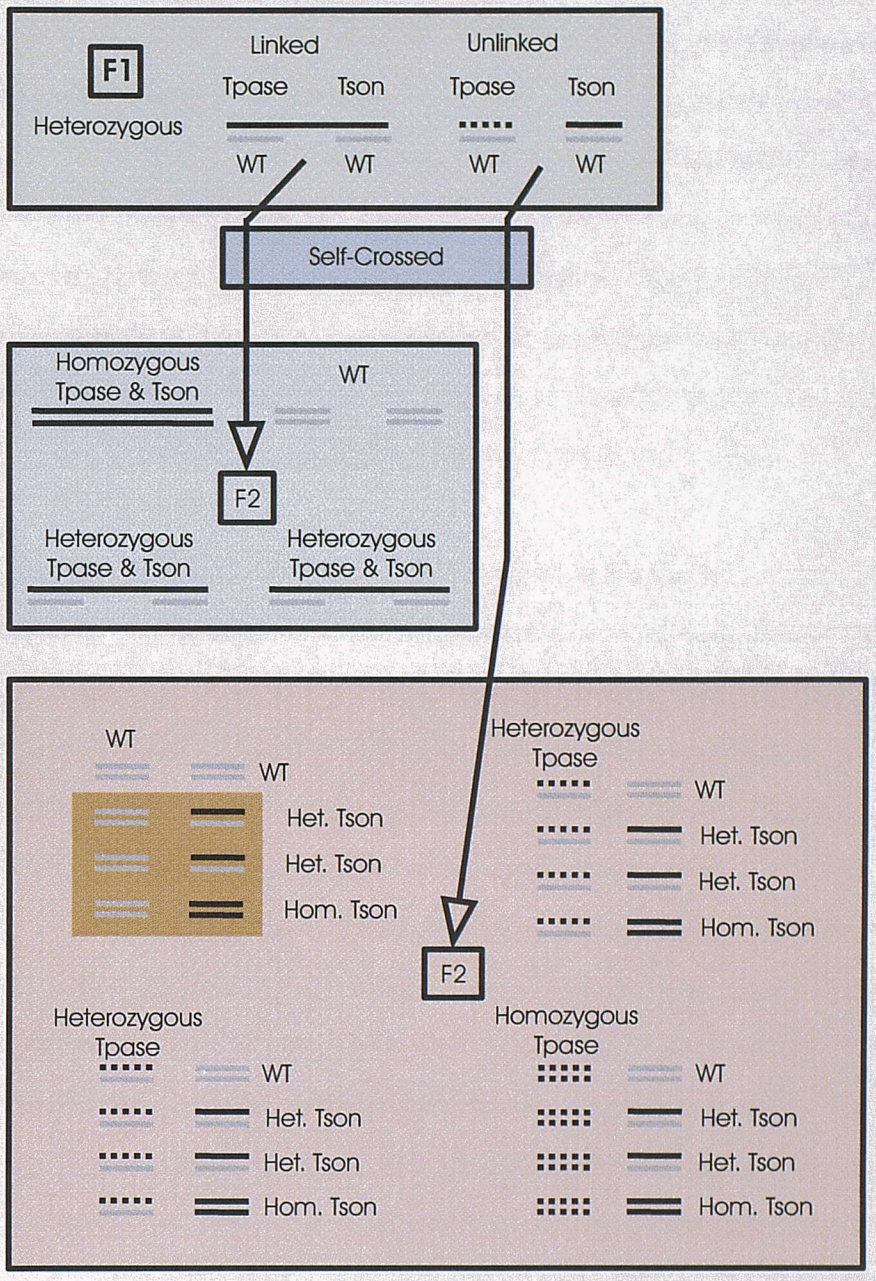


Figure 3.2. Genetics strategy used for the isolation of transformant plants with transposon (Tson) insertions without the transposase element (Tpase) that enables reallocation of the Tson. This diagram illustrates that only transposon insertions into an unlinked position of the *Arabidopsis* gDNA will enable the Tson to be isolated from the Tpase in progeny (boxed in gold).

superpools (20 x 48 = 960 pools; 48,000 transposants). Each pool was given a two-coordinate address corresponding to the superpool number and the pool number (Tissier *et al.*, 1999). Inverse PCR (IPCR) is a technique for cloning unknown DNA fragments adjacent to known sequences (Ochman *et al.*, 1988). Genomic DNA from the pools of 50 plants was digested with three different restriction enzymes (this increases the probability of amplifying the flanking DNA for each insertion present in the pool (Tissier *et al.*, 1999). The enzymes were inactivated and self-ligation was allowed to occur using T4 DNA ligase. IPCR was performed with all of the DNA pools and the resulting IPCRs were spotted in duplicates in a gridded array onto a nylon membrane (Tissier *et al.*, 1999). This IDI filter was made available to our lab for the preliminary screen. This primary screen to identify pools that contain an insertion in the gene of interest was achieved by hybridisation of the filter with a labelled genomic DNA fragment in which insertions are sought (Tissier *et al.*, 1999).

3.1.4. Screening of the IDI filter and isolation of individual insertion mutants

Prior to the start of this project, the IDI filter was screened in order to identify pools containing insertional mutants in sucrose transporters. The filter was screened at moderate stringency and several pools were identified (Marvier and Williams, unpublished). The gDNA was obtained for these pools from the Sainsbury Laboratory and was screened using PCR with primers designed to conserved regions in sucrose transporters and to the transposon sequence (Pittman, Marvier and Williams, unpublished). The primers to the conserved regions in sucrose transporters were designed to enhance the chances of identifying several sucrose transporters in the sucrose-proton transporter family (SPSF family, figure 5.1). Seed was obtained from the Sainsbury Laboratory for the pools that gave PCR products with the appropriate combination of primers. For each positive pool, 500 seeds were planted and, following germination, plants were selected for the presence of the transposon insert using PPT. Approximately 200 PPT resistant plants were grown for each pool and a screening strategy was devised to reduce the number of PCRs required to identify individual positive plants.

At the end of the PCR-based screening procedure, direct sequencing of the DNA flanking the d*Spm* insert indicated that plants AM1 and AM2 contained inserts in *AtSUC1*, and AM3 and AM4 contained inserts in *AtSUC2* (Pittman, Marvier and Williams, unpublished). AM1 and AM2 contained inserts in the same positions whereas AM3 and AM4 contained inserts at two separate positions in the *AtSUC2* gene (figure 3.3). The positions of the d*Spm* insertions were found to be within exon sequences in all four positive plants (figure 3.3). During the course of this study, different insertion mutants (T-DNA) were reported for *AtSUC2* and the mutant alleles were designated *suc2-1*, *suc2-2*

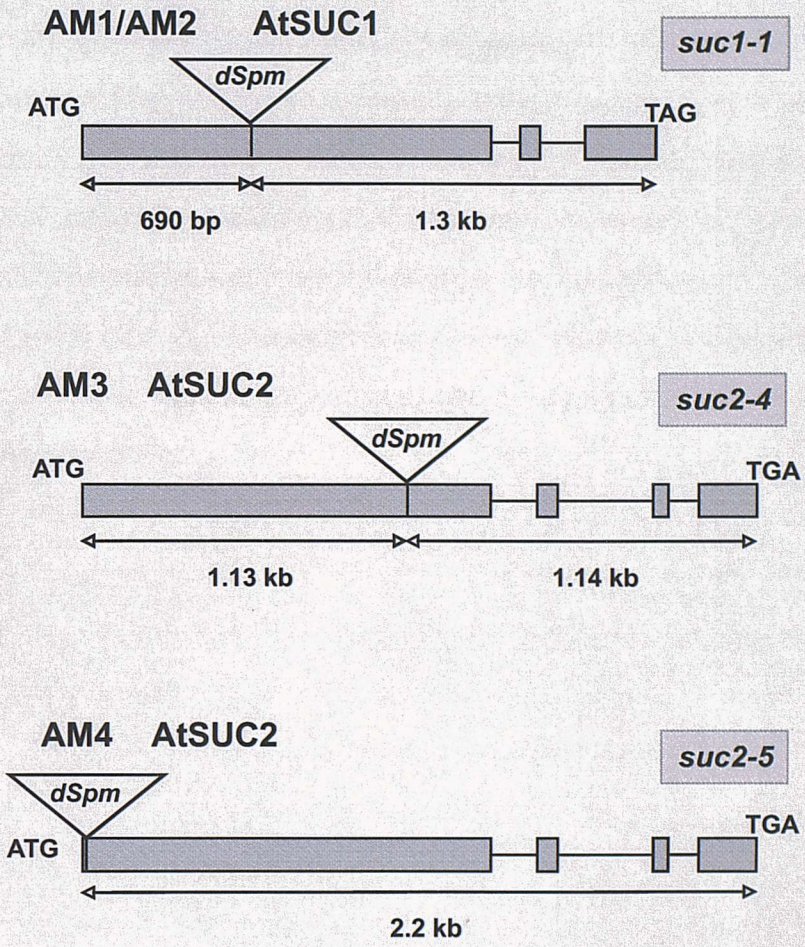


Figure 3.3. Position of d*Spm* insertions in *AtSUC1* (AM1/2 plants) and *AtSUC2* (AM3/4 plants). The insertion is in the first exon of *AtSUC1*, 690 bp downstream of the predicted start codon; the mutant allele is designated *suc1-1*. In the AM3 plant the insertion is in the first exon, 1.13 kbp downstream of the start codon; the mutant allele is termed *suc2-4*. In the AM4 plant the insertion is 70 bp downstream from the start codon in the first exon; the mutant allele is *suc2-5*.

and *suc2-3* (Gottwald *et al.*, 2000). The mutant alleles characterized in this study were generated by transposon insertion in different positions of the *AtSUC2* gene and therefore the new alleles were designated *suc2-4* and *suc2-5* (figure 3.3). The mutant allele of the *AtSUC1* gene is designated *suc1-1*. Preliminary studies investigating the PPT resistant-segregation patterns of the progeny from AM1-4 plants were carried out before the start of this project and suggested that AM1 was homozygous for the transposon while AM2 (*suc1* mutant), AM3 and AM4 (*suc2* mutants) were heterozygous. The first part of this chapter describes experiments conducted to isolate insertional mutants homozygous for the insert in *AtSUC2,* and to confirm the zygosity of the mutants used in this study. The second part provides a detailed phenotypic comparison of the *Atsuc1* and *Atsuc2* mutants showing that these transporters have quite different contributions to the growth and development of *Arabidopsis*. As mentioned above, during the course of this study a paper was published characterizing other mutant alleles of *AtSUC2* (Gottwald *et al.*, 2000), and the results from that study are discussed in comparison to those obtained in this thesis.

3.2. Results

3.2.1 Determination of zygosity of AM 1, 3 and 4

Experiments were conducted to investigate the zygosity of the mutants (AM1, 2, 3 and 4, see introduction) supplied for this project. This was carried out by determining segregation ratios of the seed from these selfed plants using PPT to select for the presence of the transposon (see introduction). According to Mendelian genetics, a selfed heterozygote should contain the following progeny: 25% WT, 50% heterozygous and 25% homozygous, while a selfed homozygote should give rise to 100% homozygous progeny. As controls, WT seeds were included which were plated with and without PPT. If the seed failed to produce a shoot or root the seeds were classified as ungerminated. Examples of the resulting seedlings are shown in figure 3.4 and graphs to illustrate the segregation ratios are presented in figure 3.5. In this chapter, heterozygous (het) is used to indicate that there is one copy of the transposon while homozygous (hom) indicates that there are two copies of the transposon present. The WT seedlings (-PPT) showed a healthy deep green colour and expanded cotyledons (determined after 7 days). They also showed the initial formations of the first true leaves (figure 3.4). Eight percent of the WT seeds grown -PPT did not germinate. Of those that did germinate, only 3% were categorized as sick (chlorotic) or dead at day 7. For WT seedlings plated with PPT (+PPT) 56% of the plated seeds did not germinate and of those that did, 96% were either sick or dead at 7 d. In this particular experiment, at day 7 only 2% managed to resist the PPT to show healthy growth. Therefore, it would appear the addition of PTT in the media was sufficient to arrest WT seeds/seedlings. Seed from the AM1 plants (putative *suc1-1* het) and AM2 plants (putative *suc1-1* hom) showed 100% germination on the +PPT media. By day 7, 28% of these putative *suc1-1* het seedlings and 3% of the putative *suc1-1* hom seedlings appeared sick indicating that these are indeed from heterozygous and homozygous lines respectively. The AM3 (putative *suc2-4* het) seeds gave a germination rate of 91% at day 3 similar to that of the WT. By day 7, only 70% of the seeds had produced healthy seedlings indicating that these are indeed from a heterozygous line. These healthy seedlings seemed to be of two different size categories; a set that were larger and more developed and a set that were stunted (figure 3.4). This was in contrast to the more uniform growth and development seen for WT (-PPT), *suc1-1* het and *suc1-1* hom lines (figure 3.4). This suggested that the more stunted seedlings could be homozygous seedlings while the larger ones were heterozygous and experiments were conducted to see if this was the case and whether homozygous mutants could be isolated (see next section).

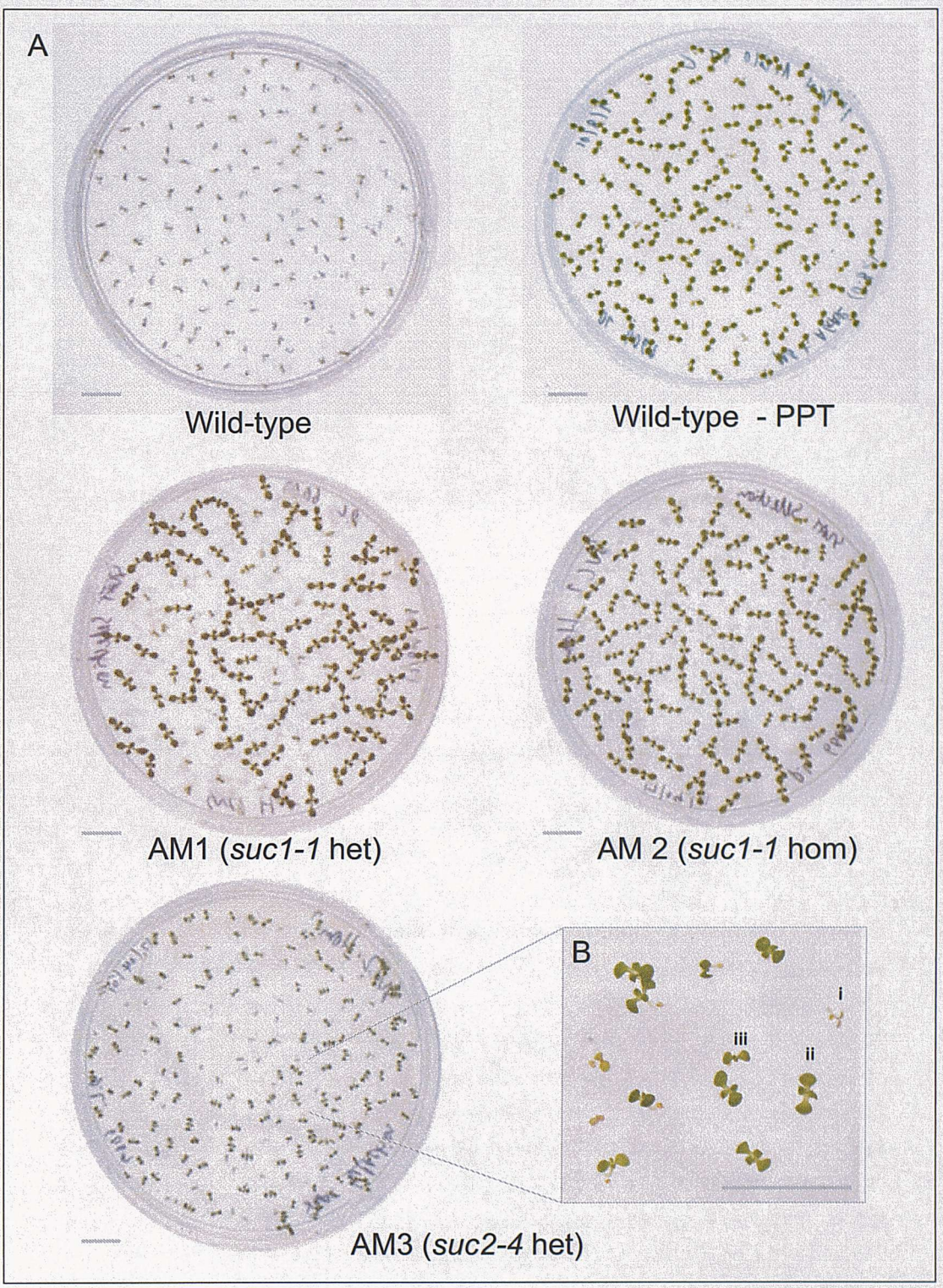


Figure 3.4. Seed germination of segregating *suc1* and *suc2* mutants. A: Following sterilization and stratification *Arabidopsis* seedlings were grown on MS plates with 10 µg/ml PPT (unless otherwise stated) with no sucrose supplementation under growth room conditions. The genotype of the selfed parent plants are indicated and not that of the progeny. Het = heterozygous, hom = homozygous. B: A magnified portion of seeds from a *suc2-4* heterozygous mutant parent showing the range of phenotypes; thought to be dead WT seedlings (i), healthy *suc2-4* het seedlings (ii) and retarded suc2-4 hom seedlings (iii) Bar = 1 cm.

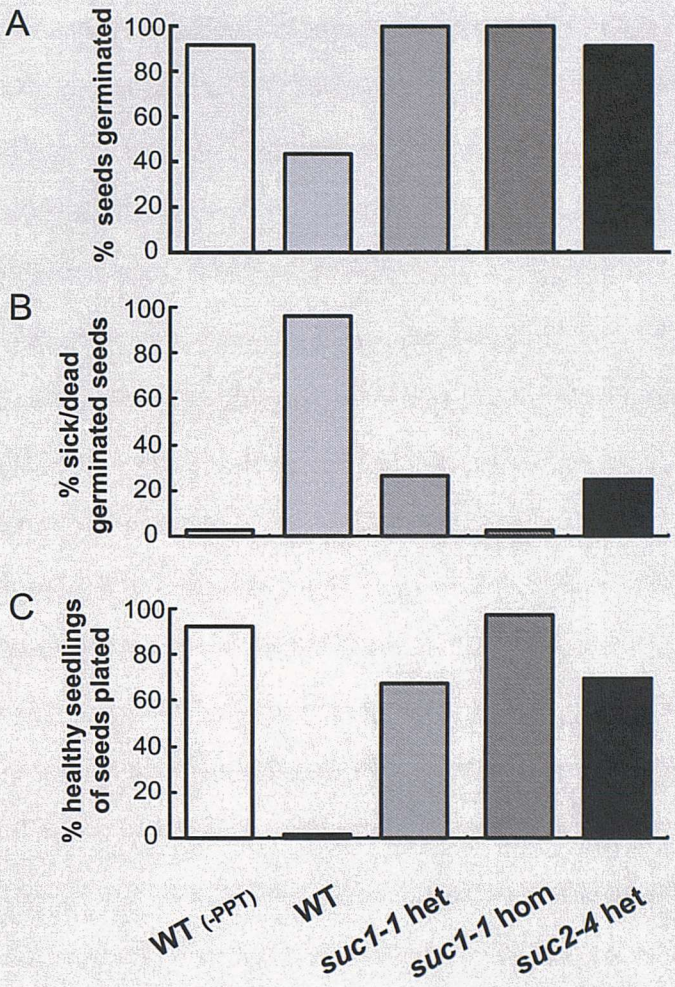


Figure 3.5. Germination and growth characteristics of *suc1-1* and *suc2-4* mutant seedlings compared to WT. Approximately 150 seeds were spread on to each plate about 1 cm apart and stratified for 48 hrs at 4°C in darkness. Seedlings were grown for 7d on MS plates (1X MS salts, 1% w/v bactoagar) with 10 µg/ml PPT (unless stated) under white light 16 h light / 8 h dark cycle (22°C in light and 20°C dark) in *Arabidopsis* growth cabinets relative humidity was kept constant at 75% while irradiance was set at 120 mol m^{-2} s^{-1}. **A,** The percentage germination shows good germination for all seed batches and high potency of PPT. **B,** PPT consistently inhibits the growth of WT seedlings. Sick seedlings were classed as those which exhibiting chlorosis. **C,** shows the affect of PPT relative to the number of seeds plated, thus the combined effects of A + B.

3.2.2. Screening and isolation of *suc2* homozygous mutants.

As a *suc2-4* hom line had not previously been found in the SLAT collection in the preliminary screening procedure, it was important to isolate homozygous mutants for the future phenotypic analysis. As both AM3 and AM4 *suc2* mutants were found to be heterozygous for d*Spm*, a screen was initiated in order to identify *suc2* homozygotes. Seed from AM3 and AM4 heterozygous plants were germinated, with the wild-type background removed by PPT selection, and visually screened for any differences within the plant population. Wild-type plants died off quickly after spraying. After several weeks, many of the remaining plants were normal in appearance but a small number of very small stunted plants also remained. All of the plants tested contained the d*Spm* insert as they amplified products following PCR with *AtSUC2* and d*Spm*-specific primers (results not shown). However, for the stunted plants, the full-length *AtSUC2* gene product could not be amplified with *AtSUC2*-specific primers indicating that these plants were homozygous mutants. The non-retarded, normal looking plants amplified a product of the correct size with *AtSUC2*-specific primers indicating that these were heterozygous for the insert (results not shown). The previous section (3.2.1) indicated that *suc2* homozygotes might also be identified at the seedling stage on plates because of their retarded growth (figure 3.4). The progeny from a *suc2-4* heterozygote plant (AM3) were plated on PPT plates containing sucrose and grown for 10 days (figure 3.6A) the wild-type died off on PPT, and the remaining seedlings again appeared to be in two size categories (figure 3.6B). These seedlings were allowed to grow further and then genotyped by PCR. The stunted seedlings were confirmed as homozygotes as they did not amplify the wild-type gene but did amplify with the combination of the *AtSUC2* and d*Spm* primers; the larger seedlings were shown to be heterozygotes as products with both sets of primers could be amplified from these (figure 3.6C). The homozygous mutants obtained in this study were consistently smaller than heterozygotes even when supplied with sucrose. However, supplementary sucrose appeared to produce consistently healthier seedlings than those grown without sucrose (figure 3.6B).

3.2.3. Phenotypic analysis of the *suc1* mutants and tissue-specific expression of *AtSUC1* in WT plants

Homozygous and heterozygous *suc1-1* mutants grown under glasshouse conditions revealed no obvious differences when compared to wild-type plants (figure 3.7). Although not carried out as part of this thesis, experiments were conducted in the Southampton laboratory to confirm that the *suc1-1* homozygote does not express the *AtSUC1* transcript (Pittman, Marvier and Williams,

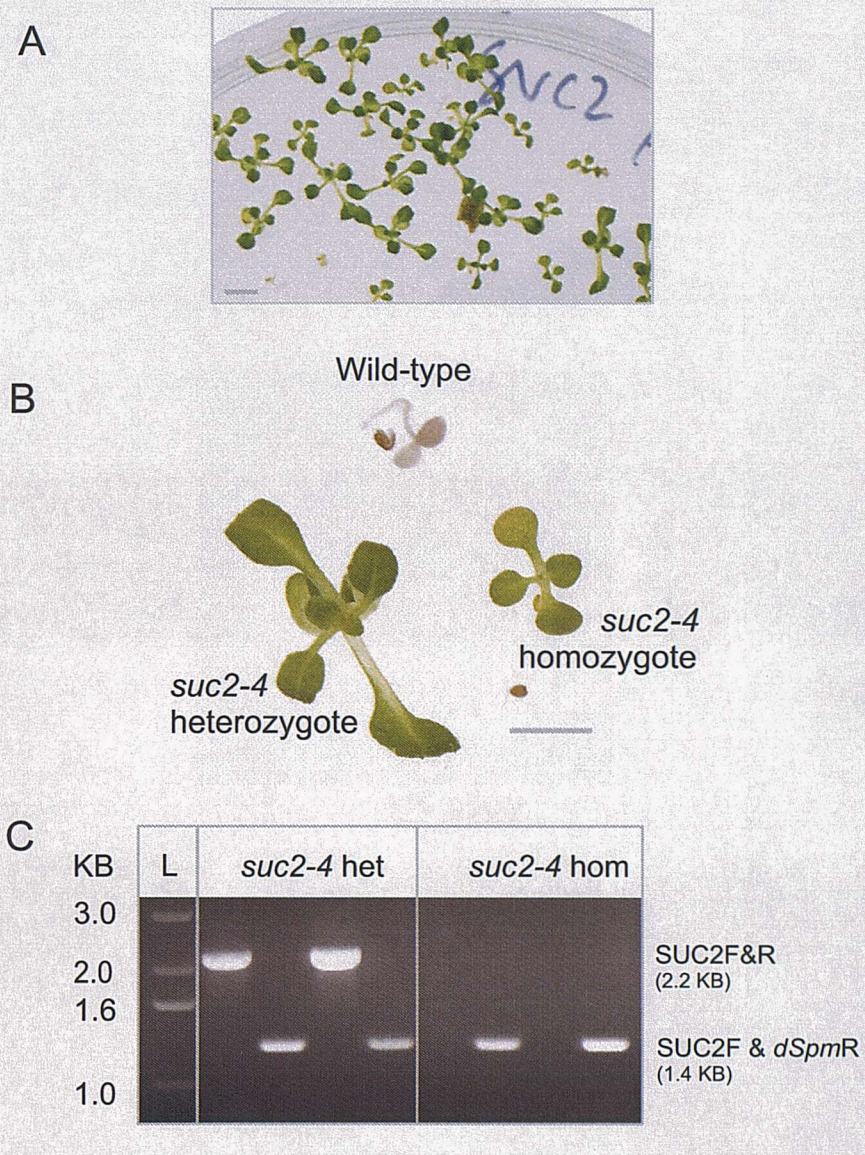


Figure 3.6. Isolation of *suc2-4* homozygous mutants. Seedlings were germinated and grown on MS agar (1% w/v) supplemented with sucrose (2% w/v) and PPT (10 µg/ml). **A**: Variation in seedling morphology within a petri dish of progeny of a segregating *suc2-4* heterozygous plant. Bars = 5 mm. **B**: The phenotype of wild-type, and *suc2-4* mutant seedlings. Wildtype seedlings die in the presence of PPT while *suc2-4* mutants survive. *suc2-4* homozygous plants are able to grow in the presence of PPT, but the absence of SUC2 results in a phenotype showing retarded growth and development. Bars = 5 mm. **C**: Representation of PCR analysis of *suc2-4* heterozygous and homozygous plants. Gene specific and transposon primers (*dSpm*R) were used to amplify from gDNA isolated from individual *suc2* mutants. Products obtained using both gene specific primers (SUC2F&R, 2.2 KB) and the gene specific and transposon primers (SUC2F & *dSpm*R, 1.4 KB) indicate a heterozygous plant. Products obtained using only the gene specific and transposon primers indicate homozygous plants.

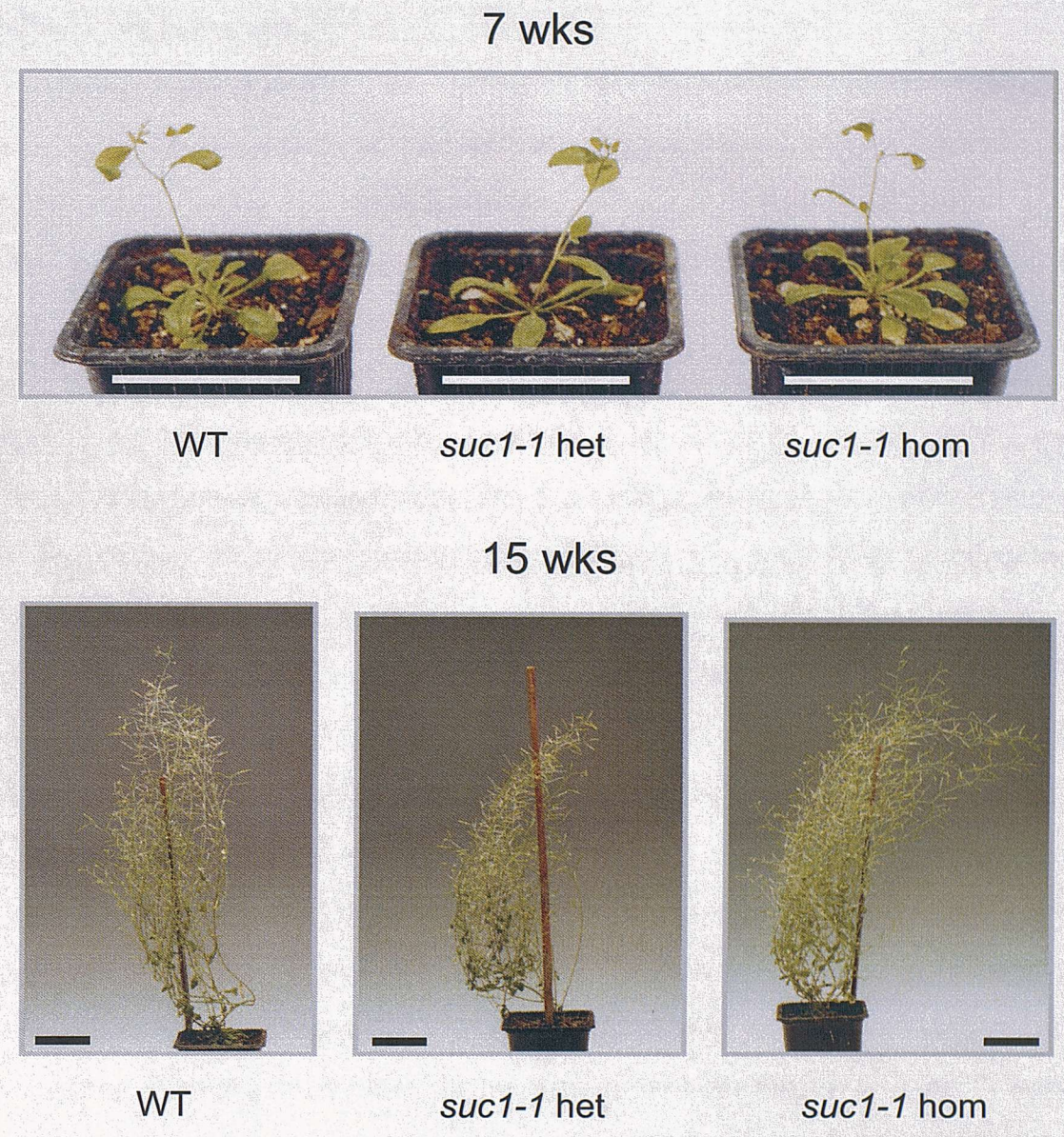


Figure 3.7. Phenotype of *suc1-1* mutants at different developmental stages. *suc1-1* mutants and wild-type (WT) plants grown on soil under glasshouse conditions. Upper panel: 7-week old *suc1-1* and WT plants. Lower panel: 15-week old *suc1-1* and WT plants. Het = heterozygous, hom = homozygous. Bars = 5 cm.

unpublished). This was carried out using RT-PCR on material prepared from floral tissue as it had previously been reported that *AtSUC1* was expressed predominantly in this material (Stadler *et al.*, 1999). RT-PCR using gene-specific primers showed that the full length *AtSUC1* transcript was not present in the putative *suc1-1* homozygotes while it was present in the putative heterozygotes; both homozygous and heterozygote *suc1-1* mutants expressed the *AtSUC2* transcript (Pittman, Marvier and Williams, unpublished data).

Although Stadler *et al.* (1999) reported that *AtSUC1* expression was confined to floral tissue (using GUS constructs), previous work in the same lab using tissue specific northern blot analysis had indicated that *AtSUC1* had a more widespread expression pattern (Sauer and Stoltz, 1994). Therefore, it was important to clarify the distribution of this transporter expression in WT plants. RT-PCR was performed with gene-specific primers for *AtSUC1* on a range of tissues isolated from WT plants. As shown in figure 3.8, *AtSUC1* expression was broad, occurring in all tissues examined but particularly in leaf, stem and flowers. Similar experiments were conducted for AtSUC5, which is the most closely related sucrose transporter to AtSUC1 (see phylogenetic analysis, figure5.1). Previously it was suggested that *AtSUC5* may be expressed in all tissues (Ludwig *et al.*, 2000) but data shown in figure 3.8 contradicts this and suggests that it has a much more limited expression pattern, being detected only in flowers and siliques. AtSUC5 is interesting as it was shown to transport biotin (vitamin H) in addition to sucrose. Thus, its localisation in the flower and siliques could suggest a role in supplying biotin to the developing embryo and seed. This remains to be investigated using immunolocalisation or promoter-GUS fusions.

A more detailed phenotypic analysis was carried out by comparing various parameters (plant height, leaf length, number of flowers per plant and silique length) in WT and *suc1-1* mutants grown under glasshouse conditions (figures 3.9 and 3.10). No major differences were observed in the *suc1-1* homozygotes compared to the WT plants for any of the parameters measured at 11 and 17 weeks in glasshouse grown plants. Although not analysed in detail in these soil grown plants, no marked difference was observed in the root system of the mutants when examined at the end of the growth period.

Plants were then grown in short- (8 h light/day) and long- (16 h light/day) day conditions this time in controlled-environment cabinets. The *suc1-1* homozygotes showed a slight increase in the time to flowering, but only in short-day conditions. However, the *suc1-1* heterozygotes were also delayed (figure 3.11).

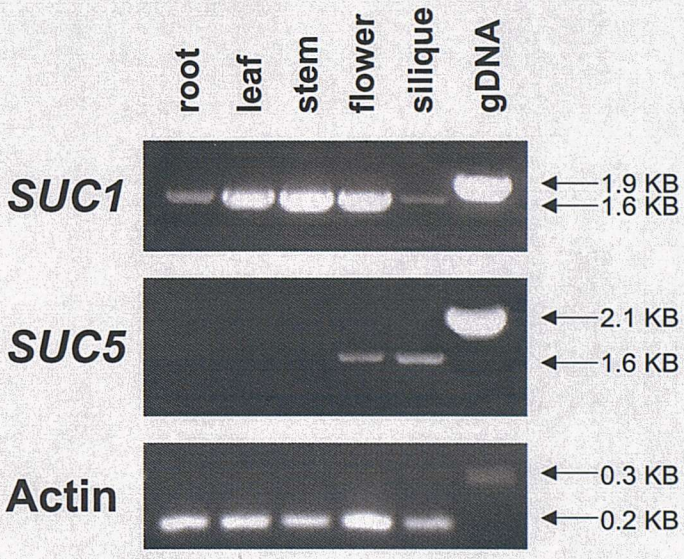


Fig. 3.8. AtSUC1 is expressed in all organs of *Arabidopsis* while AtSUC5 is found mainly in flowers and siliques. RT-PCR was used to determine transcript levels in various organs for *AtSUC1* (top), *AtSUC5* (middle) and actin (bottom). Products were obtained using genomic DNA with gene-specific primers, details of which are given in table 2.1.

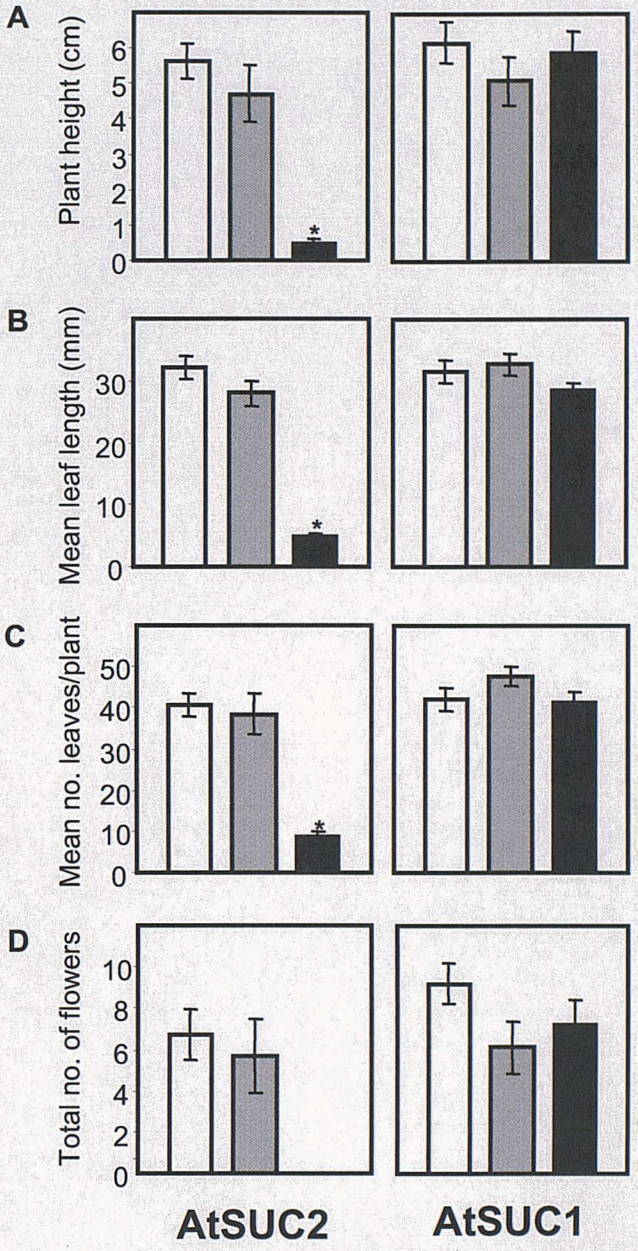
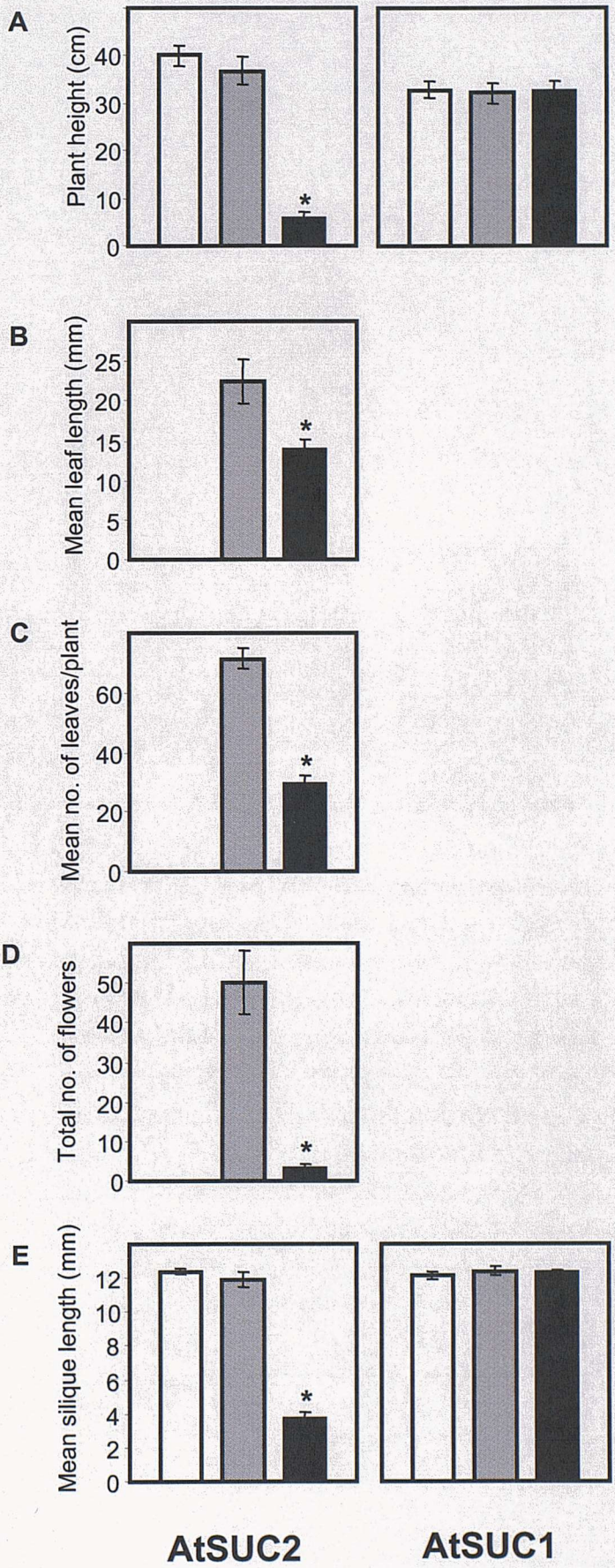


Figure 3.9. Phenotypic characterization of *suc1* and *suc2* mutants at 11 weeks post-germination. Physiological comparisons of *suc2* (left) and *suc1* (right) homozygotes (black bars) with wild-type (Col-0) (white bars) and *suc2* (left) and *suc1* (right) heterozygotes (grey bars) plants 11 weeks post germination. Plants were grown under glasshouse conditions (A) Plant height. (B) Mean leaf length. (C) Mean number of leaves per plant. (D) Total number of flowers. For each of the comparisons in (A) – (D), the *suc2* homozygotes, marked by an asterisk, were significantly different from both the wild-type and *suc2* heterozygotes ($P < 0.0001$) as determined using a two-tailed t-test. Each bar represents the mean (±SE) of 6 - 48 samples.

P.T.O.

Figure 3.10. Phenotypic characterization of sucrose-transporter mutants at 17 weeks post-germination. Physiological comparisons of *suc2* (left) and *suc1* (right) homozygotes (black bars) with wild-type (Col-0) (white bars) and *suc2* (left) and *suc1* (right) heterozygotes (grey bars) plants 17 weeks post germination. Plants were grown under glasshouse conditions. (A) Plant height. (B) Mean leaf length. (C) Mean number of leaves per plant. (D) Total number of flowers per plant. (e) Mean silique length. For each of the comparisons in (A), (C) – (E), the *suc2* homozygotes, marked by an asterisk, were significantly different from both the wild-type and *suc2* heterozygotes ($P < 0.0001$) as determined using a two-tailed t-test. For the comparison in (B), the *suc2* homozygotes, marked by an asterisk, were significantly different from both the wild-type and *suc2* heterozygotes (with a P value of $0.05 < P < 0.02$). For the comparisons in (B) – (D), all of the wild-type plants, *suc1* homozygotes and *suc1* heterozygotes and most of the *suc2* heterozygotes (nine out of twelve plants) had no leaves and flowers due to senescence. Data in (B), (C) and (D) are given for the three *suc2* heterozygotes which still retained some leaves and flowers. For the comparisons in (E), only two of the *suc2* homozygotes sampled had siliques present. In these plants the siliques were immature. Each bar represents the mean (±SE) of 3 - 36 samples.


A
Plant height (cm)
0
10
20
30
40
*
B
Mean leaf length (mm)
0
5
10
15
20
25
*
C
Mean no. of leaves/plant
0
20
40
60
*
D
Total no. of flowers
0
10
20
30
40
50
*
E
Mean silique length (mm)
0
2
4
6
8
10
12
*
AtSUC2
AtSUC1

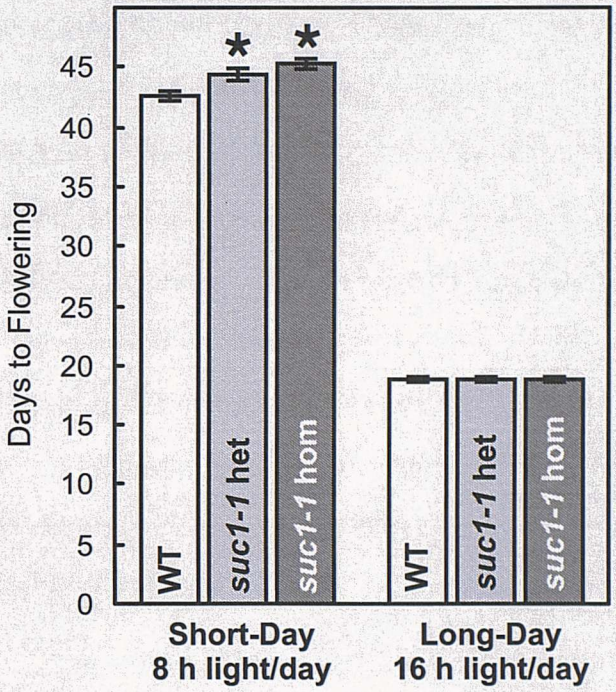


Figure 3.11. Both the *suc1-1* heterozygous and *suc1-1* homozygous lines show a delay in flowering but only when grown in short day lengths. WT, *suc1-1* het and *suc1-1* hom seeds were plated onto MS media and stratified at 4°C for 48 h and then placed in a growth cabinet for one week. Seedlings were then transferred to individual pots with sterile soil and placed in two separate environmentally controlled growth rooms one set to short day lengths (8 h L : 16 h D) and the other to long day lengths (16 h L : 8 h D). The plants were checked each morning and the appearance of the first inflorescence was noted. The bars represent the mean ±SE of 8 WT, 8 *suc1-1* het and 16 *suc1-1* hom plants.

As part of a microarray analysis of gene expression under far-red light (FR), Tepperman *et al.* (2001) demonstrated that AtSUC1 was induced by just three hours FR treatment suggesting that this gene may have an important role in de-etiolation. Therefore, the phenotype of *suc1* mutant seedlings was examined when grown under white, red and FR light. Hypocotyl length and cotyledon area were measured but there were no differences between the *suc1* mutant and the WT (figure 3.12).

Based on their localization data, Stadler *et al.* (1999) suggested that AtSUC1 might have an important role in importing sucrose into the germinating pollen grains and into growing pollen tubes. Therefore, at the end of this project, WT and *suc1* homozygous plants were grown in controlled environment cabinets in order to analyse the characteristics of the pollen. Unfortunately due to time constraints, this analysis could not be completed as part of this thesis. However, pollen from these plants was analysed subsequently (Francini and Williams, unpublished data) and the results will be described here, as there was quite a major difference in the pollen characteristics. Pollen grains were taken from flowers at similar developmental stages and applied to a solidified medium containing 20% sucrose. After 24-48 h, a marked phenotypic difference was apparent; in the WT plants many pollen had germinated and produced pollen tubes, whereas in the *suc1-1* homozygous mutants hardly any had produced pollen tubes (figure 3.13).

3.2.4. Phenotypic analysis of the *suc2* mutants

Detailed analysis for *AtSUC2* was mainly performed with *suc2-4* plants; however, *suc2-5* mutants grown under the same conditions, although not analysed in detail, displayed a similar phenotype to the *suc2-4* mutants. Examples of the mutants shown in figure 3.14 show that the *suc2-4* homozygotes were extremely stunted and retarded in their development and either did not flower or produced very few flowers. There was quite a variation in their development; some plants completed their growth cycle, although they grew at a slower rate, while other plants died before bolting or produce strange rosette shapes (figure 3.14). In the *suc2-4* homozygotes, dark red pigmentation (indicative of anthocyanin formation) occurred in the older leaves, first at the tip and then spreading to the rest of the leaf (results not shown). When analysed at 11 weeks in a batch of randomly selected WT and mutant plants grown under similar conditions (figure 3.14) *suc2-4* heterozygotes showed no significant differences to wild-type plants in terms of plant height, mean rosette leaf length, mean number of leaves per plant or total number of flowers indicating that this mutant is recessive. However, for all of these parameters, the *suc2-4* homozygotes significantly differed from the WT and heterozygous plants. Plant height, mean leaf length and mean number of leaves per plant, were all

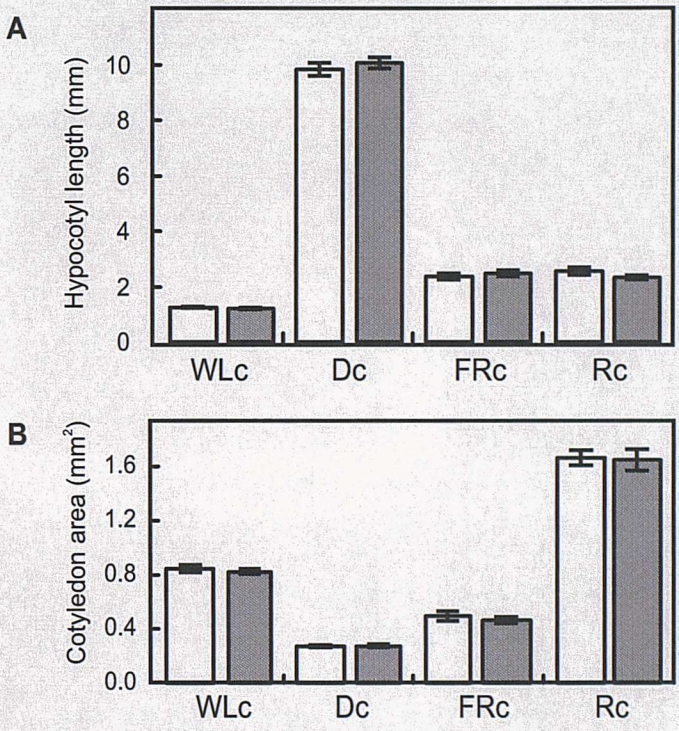


Figure 3.12. Analysis of seedling development of *suc1-1* seedlings grown in specific light treatment. Subsequent to stratification on MS media at 4°C for 48 h in darkness *Arabidopsis* seeds were given a 1hr white light (WL) treatment to initiate germination, then grown in complete darkness for 48 h before exposure to specific light treatments continuous white light (WLc), darkness (Dc), far-red light (FRc) and red light (Rc) for 48 h to investigate the affect of *suc1-1* has on the de-etiolation of seedlings. Previously, At*SUC1* was shown to up-regulated during de-etiolation under FR light (Tepperman *et al.*, 2001). The hypocotyl length (A) and cotyledon area (B) of *suc1-1* hom (grey bars) and WT (white bars) seedlings were measured from digital images using ImageJ software. Bars show the mean ±SE (*n*=25).

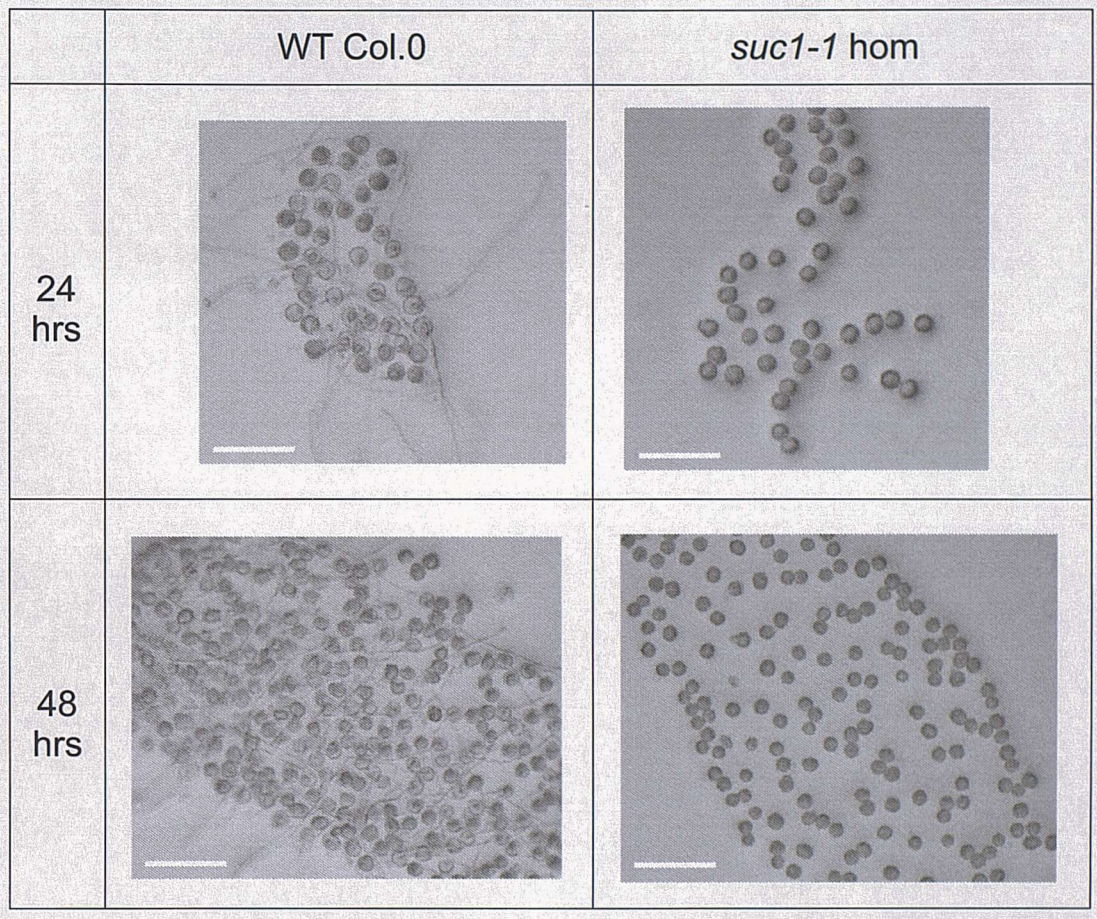


Figure 3.13. Pollen physiology of the *suc1-1* homozygote. Flowers from wild-type plants and *suc1-1* homozygous mutants at similar development stage were taken and carefully dipped into the surface of agar plates to transfer the mature pollen grains. *In vitro* germination and tube growth were assessed after incubating pollen in a modified medium (Hülskamp et al., 1995) containing 2 % sucrose (w/v), 5mM calcium chloride, 1.3 mM boric acid and 55mM myo-inositol at pH 5.9. The pollen was allowed to germinate in a humid chamber at 22°C and observed after 48 h with a Nikon E 800 Eclipse microscope. Bar = 100 µm. Results courtesy of Ms. Alessandra Francini (Francini and Williams, unpublished).

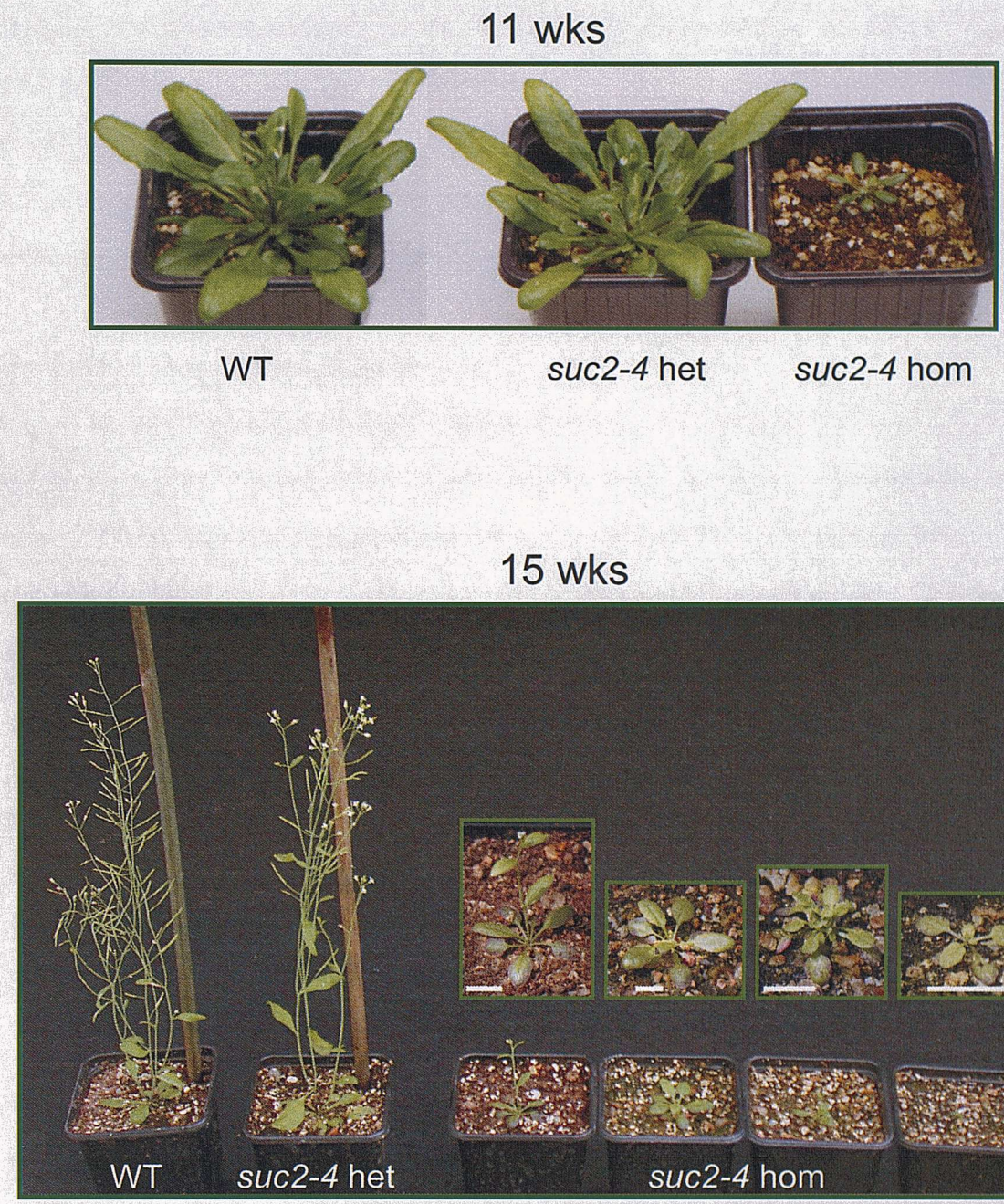


Figure 3.14. Phenotype of *suc2-4* mutants at different developmental stages. *suc2-4* and wild-type (WT) plants grown on soil under glasshouse conditions. Upper panel: 11-week old suc2-4 and wild-typet plants. Lower panel: 15-week old *suc2-4* and wild-type plants. Bar = 2 cm.

markedly reduced and none of the homozygous plants sampled at this stage had produced flowers. Detailed analysis of the root systems was not carried out on the soil-grown plants but, from visual inspection, the root systems of the *suc2-4* homozygotes were much smaller.

After 15 weeks, again there was little difference in the majority of the heterozygous *suc2-4* compared to WT with both genotypes having lost leaves and flowers (figure 3.14). It should be noted that the data shown in figure 3.10B,C,D is for three of the twelve heterozygous plants that retained some leaves and flowers. In contrast, the *suc2-4* homozygotes that survived (a proportion had died of before bolting) were still stunted and only a few had produced flowers. In those that had produced siliques, these had not matured fully and the silique size was thus significantly smaller.

3.2.5. Silique and seed characteristics

In the latter stages of development, WT plants should be investing most of their resources in reproductive sinks and therefore we investigated whether the characteristics of the siliques and seeds differed in WT plants and the sucrose-transporter mutants when each had reached maturity and the majority of the siliques had ripened. The *suc2-4* homozygotes were much older but it was important to measure the silique and seed characteristics at a time when they had matured in all plants.

The *suc2-4* homozygotes were still stunted compared to WT and heterozygous plants (results not shown). Although they were not counted, it was obvious that the number of siliques produced per plant by the *suc2-4* homozygotes was markedly lower than WT, *suc1-1* homozygotes and *suc2-4* heterozygotes. The siliques of those that did mature were significantly shorter than WT (Figure 3.10E). The seed number per silique was also reduced significantly (Figure 3.15C). Inspection of the seeds produced by *suc2-4* homozygous mutants revealed that their morphology was much different to both WT and *suc2-4* heterozygous seeds (figure 3.15D). Not only were *suc2-4* homozygote seeds significantly smaller in both their width and length (figure 3.15A,B), some also appeared slightly transparent when viewed under a light microscope (figure 3.15D). Also seen in figure 3.15B are the characteristics of siliques and seeds observed for the *suc1-1* mutants; there was a slight increase observed in seed length for both the SUC1/*suc1* and *suc1/suc1* mutants compared to WT.

The viability of the seed obtained from the mutants was compared to WT since it had previously been reported that *suc2* homozygote seed was non-viable (Gottwald *et al.*, 2000). Seed was germinated on plates with and without sucrose (2% w/v) and the percentage of healthy seedlings after 7 days was recorded (figure 3.16). The percentage of healthy *suc1-1* homozygotes after 7 days was very similar to the results seen for WT. Seed germination data for both WT and *suc1-1*

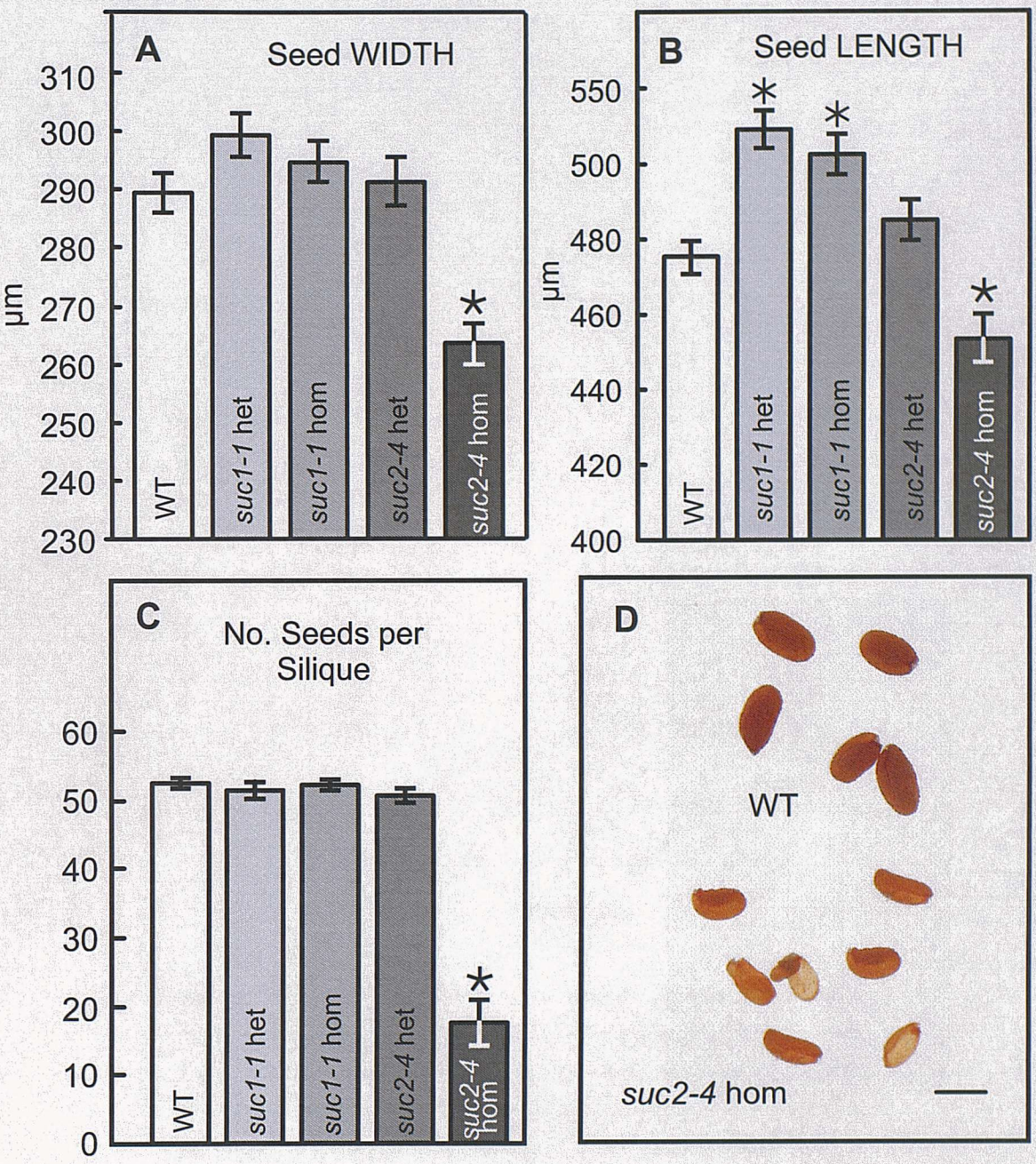


Figure 3.15. Silique and seed characteristics of *suc1-1* and *suc2-4* mutants. The data were collected from plants on which the majority of siliques had ripened. Seed width and length; each bar represents the mean (± SE) of 120-168 samples from at least 5 different plants. In all figures the *suc2-4* homozygotes, marked by an asterisk, were significantly different from both the wild-type and *suc2-4* heterozygotes (∗, $P \leq 0.05$; ∗, $P \leq 0.01$) as determined using a two-tailed t-test. Bar = 500 µm.

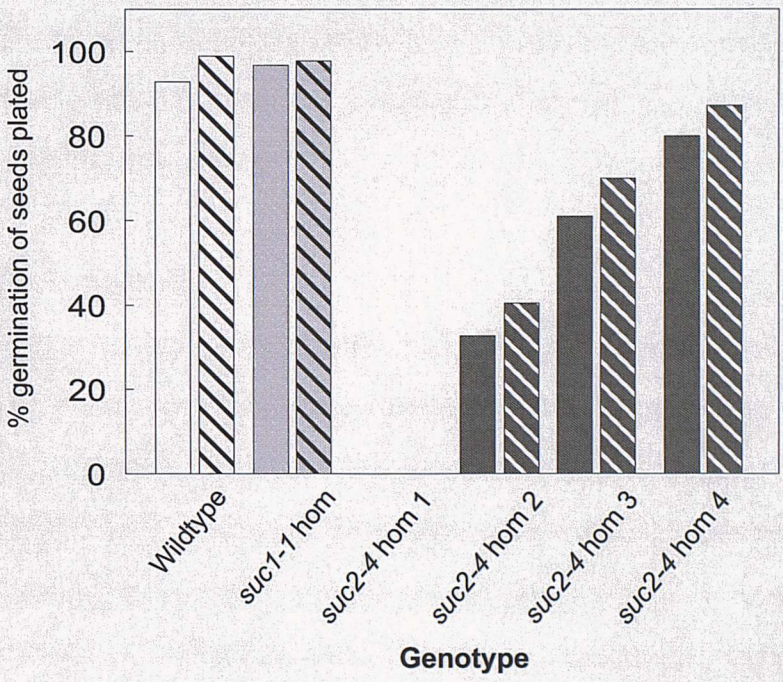


Figure 3.16. Viability of *suc1-1* and *suc2-4* mutant seeds. Seed viability of *suc1-1* (*n* = 4) and *suc2-4* homozygous mutants (results from individual plates) compared to wild-type (WT, *n* = 6) in the absence (plain bars) and presence (crossed-hatched bars) of 2% (w/v) sucrose. All seed except the WT were germinated in the presence of PPT. The seed was determined to be viable if healthy (non-chlorotic) seedlings were observed 7 d after plating. The number of healthy seedlings is shown as a percentage of the seed plated.

homozygotes are shown for one representative plant in this figure and germination rates were always high for seed from these plants. Also shown are results for *suc2-4* homozygotes and because the viability varied remarkably for seed from individual plants, the results are shown for seed from four different *suc2-4* homozygotes. The amount of seed collected per plant was much lower for *suc2-4* homozygotes compared to WT and *suc1-1* homozygotes. The percentage of healthy seedlings at day 7 varied from 0-88% in these individual plants. The seedlings were smaller than WT but they were healthy (non-chlorotic). The presence of sucrose resulted in a slight increase in the number of healthy seedlings after 7 days but it did not rescue the phenotype completely as reported by Gottwald *et al.* (2000). Some seedlings 8 out of 15 in one experiment (53%) were also able to complete their life cycle and produce some seed and so the *suc2-4* mutants are not sterile as previously reported for other *suc2* alleles (Gottwald *et al.*, 2000).

3.2.6. Isolation of SAIL mutants.

To continue the work shown in this chapter various databases produced from different *Arabidopsis* insertional mutagenesis projects were searched to identify further sugar transporter mutants. Searches of a collection of T-DNA mutants produced by the biotechnology company called Syngenta were particularly fruitful. The **S**yngenta ***A**rabidopsis* **I**nsertional **L**ibrary (SAIL formerly called GARLIC) mutants are an insertion collection which has been generated from approximately 100,000 individual T-DNA mutagenized *Arabidopsis* plants (Columbia ecotype) (Sessions *et al.*, 2002). The database is formatted to search for insertions in genes of interest by BLAST searches or gene MIPS code (currently at http://signal.salk.edu/cgi-bin/tdnaexpress). Using this facility nine SAIL mutants were discovered that were of interest; five invertase enzymes (*fruct1, fruct2, fruct5, fruct6, fruct8*), a hexose-proton transporter (*stp3*) and three sucrose-proton transporters (*suc2, suc4, suc5*) (results not shown).

Initially, homozygous mutants needed to be isolated and with this in mind an experiment was set up to study the segregation of PPT resistance to ascertain the present genotype. The T-DNA construct confers resistance to PPT therefore using Mendalian genetics described previously, if all seeds of a plant show resistance to PPT it is a good indication that the parent plant is homozygous; if 75% of the seed show resistance it suggests that the parent if heterozygous. The results of the PPT resistance segregation show that the SAIL mutant *fruct6* was supplied as a homozygous line while all other SAIL mutants were supplied as heterozygous lines (table 3.1). Theoretically the heterozygous SAIL mutants should produce a ratio of 2 heterozygous mutants to every 1 homozygous mutants,

Table 3.1. Identification of homozygous lines from SAIL (formerly GARLIC) mutant lines by PPT resistance segregation. The germination and growth of seeds from F_1 plants were studied. F_1 plants were produced from selfing the seed obtained from Syngenta (Sessions *et al.*, 2002). Between 10-18 F_1 plants were studied for each mutant line with 50-100 seeds per plate and two plates per F_1 plant. F_1 plants were considered to be homozygous if all F_2 seedlings showed resistance to the herbicide PPT, or heterozygous if 25% were arrested by PPT. The FRUCT genes encode invertase enzymes, STP3 is a hexose-proton symporter and the SUCs are sucrose-proton symporters.

Interrupted Gene	Number of F_1 SAIL Lines Isolated	
	Heterozygous	Homozygous
fruct1	6	3
fruct2	4	6
fruct5	9	1
fruct6	0	10
fruct8	4	6
stp3	6	4
suc2	11	5
suc4	3	5
suc5	6	4

however, since only 10 plants were sampled for each SAIL line (18 for *suc2)* much more variance is observed (table 3.1). This experiment has isolated homozygous lines for each SAIL mutant and in most cases has produced many homozygous lines. To confirm these lines are homozygous strategic PCR using primers designed to the gene and T-DNA with gDNA from the plant is required (described previously). Unfortunately this work could not be completed before the writing of this thesis.

During the PPT resistance studies it was noticed that the seedlings of three of the lines, *fruct6, suc2* and *suc5* were very small in comparison to the WT controls and appeared to show to a pale/yellow colouration in the cotyledon (data not shown). The *suc2* mutants also showed a purple pigmentation in the cotyledons similar to that seen with the SLAT mutant, *suc2-4,* described previously.

3.3. Discussion.

Results presented in this chapter are the first describing the characterisation of a mutant allele of the *AtSUC1* gene (*suc1-1*). A detailed phenotypic analysis of *suc2-4* mutants was also carried out as part of this project and the results are discussed in relation to the phenotypic characteristics reported for other *suc2* mutant alleles (*suc2-1*, *suc2-2* and *suc2-3*) generated by T-DNA insertional mutagenesis (Gottwald *et al.*, 2000).

3.3.1. Disrupting *AtSUC1* does not have a dramatic effect on vegetative growth and development but does have a major effect on *in vitro* pollen tube growth

Disruption of the gene encoding the sucrose transporter, *AtSUC1* has no major phenotypic effect on general growth and development (under the experimental conditions used in this project) even though *AtSUC1* is expressed in all parts of the plant (figure 3.8). It is not unheard of to find *Arabidopsis*-insertion mutants that fail to display any morphological phenotypes (Krysan *et al.*, 1996). For example, an insertion mutant of AtSTP1 the monosaccharide transporter gene, results in no obvious growth or morphological phenotype under greenhouse conditions (Sherson *et al.*, 2000). Functional redundancy may be an explanation for many of these cases as *Arabidopsis* contains a large number of multigene families. Indeed, *AtSUC1* is a member of a gene family comprising nine members (see phylogenetic analysis figure 5.1; The *Arabidopsis* Genome Initiative 2000). For such genes where redundancy is a possibility, future work may require the analysis of double, triple or even multiple gene knockout mutants. The isolation of the *suc1-1* mutant in this study is a useful starting point for such studies and possibly incorporating the SAIL *suc5* mutant might help discover the roles of AtSUC1 and AtSUC5.

Under short day conditions, a sight delay in the time to flowering was observed in the *Atsuc1* homozygous mutants; however, as a delay was also observed in the heterozygotes, the significance of this finding is not clear. In addition, a slightly longer seed was produced in the *Atsuc1* homozygotes, but again this was observed heterozygotes.

A major difference was observed in the *in vitro* pollen tube growth of *suc1-1* homozygotes compared to WT (Francini and Williams, unpublished). Although wild-type pollen germinated and produced pollen tubes when grown on sucrose, very few pollen tubes were observed in pollen from *suc1-1* homozygotes. This is the major difference observed in *suc1-1* knockout mutants and suggests that in this situation other sucrose carriers or alternative mechanism cannot compensate for the loss of *AtSUC1*. However the lack of pollen tubes produced by *suc1-1* pollen *in vitro*, may not reflect the *in*

vivo situation. Indeed no major differences were found in the amount of seed per silique in the *suc1-1* mutants compared to wild-type plants nor in the viability of the seed produced. Further work is required to determine how the pollen from these mutants performs during the fertilisation process.

The reason that no major phenotypic difference was observed during vegetative growth of the *suc1-1* mutants even though AtSUC1 is expressed widely could be due to functional redundancy, as suggested above, and it would be interesting to test whether other sucrose transporters show a higher expression in the *suc1-1* mutants. Alternatively, *AtSUC1* may play a much more subtle role that has not been highlighted in this particular analysis. For example the hexose transporter, *AtSTP4*, is considerably up-regulated in response to tissue damage and pathogen invasion (Truernit *et al.*, 1996).

The discrepancy in the tissue-specific expression pattern of *AtSUC1* shown in the present study and that reported by Stadler *et al.* (1999) is not clear. It could reflect a difference in the growth of the plant material but this seems unlikely. Interestingly, a previous report from that lab (Sauer and Stoltz, 1994) used northern analysis to show that *AtSUC1* transcripts were present in young and mature leaves, roots, with reduced levels in flowers and very low levels in stems. However, in the subsequent article from that lab which used RNase protection was used to demonstrate *AtSUC1* expression only in flowers, Stadler *et al.* (1999) stated that the previous results were not valid as the probes used in that study cross-reacted. The tissue expression pattern for *AtSUC5* shown in the present study (expressed only in flower and siliques) also contradicts the previous study showing expression in all tissues (Ludwig *et al.*, 2000). Although gene-specific primers for *AtSUC5* were used in the present study, the product should ideally be sequenced to ensure that it is indeed *AtSUC5*. The same applies for the product amplified from WT tissue with the *AtSUC1* primers, although the fact these primers do not amplify a product in *suc1* homozygotes where expression is knocked out is evidence supporting the amplification of the correct product.

Investigation of the supplementary material from Tepperman *et al.* (2001) revealed that *AtSUC1* appears to be late-induced (3-24 hrs) under the control of PHYA by far-red light. Unlike the microarray used in this thesis that utilizes the full coding sequences of genes as an oligo probe, the Affymetrix microarrays uses oligonucleotide probes that are 25 base pairs long. Thus, maybe the hybridisation of cDNAs to oligos on the Affymetrix might not be as accurate comprising the validity of the results especially with transporters such as *AtSUC1* and *AtSUC5* that have similar coding sequences. Details of the microarray construction and probe sequences used by Tepperman *et al.* (2001) were not given. No phenotype of *suc1-1* mutants were found when grown in far-red light

(figure 3.12), however, it could be that AtSUC1 is only transiently required and/or that other sucrose transporters are able to compensate. Similar light specific experiments with the *AtSUC5* SAIL knockout mutant would be the next step in exploring the finding for *AtSUC1* by Tepperman *et al.* (2001).

3.3.2. The Role of AtSUC2

Disruption of AtSUC2 *has a dramatic effect on phenotype*

Truernit and Sauer (1995) provided evidence for the role of AtSUC2 in phloem loading and unloading using *Arabidopsis* plants transformed with constructs of the A*tSUC2*-promotor linked to the β–glucuronidase (GUS) reporter gene. Following this, AtSUC2 was immunolocalised to the companion cells of the phloem (Stadler and Sauer, 1996). The work in this chapter is consistent with a proposed role for AtSUC2 in phloem loading and resource allocation (Gottwald *et al.*, 2000) and confirms its importance and unique function.

In contrast to *AtSUC1*, disruption of *AtSUC2* has a dramatic effect on phenotype. The *suc2-4* homozygous plants were severely retarded in their growth; they were significantly smaller in height, had fewer and smaller leaves and either did not flower or produced a reduced number of flowers. This phenotype is consistent with a plant that cannot move sucrose efficiently from its source leaves to sink tissues. This also is an indication that sucrose transport in *Arabidopsis* is predominately apoplastic and that a symplastic pathway is not able to compensate for this apoplastic route. These results are in broad agreement with those of Gottwald *et al.* (2000) for other *suc2* mutant alleles and thus are consistent with AtSUC2 playing a central role in phloem loading and assimilate partitioning.

Constitutive antisense repression of the sucrose transporter, *StSUT1* in *Solanum* resulted in general growth retardation together with sugar and starch accumulation in source leaves and the occurrence of lipid-storing oleosomes, results that are consistent with an important role of this transporter in phloem loading (Riesmeier *et al.*, 1994; Schulz *et al.*, 1998). The *suc2* homozygotes could now be used to investigate whether similar ultrastructural changes occur in *Arabidopsis* to those seen in *Solanum.* Interestingly, in *Solanum*, StSUT1 has been localised to the plasma membrane of sieve elements (Kuhn *et al.*, 1997) whereas in *Plantago* and *Arabidopsis*, localisation was reported in the companion cells for PmSUC2 and AtSUC2 respectively (Stadler *et al.*, 1995; Stadler and Sauer 1996). The functional significance of this is still not apparent. However, it is clear that reducing or preventing the expression of these genes has a dramatic effect on the development of the plant.

The purple pigmentation in the rosette leaves can be explained by the stress response of anthocyanin accumulation, an indication of the rosette source leaves struggling to cope with excess levels of sucrose that cannot be loaded into the phloem. Similar results were also observed in the *suc2* homozygotes obtained by Gottwald *et al.* (2000). The appearance of dark red pigments (indicative of anthocyanin formation) occurred in the older leaves, first at the tip and then spreading to the rest of the leaf. This is similar to that described in plants over-expressing cell wall invertase where presumably again there would be decreased sucrose export (von Schaewen *et al.*, 1990).

Many of the *suc2-4* homozygotes died before producing siliques but, when produced, they were generally smaller in length and contained fewer and smaller seeds. Although the results of the present study are in broad agreement with Gottwald *et al.* (2000), they differ in two important respects. The most important concerns seed viability. The seed obtained from some *suc2-4* homozygotes in this study is viable (although this varies with individual plants) whereas the previously isolated *suc2* mutants did not yield viable seed (Gottwald *et al.*, 2000) even when the plants were given supplementary sucrose. The reason for this difference is not clear but it may be due to different growth conditions. It is possible that under the growth conditions used in this project, sufficient resources were allocated in certain parent plants to produce viable seed, even though they were significantly smaller. In addition, the *suc2* mutants isolated in this study showed retarded growth at an early developmental stage even when supplementary sucrose was given to the seedlings. The *suc2-4* homozygotes (this study) were clearly smaller than the heterozygotes and WT whereas the stunted phenotype of *suc2-1* homozygous seedlings isolated previously was completely rescued by supplementary sucrose (Gottwald *et al.*, 2000). Again, this may be due to differences in growth conditions in the two studies but it would be necessary to compare mutants side-by-side in order to test whether there are any subtle phenotypic differences. It would also be valuable to extend the analysis of the *suc2-4* mutants. Microscopy of rosette leaf sections in the *suc2-1* homozygous mutants obtained by Gottwald *et al.* (2000) revealed that they contain very large starch deposits in the chloroplast suggesting a feedback effect of the build up of sucrose (Gottwald *et al.*, 2000). Furthermore, the lack of sucrose translocation in these *suc2-1* homozygous mutants was demonstrated by tracing ^{14}C-labelled sucrose fed to source leaves (Gottwald *et al.*, 2000). In contrast to the wildtype plants where most ^{14}C-labelled sucrose had been transported to the inflorescences, the *suc2-1* homozygous mutants contained the majority of ^{14}C-labelled sucrose within the rosette leaf that was initially fed the ^{14}C-labelled sucrose. A small proportion of ^{14}C-label did reach other rosette leaves and some inflorescences raising the question of how this is accomplished in the absence of

AtSUC2. One possibility proposed by Gottwald *et al.* (2000) was that under these extreme circumstances sucrose would be broken down by invertase and the hexose products transported across the membranes by hexose transporters (although at a relatively inefficient rate). Alternatively, another sucrose transporter could be induced to compensate for the absence of AtSUC2 (Gottwald *et al.*, 2000).

Differences in the phenotype of the *suc2-4* phenotype and the *suc2* mutants described by Gottwald *et al.* (2000) may be explained by the growth conditions but also may be a consequence of the different genetic backgrounds of the two mutant lines. The four mutant lines described in this chapter all contain a Landsberg *erecta* (L*er*) genetic background (Tissier *et al.*, 1999) while the mutant lines described by Gottwald *et al.* (2000) were all of the ecotype Wassilewskija (WS). It is also worth noting that the two insertional mutant lines were produced very differently. The mutant lines described in this chapter were isolated from population of transposon insertions while the mutants described by Gottwald *et al.* (2000) were produced using a insertion of transfer DNA (T-DNA) which is a segment of *Agrobacterium tumefaciens* plasmid. However, since both systems insert a long sequence of foreign DNA that contains a stop codon it is doubtfui if any variations between the mutants described in this chapter and by Gottwald *et al.* (2000) are due to the type of insertional mutagenesis.

3.3.3. SAIL Mutants.

It is hoped that the work underway to characterise the recently acquired SAIL mutants will lead to interesting results in future work. The flowering and silique development of the *suc5* mutant would be interesting to study as results shown in this chapter indicate that SUC5 expression in the mature plant are exclusive to these areas (figure 3.8). Even though expression of SUC1 appears throughout the plant (most strongly in leaf, stem and flowers, less so in root and silique; figure 3.8) a phenotype is only seen in its time to flowering (figure 3.11) and pollen development (figure 3.13). The range of different genes enables double mutants to be isolated from selective crossing. Since SUC1 and SUC5 seem to be connected not only in their flowers and silique tissue expression but in their close proximity within the phylogenetic analysis (figure 5.1) double mutant plants might further enable their roles *in vivo* to be discovered. Likewise, SUC2 and SUC4 are both sucrose-proton transporters found to be located in the companion cells of sieve elements (along with SUC3 Meyer *et al.*, 2000). Unlike SUC2, SUC4 is expressed only in the loading zone (minor veins) of source leaves (SUC2, Stadler and Sauer, 1996; SUC4, Weise *et al.*, 2000). The *suc2-4* mutant shown in this chapter

demonstrates the importance of SUC2 in the translocation of sucrose to fuel the growth and development of the plant (figure 3.14). Such a dramatic phenotype might suggest that phloem loading relies entirely on SUC2; however SUC3 and SUC4 also occur in the phloem and their roles need to be clarified. The location of SUC4 in the phloem of minor veins of source leaves and high capacity transport kinetic indicate a role in phloem loading; evertheless no mutants in SUC4 have been published at this time (Weise *et al.,* 2000). The work by Weise *et al.* (2000) would aid in designing studies to accurately characterise the phenotype of the *suc4* mutant, the results of which would advance the understanding of the role of SUC4 *in vivo*. Unfortunately at this time the SAIL collection did not contain a SUC3 insertional mutant. Then again, the possibilities of obtaining double or triple mutants incorporating the *suc2-4* mutant would be hampered by the fragile nature of *suc2-4* mutants (3.16). Also with only *SUC2* knocked-out the viability of the seed is unreliable, those seeds/seedlings that are viable may rely heavily on SUC3 and SUC4 thus double mutants may not be viable.

3.3.4. Conclusions

In summary, AtSUC1 and AtSUC2 clearly make markedly different contributions to growth and development in *Arabidopsis* with disruption of *AtSUC2* resulting in a much more severe effect on phenotype. Previous research had strongly suggested that AtSUC2 was involved in the phloem loading of sucrose at source leaves, retrieval of sucrose in its journey to sink tissue and possibly in phloem unloading in sink tissues. However, when this project was started the particular importance of this sucrose transporter in the growth and development of *Arabidopsis* was not known. The results obtained in this project, studying the *suc2-4* mutants, and the study by Gottwald *et al.* (2000) characterizing the other *suc2* mutant alleles isolated during the course of this project indicate that AtSUC2 is particularly important in growth and development of *Arabidopsis*. The dwarf and retarded phenotype of the *suc2* knockouts suggest that without AtSUC2, sucrose cannot adequately reach the sink tissues resulting in a deficiency of carbohydrates to fuel growth and to invest in seed production. AtSUC1 appears far less important in the growth and development of *Arabidopsis* possibly due to functional redundancy or it may have a more subtle role not revealed by the analysis carried out in this project. There was possibly a slight delay in the time to flowering (also seen with heterozygotes) but the major effect appeared to be on *in vitro* pollen tube growth, which was markedly reduced in the mutant (Francini and Williams, unpublished). However the lack of pollen tubes produced by *suc1-1* pollen *in vitro*, may not reflect the *in vivo* situation. Indeed no major differences were found in this study in the amount of seed per silique in the *suc1-1* mutants compared to wild-type plants nor in the

viability of the seed produced. Further work is required to determine how the pollen from these mutants performs during the fertilisation process.

As AtSUC2 appears to have a major role in phloem loading and thus carbon partitioning, the regulation of this gene was investigated further and results are described in the next chapter.

Chapter 4.

Light and sugar regulation of *AtSTP4* and *AtSUC2*

4.1. Introduction

Knowing the tissue and cellular location of individual sugar transporters and understanding their regulation helps deduce their physiological role. Plant sugar transporters could potentially be regulated by several methods including the rate of transcription; rate of translation; protein modification (covalent or allosteric modification, phosphorylation, methylation); regulation of the driving force (e.g. by regulation of the proton-motive force generated by the H^+-ATPase (Hellmann *et al.*, 2000). The tissue localization of a small proportion of sugar transporters has been determined using immunolocalisation, *in situ* hybridisation, and reporter gene technology (AtSTP1, Sherson *et al.*, 2000; AtSTP3, Büttner *et al.*, 2000; AtSTP4, Truernit *et al.*, 1996; AtSUC1, Stadler *et al.*, 1999; AtSUC2, Stadler and Sauer, 1996; AtSUC3/SUT2, Barker *et al.*, 2000/Mayer *et al.*, 2000; AtSUT4, Weise *et al.*, 2000). In the present study, the β-glucuronidase (GUS) reporter system (Jefferson, 1989) has been used to study the tissue distribution and regulation of two particular sugar transporters, AtSTP4 and AtSUC2 (Truernit and Sauer 1995, Truernit *et al.*, 1996), under various light and sugar treatments. These two sugar transporters are ideal subjects for this investigation as they transport different sugars and are expressed in different tissues, as described later.

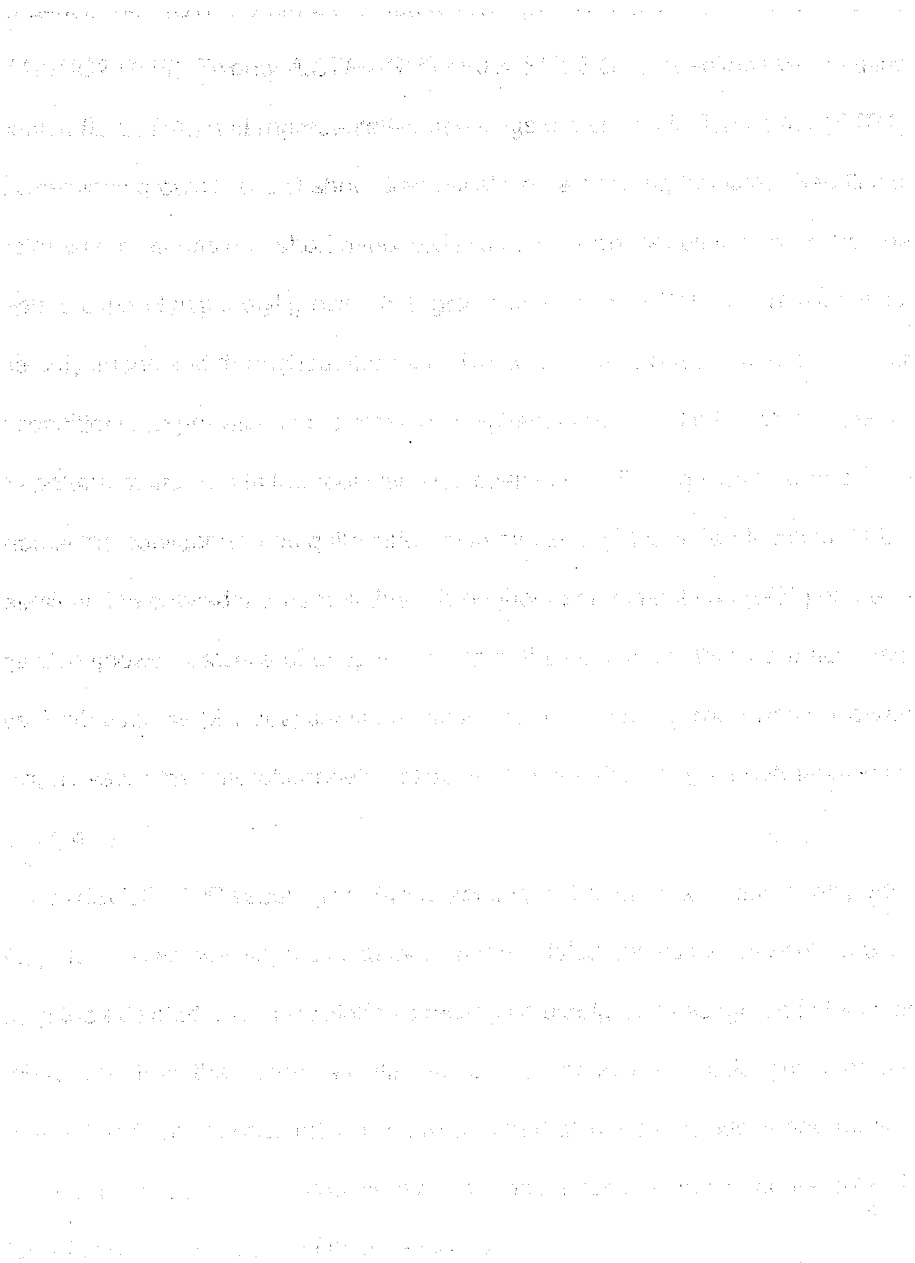
The GUS-encoding gene (*uid*A) originates from the bacterium, *Escherichia coli,* and has been developed to be an effective tool to study gene expression in plants (Jefferson *et al.*, 1987b). For such analysis the promoter of the gene of interest is fused to the coding sequence of the *GUS* gene and plants are transformed with the construct. This allows gene expression to be studied without affecting the wild-type genes and thus the growth and development of the plant. The GUS protein is a hydrolytic enzyme (EC 3.2.1.31) that catalyses the cleavage of a wide range of β-glucuronide substrates. Many plants contain no detectable β-glucuronides thus providing a null background (Jefferson *et al.*, 1987a). The enzyme is very stable within cells and extracts, and is tolerant to many detergents, ionic conditions, thiol-reducing reagents (e.g. β-mercaptoethanol) and temperatures (Jefferson, 1989). The wide range of substrates enables both histochemical localisation and quantification assays to be performed. In the histochemical assay, the detection of GUS activity occurs in two steps. In the first step, GUS catalyses the hydrolysis of the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and liberates one molecule of 5-bromo-4-chloro-indoxyl (indoxyl ester)

that is not coloured; the indoxyl then undergoes an oxidative dimerization producing a blue coloration. For quantification of GUS activity, a fluorimetric assay can be used that is highly sensitive. The substrate, 4-methyl umbelliferyl ß-D-glucuronide (4-MUG), is broken down by the GUS enzyme to produce D-glucuronic acid and 4-methylumbelliferone (4-MU). The latter can be measured fluorimetrically with excitation at 365 nm and emission at 455 nm (Jefferson, 1989). The rate of accumulation of 4-MU is linearly related to the concentration of GUS enzyme while substrate is in excess and thus can be used to quantify the level of transcription. The assay has the advantages of a high signal-to-noise ratio and is extremely sensitive due to amplification of the enzymatic reaction. Both procedures (histochemical and quantification assays) are comparatively inexpensive and rapid procedures that require only a few pieces of specialized equipment.

In this study, two transgenic plant lines have been used that contain a construct fusing the promoter of either *AtSTP4* or *AtSUC2 (*Truernit and Sauer 1995, Truernit *et al.*, 1996*)* to the *GUS* gene. *AtSTP4* and *AtSUC2* are two sugar transporter genes reported to have very different functions. *AtSTP4* encodes a hexose transporter, most abundant in the roots and flowers of *Arabidopsis* with reduced levels in the leaves, and lower levels still in the stems and fruits (Truernit *et al.*, 1996). Wounding induces *AtSTP4* transcription, as does pathogen infection (Truernit *et al.*, 1996; Fotopoulos *et al.*, 2003). Thus, *AtSTP4* is located in specific tissues and would seem to be a highly responsive sugar transporter. AtSUC2 is a sucrose transporter found throughout the vascular system of *Arabidopsis* (Truernit and Sauer, 1995). AtSUC2 has been localised to the companion cells of the phloem and is found in all green tissues such as rosette leaf, stems, sepals and roots (Stadler and Sauer, 1996). Therefore, it has been suggested that it has a role in sucrose loading of the phloem for transport to sink tissues and possibly participates unloading (Truernit and Sauer, 1995). The role in loading is consistent with studies on plants in which *AtSUC2* has been knocked out producing plants which are stunted and retarded in their development (figure 3.14; Gottwald *et al.*, 2000). The functional significance of AtSUC2 in sink tissues has yet to be elucidated.

In order to understand the function of *AtSTP4* and *AtSUC2* more fully, this chapter investigates the regulation of these two transporter genes by light and sugars. Growing seedlings in different light conditions provides a way of manipulating the source/sink relationship of the different tissues. Similarly, supplementing the growth media with sugars affects the demand of sink tissues such as roots for sugars synthesized or stored within the cotyledons and permit the investigation of whether the sugar transporters are responsive to sugar status. Both histochemical assays and

quantitative fluorometic assays were used to investigate the effect of light and sugars on *AtSTP4* and *AtSUC2* transcription in seedlings.



4.2. Results

4.2.1. The effect of light on the pattern of *AtSTP4* and *AtSUC2* expression

The seedlings used in this study were grown under three different light conditions: light for 7 days (L), dark for 7 days (D) and finally dark for 6 days followed by light for 1 day (D-L). The seedlings either contained the *AtSTP4* promoter linked to GUS (*AtSTP4-GUS*) or the *AtSUC2* promoter linked to GUS (*AtSUC2-GUS*). Twenty *AtSTP4-GUS* and *AtSUC2-GUS* seedlings were stained from each light condition (L, D, D-L) and representative seedlings are shown in figures 4.1 (STP4) and 4.2 (SUC2). Seedlings grown L and D show dramatically different morphologies. Seedlings grown L have fully developed cotyledons and short hypocotyls while D-grown seedlings divert the energy reserves of the seed to expand hypocotyl growth. In L-grown seedlings, *AtSTP4* expression was seen at the tips of the cotyledons and throughout the roots. No staining was observed in the hypocotyls in any of the light conditions. Expression in the roots was highest at the root tip but also strong at the top of the root. This pattern of staining in the root was also seen in seedlings grown in D and D-L conditions. Expression in the cotyledons was quite different in etiolated (D) and de-etiolating (D-L) seedlings. In these seedlings the cotyledons were stained throughout and almost half (9/20) of the de-etiolating seedlings also showed patches of deeper staining in the cotyledons that were not seen in etiolated seedlings. With sucrose (2% w/v) supplementation an increase in growth was observed in all light conditions, however their morphogenetic response to the different light treatments was similar (figures 4.1, 4.2, 4.6, 4.8).

The *AtSUC2-GUS* seedlings showed staining in the vascular tissue of all parts of the seedling (figure 4.2). In L-grown seedlings, the staining in the cotyledons was visible only in the vascular tissue however, in the etiolated and de-etiolating seedlings a blue/green background stain was seen, but this was much weaker than that in the vascular tissue. Staining in the vascular tissue of the hypocotyl in etiolated and de-etiolated seedlings was weaker than that in the cotyledons and roots. The addition of sucrose in the media seemed to produce a more pronounced staining in the vascular tissue of the hypocotyls of etiolated and de-etiolating seedlings.

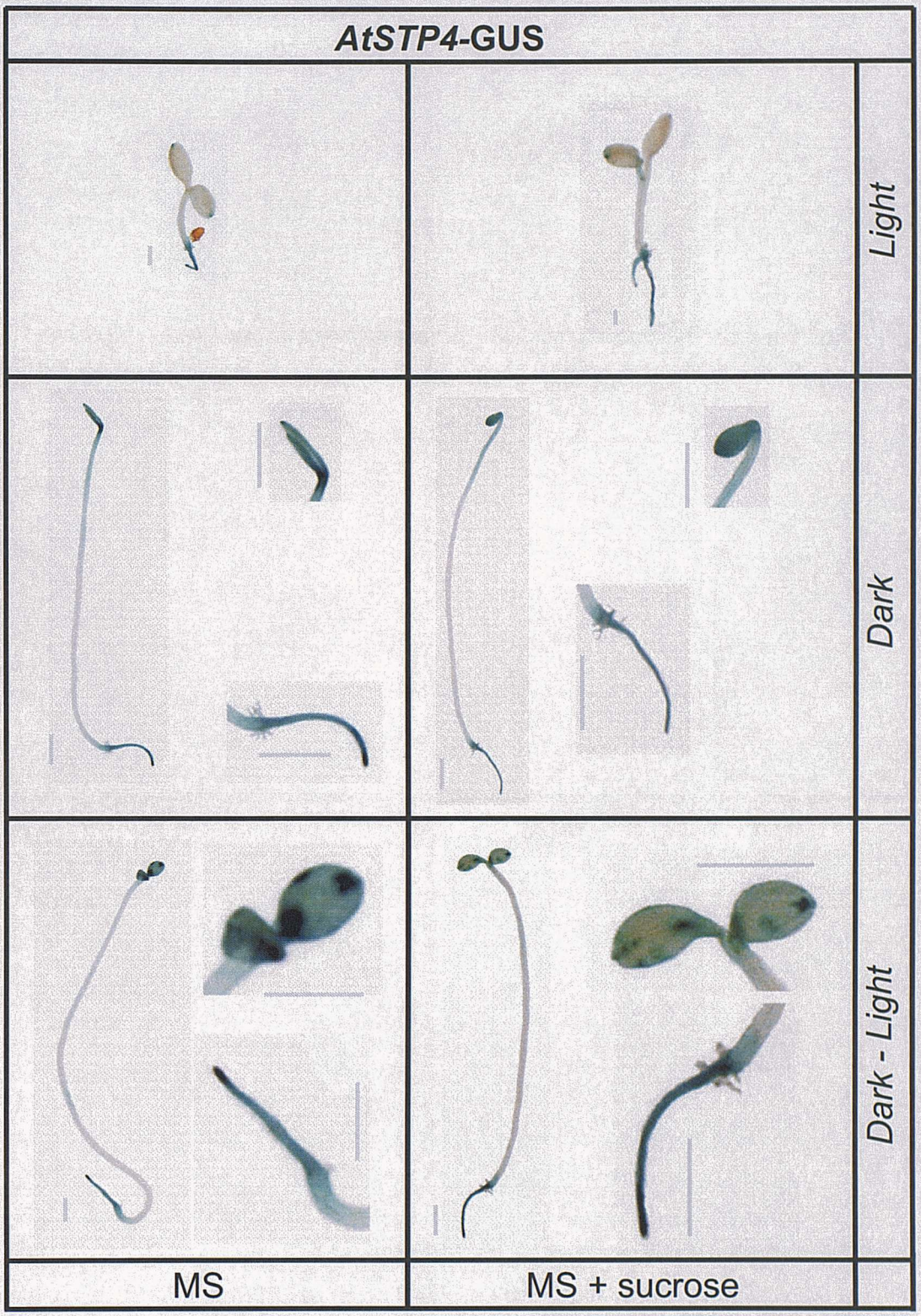


Figure 4.1. Histochemical staining of *AtSTP4*-GUS seedlings grown under different light conditions. Pictures show representative seedlings stained for GUS driven by the *AtSTP4* promoter. Seeds were germinated on MS medium (± 2% w/v sucrose) and grown in either light (7 d), dark (7 d) or dark (6 d)-light (1 d). Twenty seedlings were examined for each condition and representative samples are shown. Bars = 1 mm.

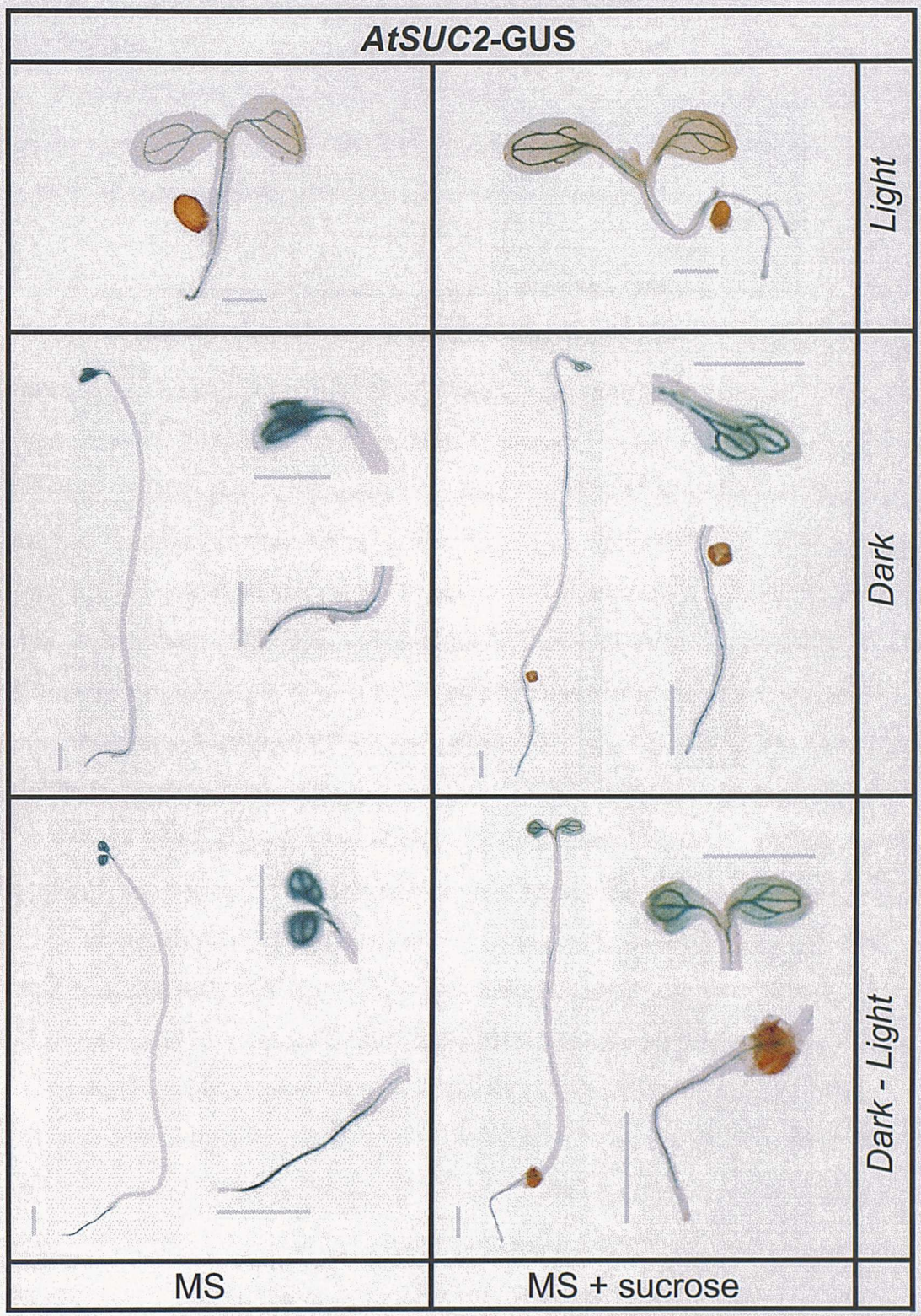


Figure 4.2. Histochemical staining of *AtSUC2-GUS* seedlings grown under different light conditions. Pictures show representative seedlings stained for GUS driven by the *AtSUC2* promoter. Seeds were germinated on MS medium (± 2% w/v sucrose) and grown in either light (7 d), dark (7 d) or dark (6 d)-light (1 d). Twenty seedlings were examined for each condition and representative samples are shown. Bars = 1 mm.

4.2.2. Quantification of *AtSTP4* and *AtSUC2* expression in seedlings grown under different light conditions

In order to quantify the expression levels of *AtSTP4* and *AtSUC2* expression in cotyledons, hypocotyls and roots in the different light conditions the GUS fluorometric assay was used. Initial experiments were conducted to optimise the assay for this investigation.

4.2.2.1. Optimization of the GUS fluorometric assay for *Arabidopsis* seedlings

It is essential that when the production of 4-MU (the product of the substrate 4-MUG) is assayed at a set time point that it is measured when the substrate is still in excess and the substrate catalysis verses time is linear, otherwise the result obtained is not a true reflection of the amount of GUS activity present. Jefferson (1987) stated that using 1 mM 4-MUG, GUS activity remained linear even in crude extracts for many hours, even days. The rate of catalysis of 4-MUG depends on the amount of GUS present in the reaction, which depends on the level of gene transcription and amount of tissue that was used for extraction. Therefore, a time course was conducted using *AtSUC2-GUS* seedlings. Reactions with 5 and 10 seedlings with 0.5 mM 4-MUG (half the substrate concentration used by Jefferson, 1987) showed that the rate of catalysis is linear at least up to 120 minutes in the 10 seedling reactions (see figure 4.3).

Jefferson (1987) suggested that 4-MU is sufficiently stable at <10 °C in darkness to allow aliquots to be stored at least overnight for measurement the next morning. Aliquots were assayed on the day of the experiments and seven days later, degradation of 4-MU had not occurred and all readings were still relative to the standard (results not shown). This enables large numbers of reactions to be measured in the same experiment without degradation interfering with the results.

As *AtSUC2* is expressed in the vascular tissue (figure 4.2; Truernit and Sauer, 1995) sufficient disruption to release the GUS enzyme is essential to obtain accurate data. Initially the GUS was released from the plant tissue by rapid freeze-thaw in liquid nitrogen, which ruptures the cell membranes and cell walls of the tissue. This was compared to mechanical disruption by homogenisation with a plastic pestle. Table 4.1 shows that tissues that were ground with a pestle gave a higher yield of the GUS enzyme indicated by a higher rate of substrate catalysis. Furthermore tissue disruption by grinding with a pestle also incurred a reduced variation between samples as indicated by the lower percentage standard error of the mean.

The breakdown of 4-MUG to 4-MU due to the presence of plant tissue was investigated to ensure that catalysis was purely a result of the presence of the introduced GUS enzyme and not a

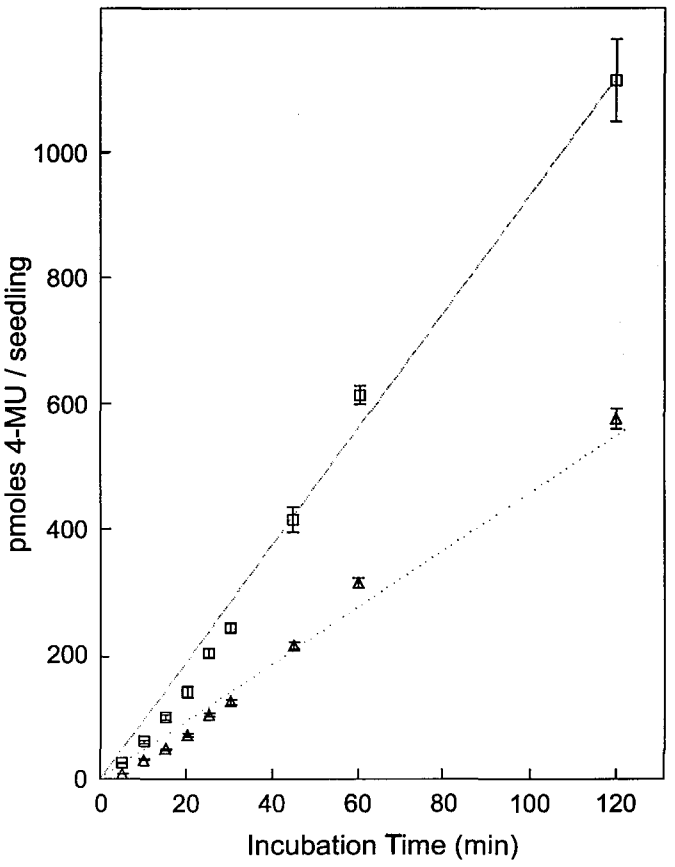


Figure 4.3. Time course for GUS activity extracted from *AtSUC2-GUS Arabidopsis* seedlings. GUS activity was measured in from *AtSUC2*-GUS *Arabidopsis* seedlings grown for 7 d in white-light. Assays contained GUS protein isolated from 5 (▲) or 10 (■) seedlings and values shown are the mean ± SE (n=6).

Table 4.1. Comparison of two methods of GUS extraction from *Arabidopsis* tissue. Ten 7 d *AtSUC2*-GUS *Arabidopsis* seedlings were harvested into GUS extraction buffer and disrupted by either rapidly thawing and refreezing, three times in liquid N_2 or ground using a pellet pestle.

	Mean ± SE pmoles 4-MU / seedling / min		SE (% of the mean)	
Time of assay (min)	30	60	30	60
Freeze / thaw	10 ± 0.7	17 ± 1.3	7.5	7.2
Ground	49 ± 3.2	96 ± 4.6	6.5	4.8

result of the plant tissue constituents. Six replicates of ten seedling reactions were assayed incorporating 7d old seedlings of *Arabidopsis* WT and *Arabidopsis STP4-GUS* (figure 4.4). Reactions with the WT seedlings throughout the time course consistently gave values between 0-10 pmole 4-MU per seedling per min, while *STP4-GUS* seedling reactions produced a positive correlation between 4-MU production and time. This experiment indicates that WT plant tissue does give a slight background reading but the rate is minor in comparison with the positive controls. To reduce the interference of this background, longer time points could be selected resulting in the background levels becoming a smaller percentage of the experimental values. To obtain the true experimental values the background levels of WT *Arabidopsis* can simply be subtracted from the values obtained and this was carried out in subsequent experiments.

4.2.2.2 Effect of light on *AtSTP4 expression*

Due to the large morphological differences of seedlings grown in the light and dark and consequently the signifiifcant change in fresh weight under the different light conditions, *AtSTP4* transcription was not only studied on a per tissue per seedling basis but also calculated per mg FW (figure 4.5). When GUS activity was expressed per seedling, *AtSTP4* transcription was higher in the cotyledons than in the hypocotyls and roots under all light and sucrose conditions (figure 4.5). It should be noted that while the hypocotyls did not show as strong staining as the roots in the histochemical method (figure 4.1), the fluorometric assay indicated that *AtSTP4* transcription is higher in the hypocotyls than in the roots when results are expressed on a tissue basis.

AtSTP4 transcription in cotyledons did not show a significant response to light under the conditions of these experiments (figure 4.6A), but the addition of sucrose to the media dramatically reduced *AtSTP4* transcription to a similar degree in all light conditions. In the hypocotyls of seedlings grown in the D-L conditions there was a significant decrease in *AtSTP4* transcription compared to seedlings grown in the D suggesting a slight down-regulation of *AtSTP4* transcription by light. Seedlings grown in the L did not show a significant difference in expression with or without sucrose. In the presence of sucrose, the hypocotyls of seedlings grown in L showed a significant increase in *AtSTP4* transcription compared to both the D and D-L conditions. Thus when the seedlings are supplemented with sucrose in the media, *AtSTP4* transcription is highest in the hypocotyls of L grown seedlings.

AtSTP4 transcription in the roots of seedlings was highest when grown in the L compared to seedlings grown in D or D-L (figure 4.6C). If the seedlings strategy for growth in light is to grow and

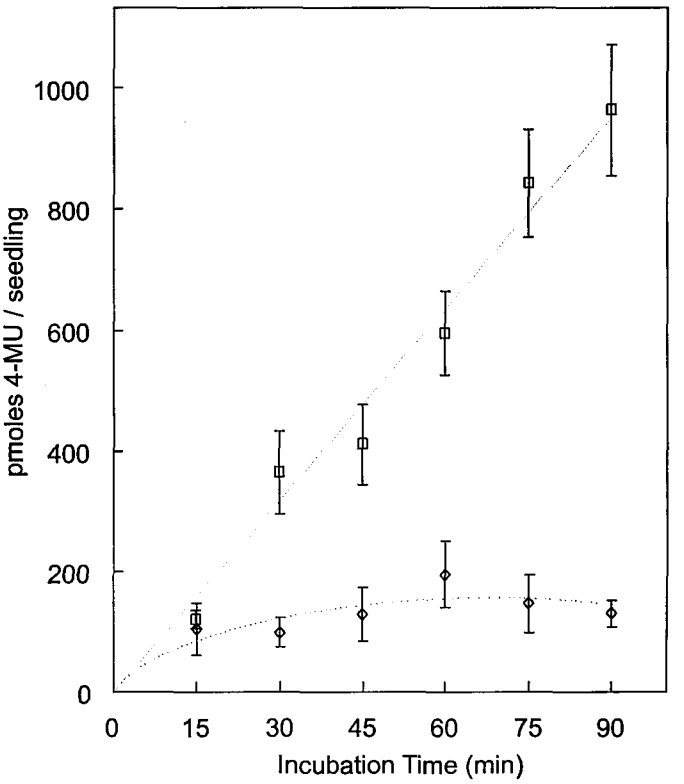


Figure 4.4. Time course for GUS activity in *AtSUC2-GUS* and WT plants. GUS activity was measured in 10 *AtSUC2-GUS* (□) or WT(◇) seedlings at 15 minutes intervals. Values shown are the mean ± SE (*n*=6).

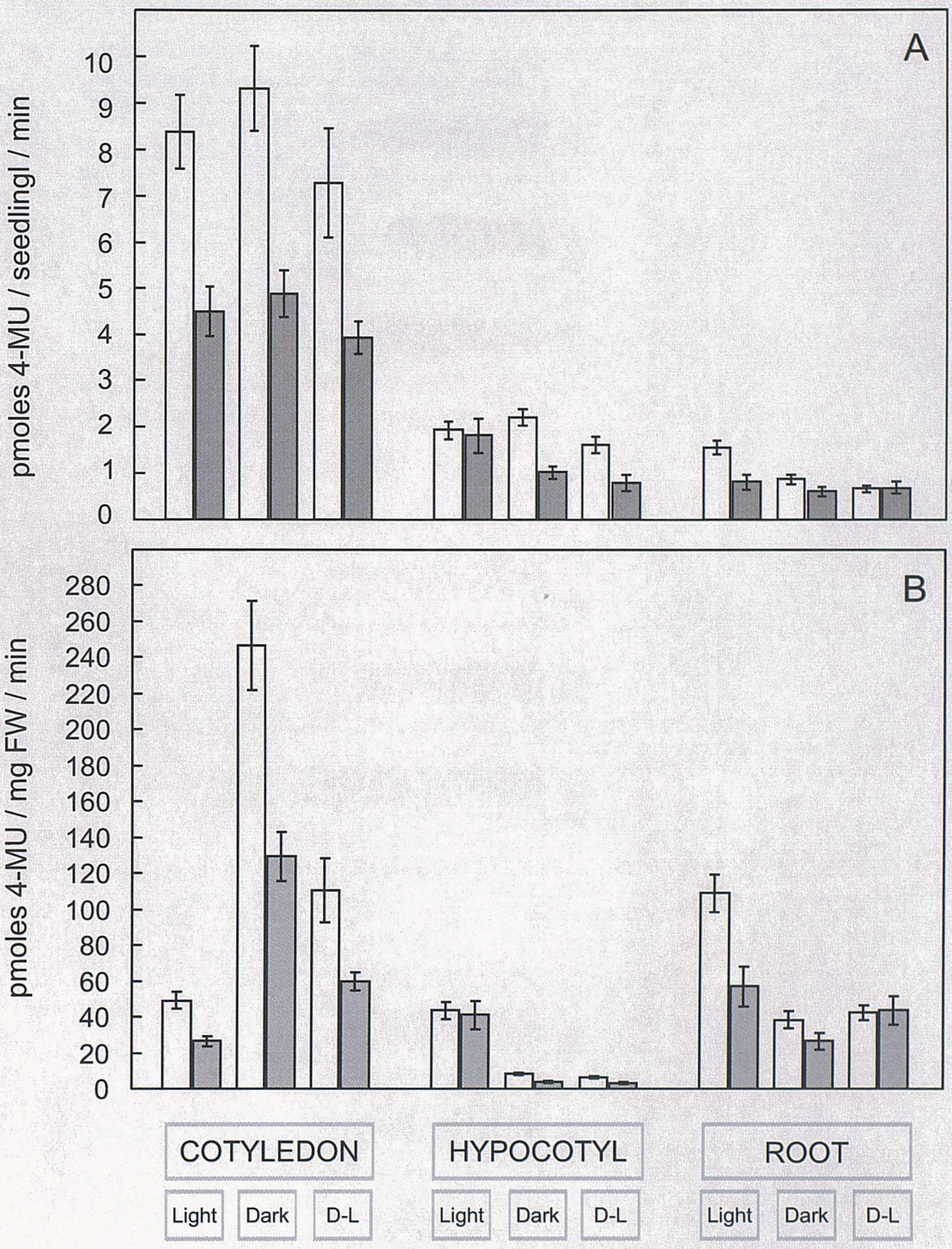


Figure 4.5. Tissue-specific *AtSTP4* Transcription in *Arabidopsis* seedlings. GUS activity was measured in extracts from cotyledons, hypocotyls and roots of 7 d *AtSTP4-GUS Arabidopsis* seedlings grown in continuous white light, darkness or 6 d darkness followed by transfer to white light for 1 d (D-L). Seedlings were grown with (grey bars) or without (white bars) 2% (w/v) sucrose. A) Data presented on a per seedlings basis. B) Data presented per mg fresh weight (FW). Values shown are the mean ± SE (n=18) with each replicate representing 10 seedlings.

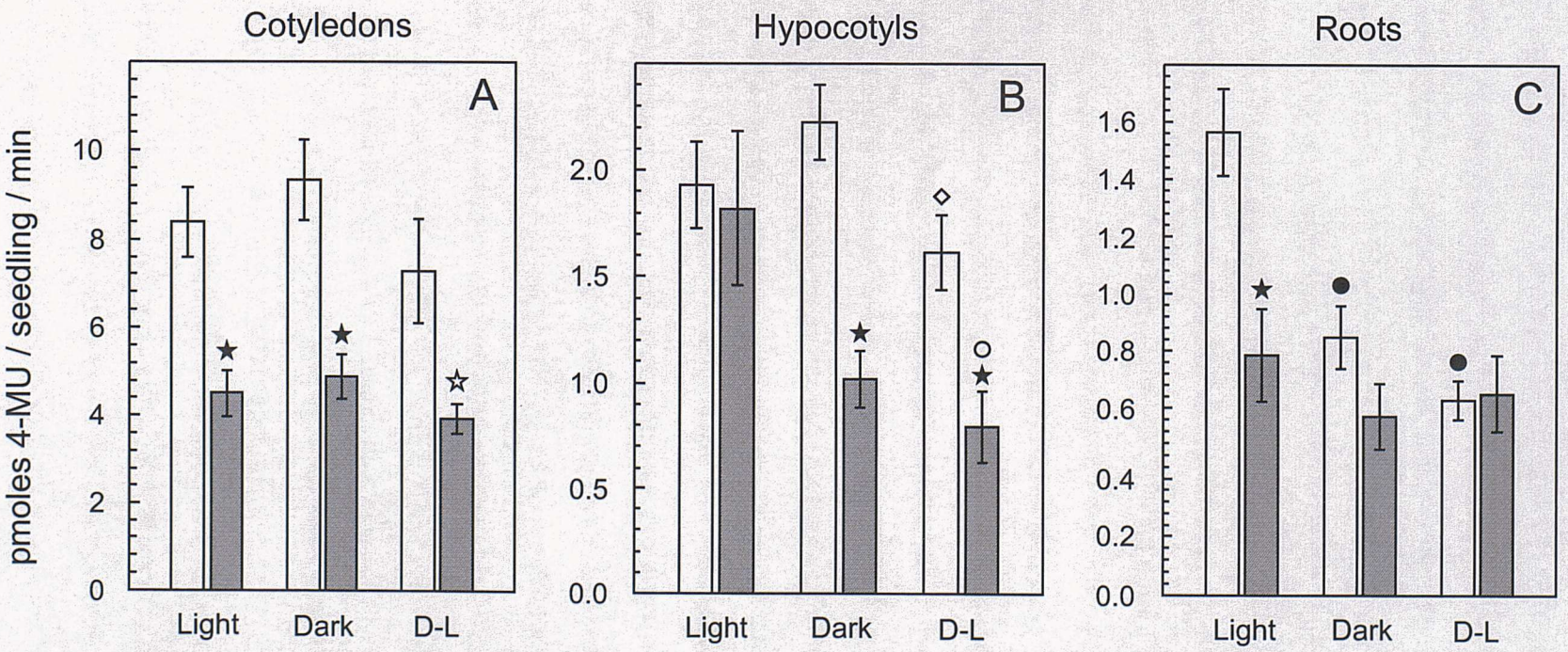


Figure 4.6. The Effect of Light on Tissue-Specific *AtSTP4* Transcription. The data shown are the same as for figure 4.5A but are redrawn to enable the light-effects in the hypocotyls and root tissues to be seen. Seedlings were grown with (grey bars) or without (white bars) 2 % w/v sucrose for 7 days and GUS activity was measured in A) Cotyledons, B) Hypocotyls and C) Roots. D-L: Dark 6 days, light 1 day. The significance levels and specific comparisons of the t-tests are given in the key.

★..... Effect of adding sucrose is significant in the same light condition
●..... Significant vs. light in the same tissue and media
◆..... Significant vs. dark in the same tissue and media
No-fill..... Significant to P value ≤ 0.05 (5% confidence level)
Fill..... Significant to P value ≤ 0.01 (1% confidence level)

develop the roots to increase the supply of nutrients from the soil whereas seedlings in the dark might continue to concentrate most of its resources to hypocotyl growth to find light, then an increase in the sink hexose transporter, AtSTP4 might be expected. Sucrose supplementation of the medium appears to abolish the increase in *AtSTP4* transcription in roots of seedlings grown in the L as a significant difference in *AtSTP4* transcription is seen in the roots of seedlings grown with sucrose supplementation.

AtSTP4 transcription in the cotyledons was higher than that in the hypocotyls and roots (figure 4.6). Seedlings grown in the light showed higher *AtSTP4* transcription in the roots than in the dark, however no increase was seen in seedlings that were transferred from darkness to light compared to dark-grown seedlings (figure 4.6C). In all tissues, *AtSTP4* transcription was suppressed by the supplementation of sucrose in the growth medium, but in the hypocotyls and roots this was only significant in specific light conditions: in the dark in the hypocotyls and in the light in the roots (figure 4.6A,B,C). Because of the significant differences in growth morphology between seedlings grown under the different light conditions, it is also informative to consider the results on a fresh weight basis. From these calculations, the cotyledons of seedlings showed a remarkable difference between *AtSTP4* transcription in different light conditions both with and without sucrose in the medium (Figure 4.5B). *AtSTP4* transcription was highest in the cotyledons of seedlings grown in the D, followed by those grown in D-L, which showed a 2-fold decrease, and lowest in the cotyledons of seedlings grown in the L that had a 4-fold decrease in *AtSTP4* transcription relative to D. In the hypocotyls, *AtSTP4* transcription was highest in seedlings grown in L with an increase of over 6-fold the *AtSTP4* transcription as measured in seedlings grown in the D and D-L (Figure 4.5B). The presence of sucrose in the medium resulted in reduced *AtSTP4* promoter activity in the D and D-L. In agreement with the result expressed on a per seedlings basis, *AtSTP4* transcription in the roots was highest in seedlings grown in the L (Figure 4.5B). When sucrose was present, the levels of GUS activity in the roots subjected to the D-L treatment were not significantly different from either the D or L treatments and were between these mean values.

In summary, calculation of GUS activity per mg FW revealed a light modulated down-regulation of *AtSTP4* in the cotyledons and a light mediated up-regulation of *AtSTP4* in the hypocotyls. Both these effects were present with and without sucrose supplementation. In the roots a light-dependent increase in *AtSTP4* activity was found, which was decreased by the presence of sucrose in the medium.

4.2.2.3 Effect of light on *AtSUC2* expression

As with *AtSTP4* transcription, *AtSUC2* transcription appeared to be highest in the cotyledons of the seedlings (figure 4.7A). Expression of *AtSUC2* in the cotyledons of seedlings grown without sucrose supplementation was higher in the L compared to the D and D-L conditions (figure 4.8A). However, with sucrose in the media this increase was abolished. Unlike the repression of *AtSTP4* transcription with sucrose supplementation in all light conditions, sucrose supplementation represses *AtSUC2* transcription only in continuous light. *AtSUC2* transcription is highest in the cotyledons grown in the light as these seedlings are rapidly developing into a mature plant thus there is a strong demand for carbon nutrition from the cotyledons to fuel this development. Hypocotyls and roots also show the highest *AtSUC2* transcription in the light and this is also abolished with sucrose supplementation.

In summary, *AtSUC2* transcription was highest in the cotyledons (figure 4.7A). In all tissues *AtSUC2* transcription was highest in seedlings grown in the light compared with the D, however, with sucrose supplementation this response was abolished (figure 4.8). Light grown seedlings showed down regulation by sucrose in all tissues (to $p \leq 0.01$) (figure 4.8).

Promoter activity expressed per mg FW basis, dramatically alters the interpretation of the results (figure 4.7B). *AtSUC2* transcription per mg FW shows significant differences between the light conditions. Highest *AtSUC2* transcription was shown in the cotyledons of seedlings grown in the D, the difference in *AtSUC2* cotyledon transcription between L and D-L seedlings was not significant (figure 4.7B). A significant difference in hypocotyl *AtSUC2* transcription was apparent between both L and D and L and D-L grown seedlings but not between D and D-L grown seedlings (figure 4.7B). Unlike the result shown on a per seedling basis there is the same relationship in the presence of sucrose.

The relationship between the light and medium conditions for *AtSUC2* transcription in the roots were the same when calculated both on per seedling (figure 4.8C) and per mg FW (figure 4.7B) basis. Expressed per seedling hypocotyl *AtSUC2* transcription in the L is approximately 4 fold compared to that in the roots for the same conditions (figure 4.7A). However the results shown per mg FW make the *AtSUC2* transcription in these two tissues almost equal (figure 4.7B).

In summary, when AtSUC2-GUS transcription is expressed per mg FW, it appears that cotyledon *AtSUC2* transcription is down regulated in the L. The opposite relationship is shown in the hypocotyls; L up regulates transcription both with and without supplementary sucrose. Light up-regulation is also present in the roots but is abolished in the presence of sucrose. Only L grown

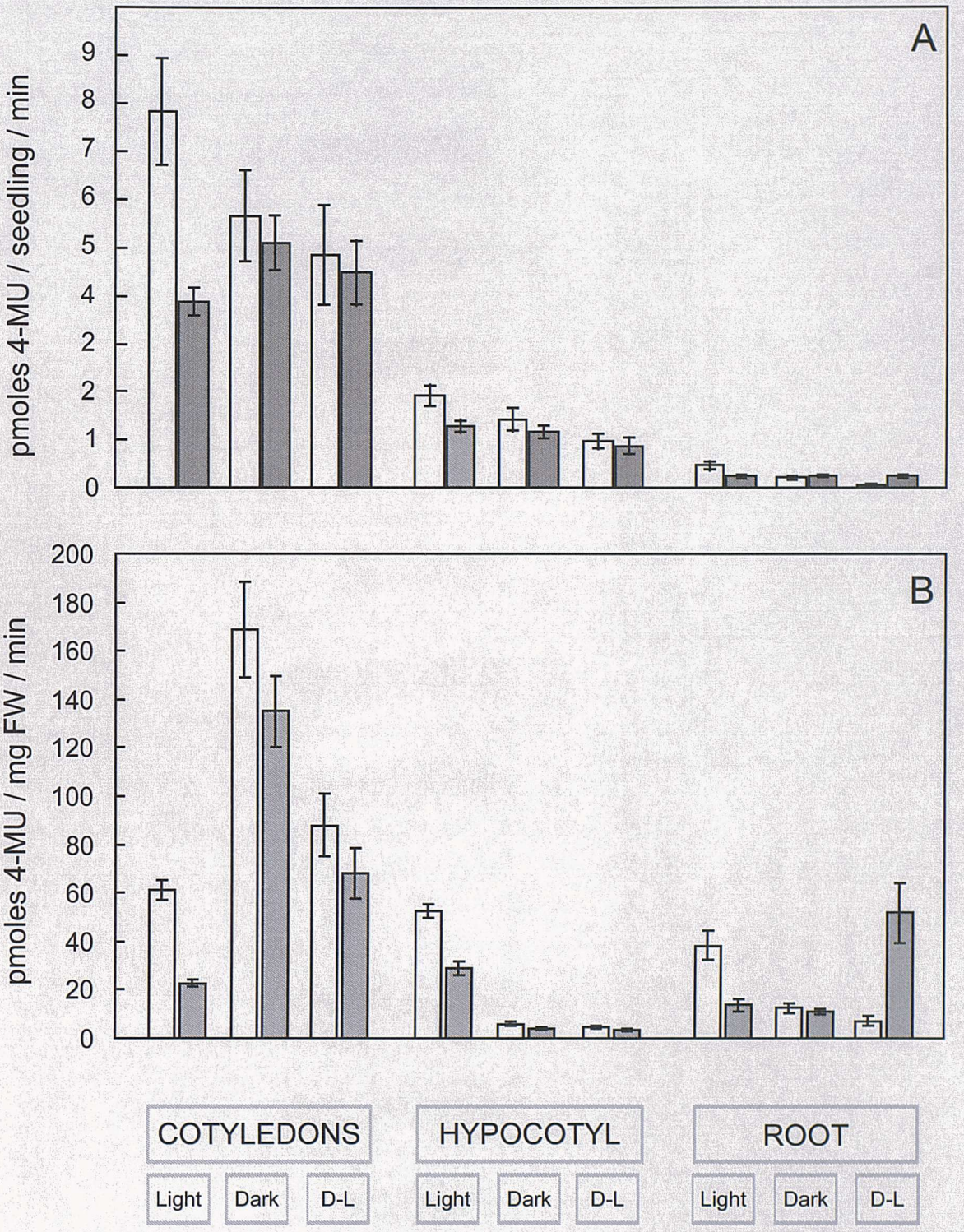


Figure 4.7. Tissue-specific *AtSUC2* Transcription in *Arabidopsis* seedlings. GUS activity was measured in extracts from cotyledons, hypocotyls and roots of 7 d *AtSUC2-GUS Arabidopsis* seedlings grown in continuous white light, darkness or 6 d darkness followed by transfer to white light for 1 d (D-L). Seedlings were grown with (grey bars) or without (white bars) 2% (w/v) sucrose. A) Data presented on a per seedlings basis. B) Data presented per mg fresh weight (FW). Values shown are the mean ± SE (n=18) with each replicate representing 10 seedlings.

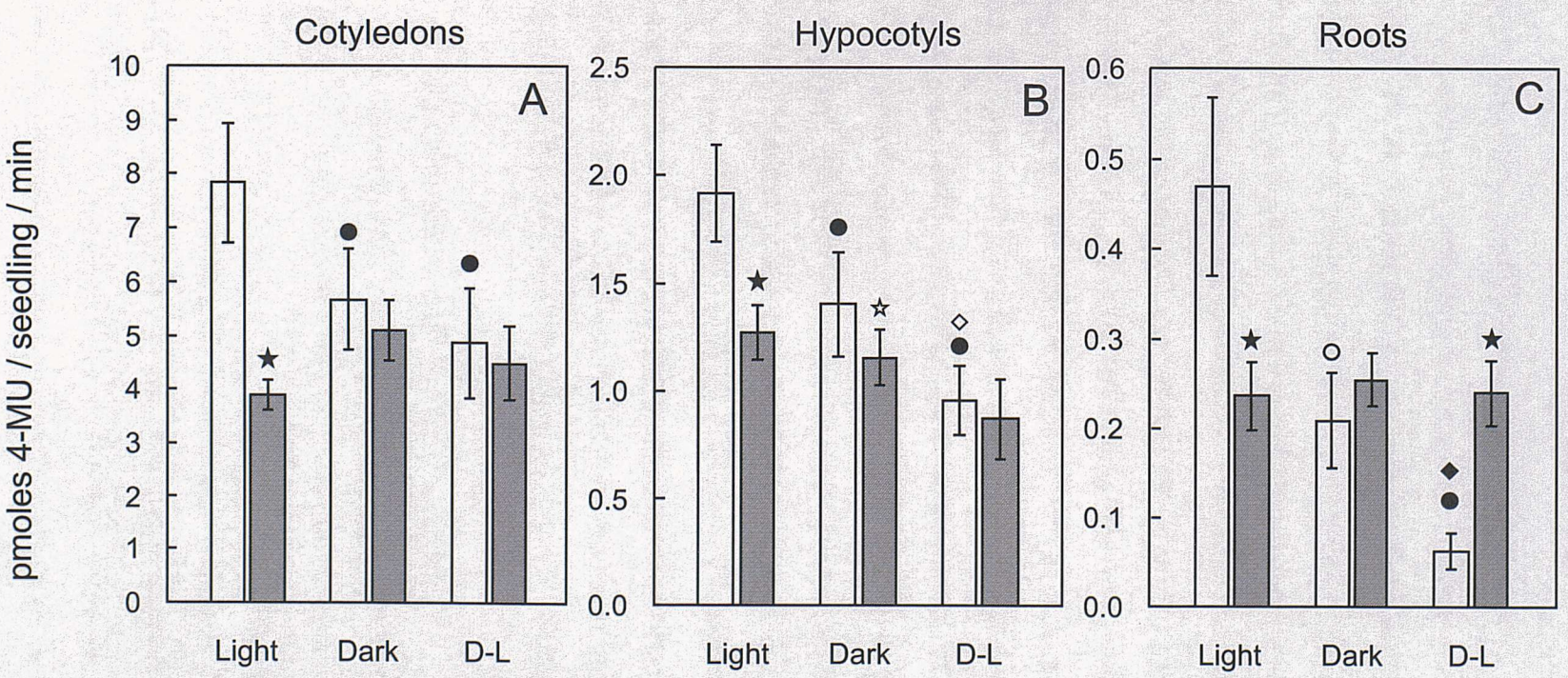


Figure 4.8. The Effect of Light on Tissue-Specific *AtSUC2* Transcription. The data shown are the same as for figure 4.7 but are redrawn to enable the light-effects in the hypocotyls and root tissues to be seen. Seedlings were grown with (grey bars) or without (white bars) 2 % w/v sucrose for 7 days and GUS activity was measured in A) Cotyledons, B) Hypocotyls and C) Roots. D-L: Dark 6 days, light 1 day. The significance levels and specific comparisons of the t-tests are given in the key.

★..... Effect of adding sucrose is significant in the same light condition
●..... Significant vs. light in the same tissue and media
◆..... Significant vs. dark in the same tissue and media
No-fill..... Significant to P value $\leq$0.05 (5% confidence level)
Fill..... Significant to P value $\leq$0.01 (1% confidence level)

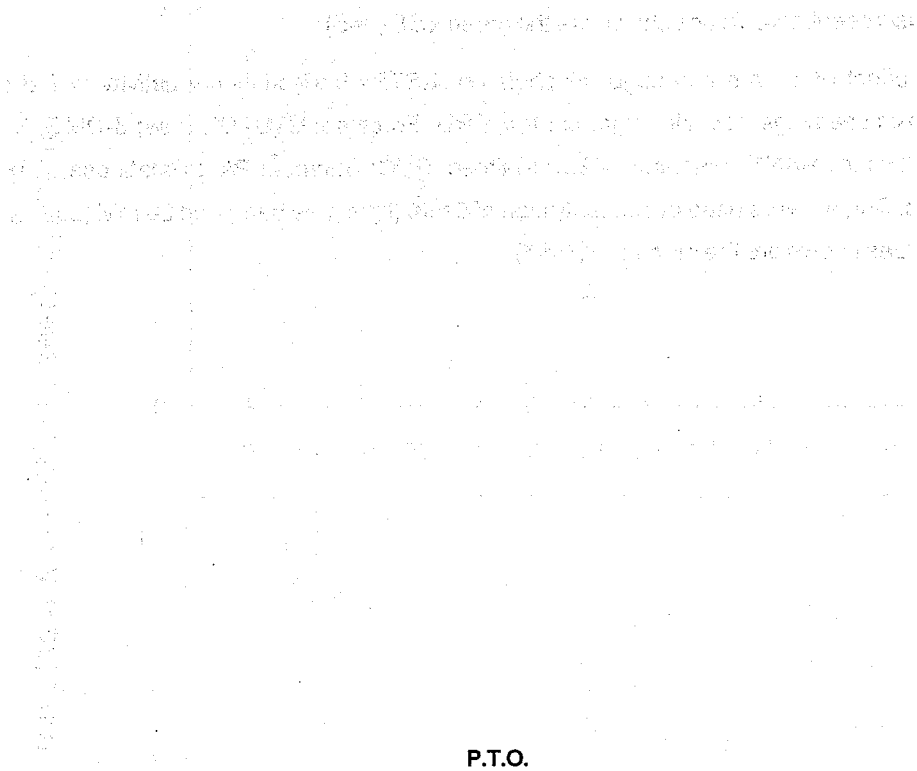
seedlings show down-regulation of AtSUC2 in response to sucrose supplementation, but this occurs in all tissues (cotyledon, hypocotyl and root).

4.2.4.1 Regulation of *AtSTP4* transcription by sugars.

To further understand the down-regulation of *AtSTP4* transcription by sucrose observed previously (figure 4.6A), the concentration dependence of the sucrose response was investigated. Inhibition by sucrose is seen at 30 mM, where there is an approximate 50% reduction in the transcriptional activity of *AtSTP4* (figure 4.9B). 30 mM sucrose in the media is equivalent to 1 % w/v. Experiments carried out subsequently to these appeared to contradict what had previously been seen. When a range of sugars were tested it was noted that supplementation of the media with 30 mM sucrose seemed to double *AtSTP4* transcriptional activity. Indeed all monosaccharide and disaccharide sugars seemed to increase the *AtSTP4* transcriptional activity although levels were not significantly higher than the mannitol treatment, which was used as an osmotic control. However, the oligosaccharide, raffinose, did not have any significant effect on *AtSTP4* transcription. As the data from the later GUS experiments was different from those carried out earlier, the sugar regulation was investigated by measuring transcript levels using a semi-quantitative RT-PCR approach. The normalized IDVs for the cDNA bands corresponding to *AtSTP4* show that sucrose but not glucose supplementation of the media at 30 mM produced a ~25% reduction of *AtSTP4* transcripts (figure 4.11B) consistent with earlier experiments using the GUS assay (figure 4.5A). Interestingly the 2-fold increase in *AtSTP4* transcription seen with 30 mM glucose in the later GUS assays (figure 4.9B) was not observed when transcripts were measured using RT-PCR (figure 4.11A,B).

4.2.4.2 Regulation of *AtSUC2* transcription by sugars.

Compared to *AtSTP4* transcription, *AtSUC2* transcription was more sensitive to the concentration of sucrose supplemented to the media. The initial range of sucrose concentrations showed that the lowest amount of sucrose added to the media elicited a sharp down-regulation in *AtSUC2* transcriptional activity (figure 4.10A). The experiment was modified to include lower concentrations of sucrose to the media with the aim of determining the minimum sucrose concentration that can mediate this response (figure 4.10B). The results of these experiments showed that the down-regulation started at a concentration as low as 5 mM with *AtSUC2* transcription reduced by a third. At 10 mM down-regulation was approximately half of the no sucrose control. Higher concentrations of sucrose supplementation in the media did not down-regulate *AtSUC2*

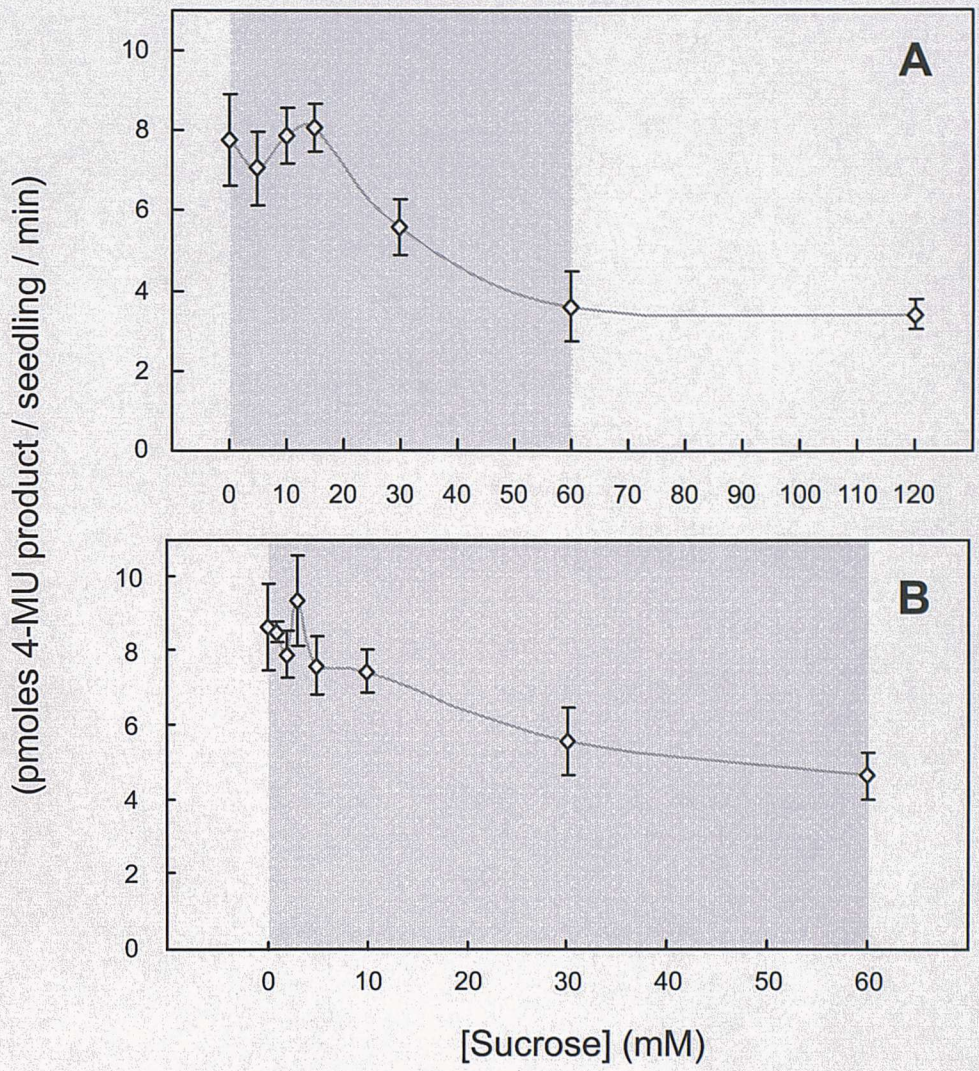


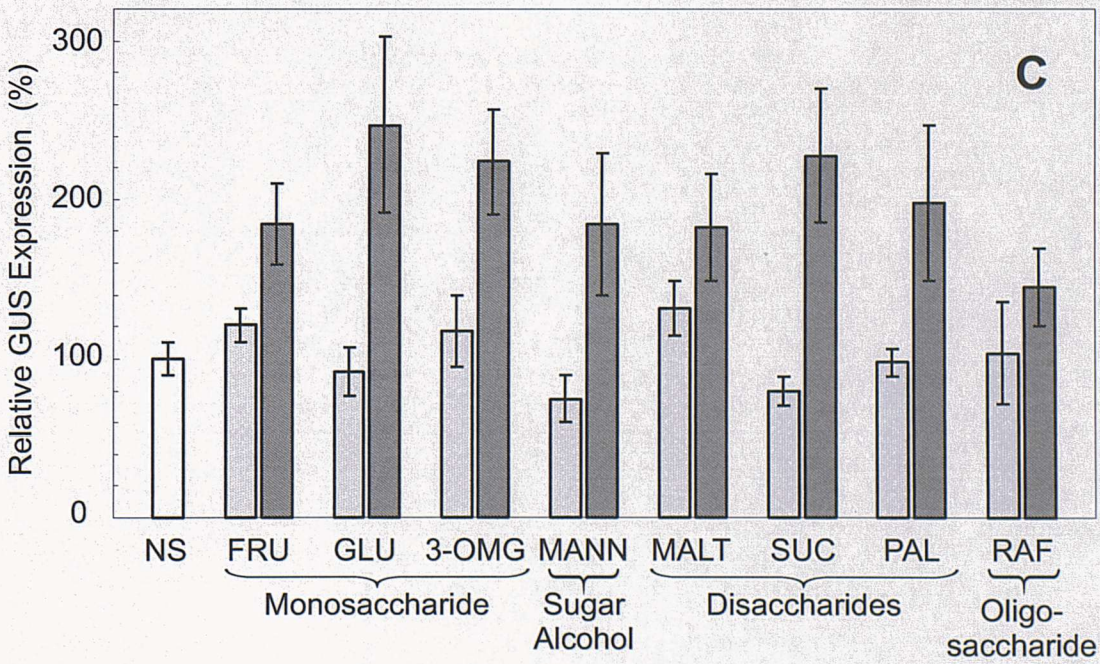
P.T.O.

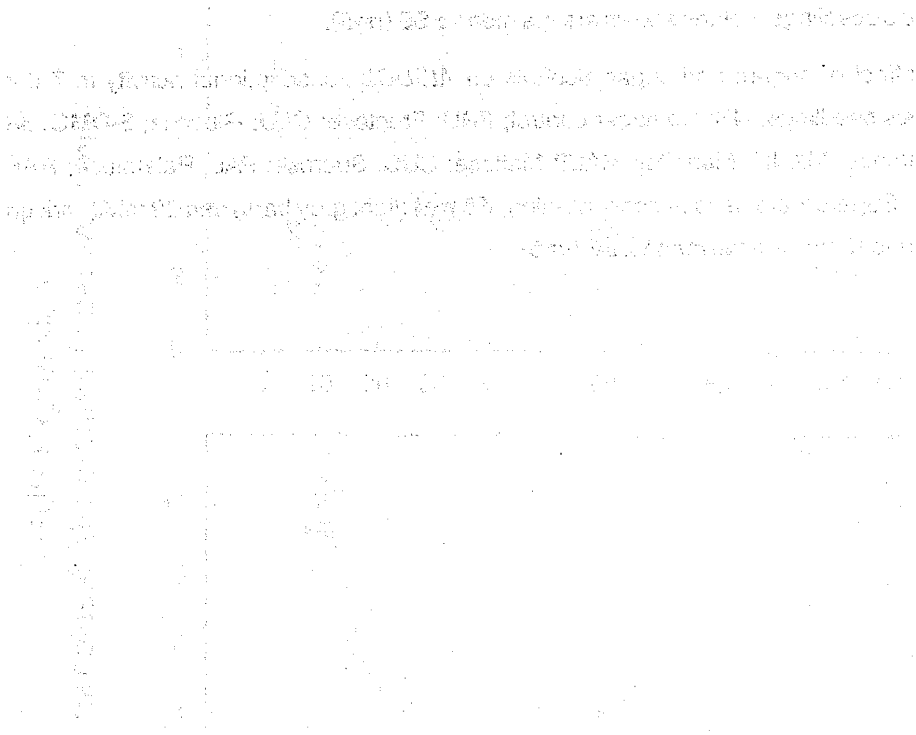
Figure 4.9. The effect of sugar on *AtSTP4* expression

A & **B**: The effect of sucrose concentration on *AtSTP4* transcriptional activity in 7 d light-grown *Arabidopsis* seedlings. Values shown are the mean ± SE (*n*=5).

C: The effect of sugars and sugar alcohols on *AtSTP4* transcriptional activity in 7 d old *Arabidopsis* seedlings. NS: No sugar control; FRU: Fructose; GLU: Glucose; 3-OMG: 3-O-methyl-glucose; MANN: Mannitol; MALT: Maltose; SUC: Sucrose; PAL: Palatinose; RAFF: Raffinose. Sugars were used at concentration of 5 mM (light grey bars) and 30 mM (dark grey bars). Values shown are the mean ± SE (*n*=5)


A
B
(pmoles 4-MU product / seedling / min)
[Sucrose] (mM)
0
2
4
6
8
10
0 10 20 30 40 50 60 70 80 90 100 110 120
0 10 20 30 40 50 60


C
Relative GUS Expression (%)
0
100
200
300
NS
FRU
GLU
3-OMG
MANN
MALT
SUC
PAL
RAF
Monosaccharide
Sugar Alcohol
Disaccharides
Oligo-saccharide

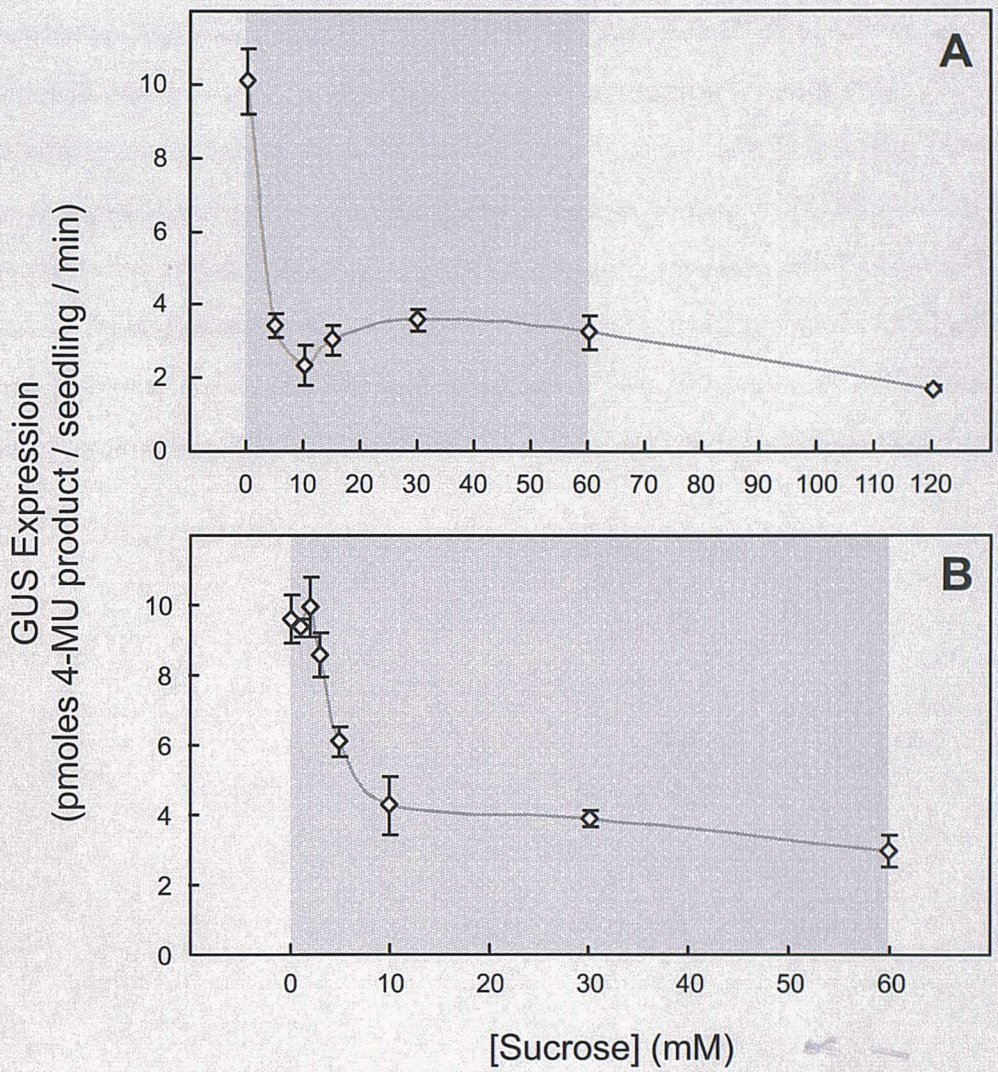


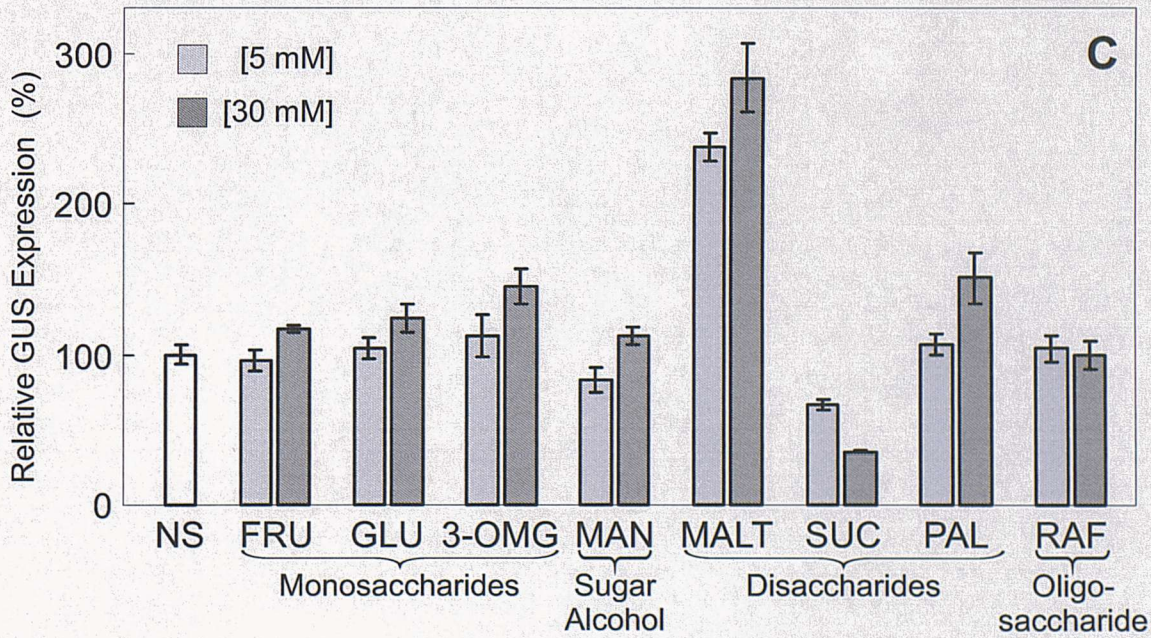
P.T.O.

Figure 4.10. The effect of sugar on *AtSUC2* expression

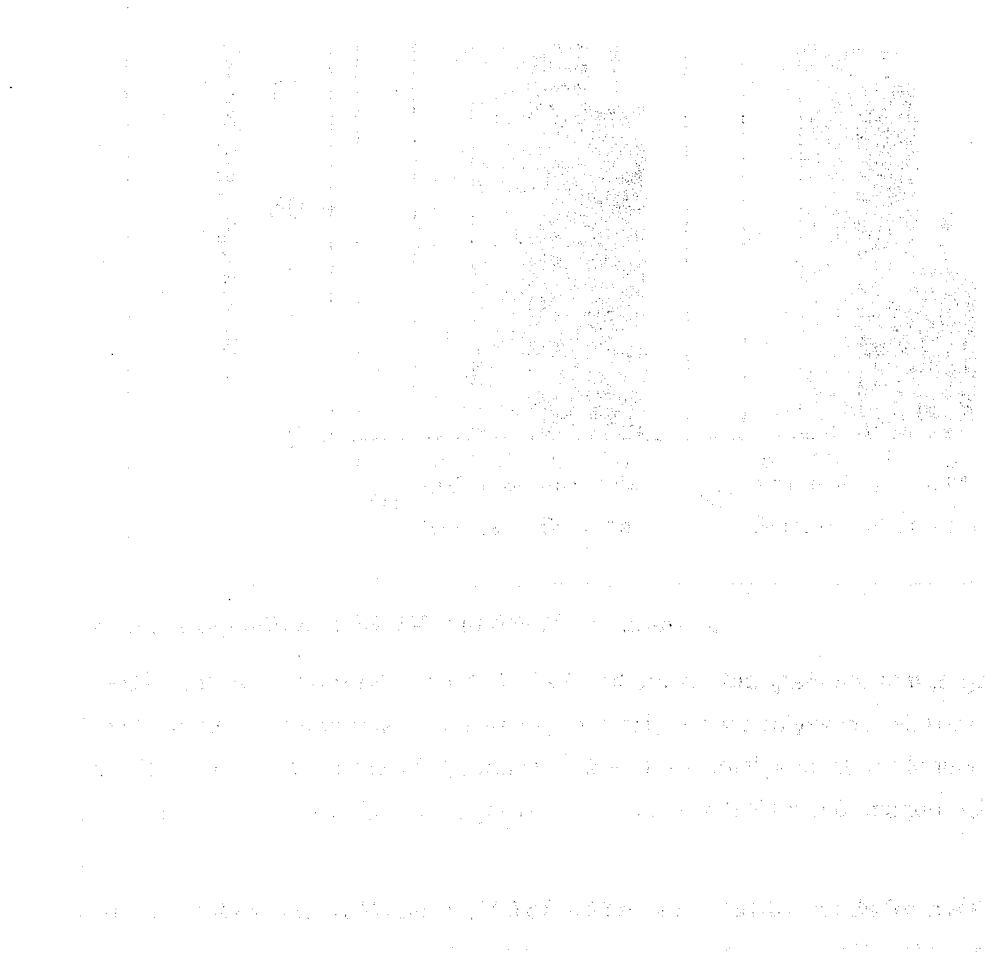
A & **B**: The effect of sucrose concentration on *AtSUC2* transcriptional activity in 7 d light-grown *Arabidopsis* seedlings. Values shown are the mean ± SE (*n*=5).

C: The effect of sugars and sugar alcohols on *AtSUC2* transcriptional activity in 7 d old *Arabidopsis* seedlings. NS: No sugar control; FRU: Fructose; GLU: Glucose; 3-OMG: 3-O-methyl-glucose; MANN: Mannitol; MALT: Maltose; SUC: Sucrose; PAL: Palatinose; RAFF: Raffinose. Sugars were used at concentration of 5 mM (light grey bars) and 30 mM (dark grey bars). Values shown are the mean ± SE (*n*=5)


A
B
GUS Expression
(pmoles 4-MU product / seedling / min)
[Sucrose] (mM)


C
[5 mM]
[30 mM]
Relative GUS Expression (%)
NS
FRU
GLU
3-OMG
MAN
MALT
SUC
PAL
RAF
Monosaccharides
Sugar Alcohol
Disaccharides
Oligo-saccharide

transcription further. The results of the RT-PCR experiments agree with the observations seen using the *AtSUC2-GUS* plants. The mean normalized IDVs show that both 5 mM and 30 mM sucrose supplementation cause a decrease in *AtSUC2* transcription. In accord with the GUS assays from the cotyledons of seedlings grown on a range of sucrose concentration (figure 4.10B) the RT-PCR results (figure 4.11) show that the sucrose concentration correlates to the degree of *AtSUC2* transcription down-regulation. Sucrose supplementation of 5 mM produced a decrease in *AtSUC2* transcription of ~25% where as 30 mM sucrose supplementation reduced *AtSUC2* transcription by ~60%. The reasonable correlation suggests that regulation is primarily at the transcriptional level rather than RNA stability.



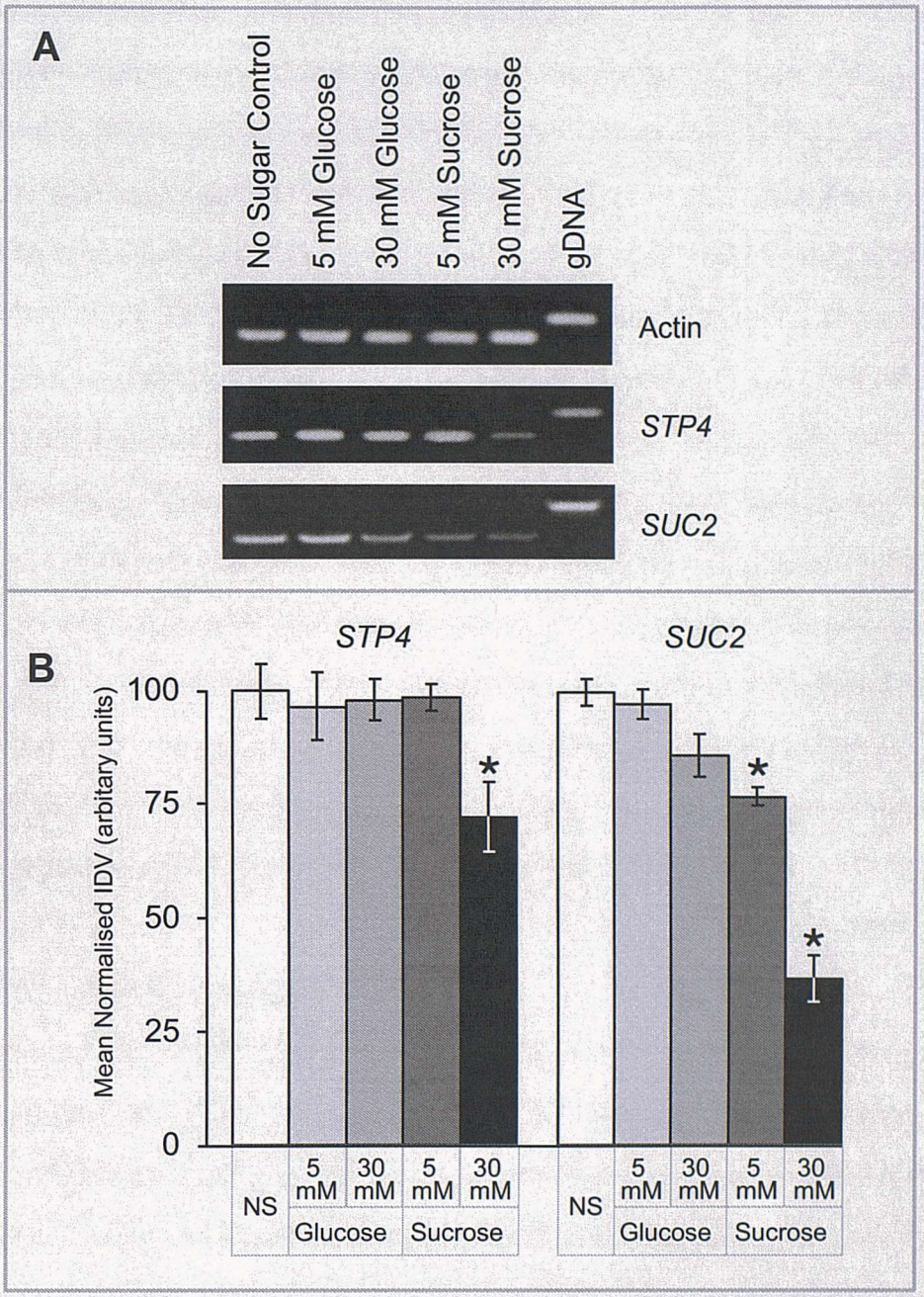


Figure 4.11. Regulation of *ASTP4* and *AtSUC2* by sucrose.

A: RT-PCR analysis of *AtSTP4* and *AtSUC2* in 7d old *Arabidopsis* seedlings grown on MS media with sugar supplementation. Seedlings had fully opened cotyledons with no obvious signs of rosette leave formation (stage 1.0, Boyes *et al.*, 2001). The total product was loaded onto a 0.8% (w/v) agarose gel, 2% (w/v) for actin agarose gel stained with EtBr and imaged using an Alpha Imager.

B: Mean Integrated Density Values (IDVs) of *AtSTP4* and *AtSUC2* bands from RT-PCRs. PCR was performed as described in A. Values shown are the mean ± SE (n=3) of three separate IDV calculations and were normalized to the actin control. * denotes statistically significant differences (2-tailed t-test, $P < 0.05$) with respect to the no sugar control (NS).

4.3. Discussion.

Evidence from a number of studies is accumulating to show that the activity of sugar transporters is likely to be tightly controlled. Several levels of regulation may occur (Delrot *et al.*, 2000; Williams *et al.*, 2000a) including regulation by the proton-motive force for proton-coupled transporters. Transcriptional regulation of sucrose transporters appears to be particularly important and is seen for example during seed development (Weber *et al.*, 1997), pollen maturation and germination (Stadler *et al.*, 1999), in leaves undergoing sink-to-source transitions (Truernit and Sauer, 1995; Riesmeier *et al.*, 1994) and in response to various stimuli; for example salt, (Noiraud *et al.*, 2000) wounding and pathogen attack (Truernit and Sauer, 1995;Truernit *et al.*, 1996; Sakr *et al.*, 1997), sugars (Aoki *et al.*, 1999; Barker *et al.*, 2000; Matsukura *et al.*, 2000; Vaughan *et al.*, 2002; Barth *et al.*, 2003) and light (Hirose *et al.*, 1997; Kühn *et al.*, 1997; Aoki *et al.*, 1999; Shakya and Sturm, 1998; Matsukura *et al.*, 2000). Examples of post-transcriptional control mechanisms have also been reported such as mRNA stability, mRNA translation and post-translational control (Kühn *et al.*, 1997; Hirose *et al.*, 1997; Delrot *et al.*, 2000, Williams *et al.*, 2000a). The latter includes possible phosphorylation/dephosphorylation control (Roblin *et al.*, 1998). The present study investigated transcriptional regulation of *AtSTP4* and *AtSUC2* using the reporter gene GUS.

4.3.1 Anaiysis of the GUS Assay Data

Growing seedlings in such dramatically different light conditions resulted in plants with quite different morphology. Investigating the transcriptional activity in such seedlings is thus quite problematic. Therefore results were expressed both per seedling and per mg FW. The FW of plant cells consists mainly of their water content and when tissues grow by cell elongation the FW would increase when the number of cells and thus cell membranes that a sucrose molecule would have to negotiate would remain the same. Therefore, it would be more appropriate to obtain a measure of the number of cells. A more accurate method of reducing the effect of morphology might be to measure the protein of each section of the seedling. However, light is also know to initiate the production of the protein machinery required to make a cell autotrophic in the development of a light-grown seedling (McCormac *et al.*, 2001) and thus this would not reflect the number of cells in each tissue of the seedlings grown under different light conditions. A method to measure cell number might be to extract total gDNA and, using a specific probe, assay the relative proportions of gDNA from each tissue of the seedlings grown in each light condition. The theory behind this method being that each cell has its own copy of gDNA thus the relative amounts of gDNA extracted would be proportional to the number

of cells from the tissue. However, this approach is far from perfect as this investigation utilizes specific tissues of seedlings the samples from which gDNA were to be obtained would be minute. In addition there is a potential problem of DNA degradation during the isolation of the gDNA and the subsequent southern blot or PCR, which would prevent accurate quantification.

Before going down this route it would be more useful to compare the results obtained by using the GUS lines with a different approach such as quantitative RT-PCR. If the RT-PCR results agreed with the results using the GUS lines then this would suggest that measuring the transcriptional activity/GUS activity on a per seedlings basis to be realistic to the physiological state of the tissue.

While studying the tissue-specific transcriptional activity of *AtSTP4* and *AtSUC2* in 7 d seedlings grown in different light conditions it was found that sucrose supplementation affected the cotyledon transcriptional activity of both sugar transporters. When comparing the effect of sucrose supplementation, the light treatments were constant and thus this direction did not require the development of a method for cancelling any artefacts in the data due to photomorphogenesis. If more time were allowed, improving the accuracy of measuring the transcriptional activity of *AtSTP4* and *AtSUC2* in different seedlings tissues in different light conditions using the relative amounts of gDNA could be taken further.

4.3.2. Localization of *AtSTP4* Transcription

The histochemical staining indicated that *AtSTP4* transcription occurred in the cotyledons and in the roots of *Arabidopsis* seedlings (figure 4.1). This is in agreement with the results presented by Truernit *et al.* (1996). Their paper only reported on light-grown seedlings whereas in the present study it has also been investigated using etiolated seedlings and seedling in the process of de-etiolation. It is interesting to note that a cloudy staining in the cotyledons of seedling was also observed by Truernit *et al.* (1996). Truernit *et al.* (1996) proposed that *AtSTP4* was a sink-specific monosaccharide transporter. It is hard to postulate a role for such a transporter in the cotyledon of seedlings especially since the staining is variable and "patchy". The role in the roots of *Arabidopsis* is easier to understand. Once sucrose is broken down at sink sites by invertase into its constituents, glucose and fructose, carriers such as AtSTP4 are required for the import of these sugars into sink cells. Its expression in the roots seems to be down-regulated in the dark, which might be a consequence of the reallocation of sugars to the hypocotyls in dark-grown seedlings. The fact that de-etiolating (D-L) seedlings do not show an increase in *AtSTP4* transcription relative to etiolated (D) seedlings maybe a consequence of insufficient time to adapt to a growth strategy in the light. As the root is the only

tissue in which AtSTP4 seems to have an obvious role it is puzzling that the GUS assays shown here (figure 4.5) suggest that *AtSTP4* transcription is lowest in the roots when compared to the relevant values for the cotyledons and hypocotyls in all light conditions both with and without sucrose supplementation. In mature plants, Truernit *et al.*, (1996) showed using northern blot analysis that *STP4* transcripts were most abundant in the roots, then the flowers and least in the leaves. It was also stated that little was detected in stems and fruits. Light-induced changes in *AtSTP4* transcription were not observed in the present study (figure 4.6). Since hypocotyl extension is predominantly due to cell elongation, AtSTP4 might import hexoses into hypocotyl cells thus decreasing the water potential of the hypocotyl cells encouraging water to enter the cell causing them to expand. However, support for this is not apparent, as hypocotyl *AtSTP4* transcription was unchanged between light conditions and, with sucrose supplementation, reduced in etiolated and de-etiolated seedlings.

In summary, it has been shown *AtSTP4* transcription is located in the roots of *Arabidopsis* in agreement with its stated role as a sink-specific monosaccharide transporter (Truernit *et al.*, 1996). The role of this transporter in the cotyledons is still unknown.

4.3.3. Localisation of *AtSUC2* Transcription

The localisation of SUC2 in light-grown plants has been studied in some detail and in various plant species. *AtSUC2* was first discovered along with *AtSUC1* from an *Arabidopsis* cDNA library using a spinach sucrose transporter probe (*pS21*) and both were shown to transport sucrose and to a lesser extent maltose (Sauer and Stolz, 1994). Using a *SUC2-GUS* line (also used for this study), *AtSUC2-GUS* was found in the vascular tissues of all organs of the plant indicating that this sucrose transporter has a role in phloem loading and possibly unloading (Truernit and Sauer, 1995). The localisation of StSUT1 (thought to be the orthologue of AtSUC2 in potato) was investigated using immunofluorescent imaging and they were found in enucleate sieve elements (Kühn *et al.*, 1997). In the present study the localization of expression is found in the vascular system throughout the seedlings and does not change in etiolated or de-etiolated seedlings compared to light-grown seedlings (figure 4.2).

4.3.4. The effect of sucrose supplementation on *AtSTP4* transcription

The GUS staining shown in the present study (figure 4.1) was not obviously affected by sucrose supplementation and Truernit *et al.* (1996) reported similar results in leaves of *Arabidopsis*. However using the fluorometric quantitative technique and RT-PCR, this study has indicated that

AtSTP4 is responsive to sucrose supplementation. Results shown in this chapter show that 30 mM sucrose, but not glucose, produced a down-regulation in the promoter activity of *AtSTP4* in 7 d old *Arabidopsis* seedlings (figures 4.10 and 4.11). It is important to relate the degree of *AtSTP4* transcriptional activity in each tissue (figure 4.6) to its possible physiological role in the seedling. The down-regulation of *AtSTP4* transcriptional activity in the cotyledons by sucrose supplementation in the media is consistent in seedlings in all light conditions with very different growth strategies. As a suggested sink-specific hexose transporter (Truernit *et al.*, 1996) the opposite relationship might be expected. With a source of sucrose present at the roots the cotyledons would not be required to export so much sucrose and thus more would be available to fuel cotyledon development. This increase in sucrose allocation for the cotyledons might bring an increase in hexose transporters in the cotyledons. However, the results here (figure 4.6) show that this is not the case or that this hexose transporter does not have a role in this process. At present, a reasonable hypothesis to explain down-regulation of *AtSTP4* transcriptional activity in the cotyledons when seedlings are grown on sucrose cannot be suggested. Repeats of the *AtSTP4*-GUS assays are required to investigate which sugars elicit a response and to what degree (4.9B), especially since this experiment produced results contradictory to those previously obtained when looking at the sensitivity of the sucrose down-regulation (figure 4.9A).

The *AtSTP4* transcriptional down regulation by sucrose supplementation in the hypocotyls appears to be affected by the light environment in which the seedlings are grown. Only those seedlings that have been subjected to long periods of growth in complete darkness show this sucrose-supplementation down regulation of *AtSTP4* transcriptional activity. If light produces an up-regulation of *AtSTP4* transcriptional activity in the hypocotyls of seedling, this is not seen within 24hrs of de-etiolation in the light. Since no staining was observed in the hypocotyls of seedlings further experiments would be required before a satisfactory theory as to the role of AtSTP4 in the hypocotyls of seedlings.

The sucrose supplementation down-regulation of *AtSTP4* transcription in the roots of light-grown seedlings might be due to roots having a readily available sugar supply and thus reducing its demand on the source tissues. However, since AtSTP4 is a hexose transporter and sugar is thought to arrive at sink sites as sucrose the reason why transcription of a hexose transporter is down-regulated is not clear. As to why *AtSTP4* transcription in the roots is affected by the location of the source (the media as opposed to the cotyledons), an explanation cannot be offered at this time.

In summary, *AtSTP4* transcription appears to be down-regulated in the cotyledons in response to sucrose supplementation, but not glucose supplementation (figure 4.10 and 4.11). However, a role for AtSTP4 in the cotyledons has yet to be deduced.

4.3.5. The effect of sucrose supplementation on *AtSUC2* transcription

Evidence for sugar regulation of expression of sugar transporters is limited and does appear to be dependent on the particular sugar transporter under investigation (Williams *et al.*, 2000a; Atanassova *et al.*, 2003). The expression of the companion cell-specific sucrose transporter in rice (OsSUT1) was enhanced by both glucose and sucrose (100mM) whereas BvSUT1, a sugar beet leaf sucrose transporter also localised to the companion cells was down-regulated specifically by sucrose (100 mM), but not glucose (Matsukura *et al.*, 2000; Vaughn *et al.*, 2002). Sucrose-feeding experiments with detached maize leaves resulted in an increase in *ZmSUT1* transcripts (Aoki *et al.*, 1999). Although AtSUC2 was previously reported as not being sugar regulated (Truernit and Sauer 1995), this present study using the more sensitive quantitative fluorescent assay has shown that AtSUC2 is down-regulated by sucrose, but not by other sugars. Down-regulation also occurred at relatively low concentrations compared to those shown previously to down-regulate expression of sucrose transporters. For example, VfSUT1, a sucrose transporter expressed in developing embryos of fava bean seed showed a decrease in expression at 150mM sucrose or glucose whereas lower levels (10mM) had no effect on transcript levels. Thus, this study has indicated that the sucrose regulation of sucrose transporter gene expression could play a key role in the control of sugar partitioning and it would be interesting to investigate the signalling pathway involved in this response.

The studies regarding the regulation of sucrose transporters by its substrate pose the questions how is sucrose sensed in the cells and how is it monitored. This area of plant metabolism is a rapidly growing area due to the expanding genetic tools and approaches that can be utilized. Interestingly, a member of the sucrose transporter family has been suggested to be a sensor and thus have a role in sucrose regulation (Barker *et al.*, 2000). SUT2/SUC3 was first proposed to have a sensing function from its hydropathy plot that revealed an extended cytosolic loop in the middle of the protein between transmembrane domains 6 and 7, which were thought to be similar to the extended N-terminal regions of the yeast sensors RGT2 and SNF3 (Lalonde *et al.*, 1999). Initially no transport was detected for SUT2/SUC3 (Barker *et al.*, 2000), as had been seen with the yeast sensors, but later studies found that it did transport sucrose albeit with a lower affinity for sucrose (Schulze *et al.*, 2000). This low sucrose affinity was defined not by the extended central cytosolic loop, but by the N-

terminal region (Schulze *et al.*, 2000; Meyer *et al.*, 2000). A sugar sensing capability for AtSUT2/AtSUC3 has not been ruled out as it has yet to be fully investigated.

The discovery that LeSUT1, LeSUT2 and LeSUT4 were co-localized in the enucleate sieve elements in tomato (and other solanaceous species such as potato and tobacco) prompted the question of whether these sucrose transporters interacted together to regulate sucrose influx, especially since nuclear regulation would be more problematical (Reinders *et al.*, 2002). Using the split-ubiquitin system, it was found that SUT1/SUC2 and SUT2/SUC3 have the potential to form homooligomers (Reinders *et al.*, 2002). Maybe the extended cytosolic central loop of SUT2/SUC3 is required during this interaction.

It is now apparent that the possibilities of sugar transporter regulation also include a putative sucrose transporter/sensor and protein-protein interactions between sucrose transporters in addition to the various other points of regulation summarized at the start of this discussion. As research continues in this field of sugar transporter regulation and more studies are published a better understanding of carbon partitioning will emerge.

Chapter 5

Identification of genes responsive to far-red light using an *Arabidopsis* membrane transporter microarray

5.1. Introduction

The previous chapters demonstrate that sugar transporters have specific locations and functions and are differentially regulated. *Arabidopsis* has a high number of putative sugar transporters (The *Arabidopsis* Genome Initiative, 2000). Therefore, revealing their roles will require a lot of experimental data. The development of microarray technology has enabled researchers to compare the transcriptional regulation of thousands of genes simultaneously in response to an experimental variable (Aharoni and Vorst, 2002). This high throughput, economical method for a comprehensive global analysis of gene expression is now becoming the preferred method of transcript profiling (for a review of gene expression analysis by transcript profiling see the comprehensive review by Donson *et al.*, 2002). This chapter presents novel data on the transcriptional regulation of *Arabidopsis* transporter genes in seedlings in response to far-red light. Sugar transporter genes in the *Arabidopsis* genome, which are present on the custom-made *Arabidopsis* membrane transporter (AMT) array were also subjected to phylogenetic analysis to investigate how the genes grouped together in families. As well as suggesting novel roles for sugar transporters in the far-red de-etiolation of seedlings, the microarray data was also utilized in investigating putative *cis*-regulatory elements that may be targets in light signalling.

5.1.1. The development of microarray technology

Although the principle used in microarrays was published many years ago (Kafatos *et al.*, 1979), it took a long time for microarray technology to be used for the first time. The first microarray was developed at Stanford and utilized a set of ESTs (Schena *et al.*, 1995). Two types of chips are commonly used in microarray technology: those containing DNA fragments (usually PCR-amplified DNA sequences) immobilized on a solid surface; or those containing oligonucleotides synthesized *in situ* on a glass surface. The chips are hybridized with two fluorescently-labeled probes derived from the mRNA population present in the samples. The hybridized microarray is then scanned with a laser and the signal reflects the relative mRNA abundance for each gene represented on the array. The use of two differently labeled samples allows the quantitative comparison of gene expression between

a control and a test experiment. With the completion of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000) this technology has begun to be exploited near to its full capacity as DNA oligonucleotide microarray chips can now be produced which contain sequences representing the full genome (Aharoni and Vorst, 2001; Wisman and Ohlrogge, 2000; Wu *et al.*, 2001). Affymetrix® has brought together the development in microarray technology and sequencing of the *Arabidopsis* genome to produce the GeneChip® *Arabidopsis* ATH1Genome Array (www.affymetrix.com). The array contains probes synthesized *in situ* and designed to measure temporal and spatial gene expression in over 24,000 gene sequences. Other companies now offer custom-made DNA oligonucleotide microarray chips, which enable laboratories to tailor the selection of genes to their particular research interests. Plant molecular biologists have been quick to capitalise on the efficiencies of microarrays in the screening of responsive genes and have provided valuable insights into the regulation of plant development, metabolism and physiology by nuclear gene expression (Buckhout and Thimm, 2003; Mele and Hake, 2003; Tabata, 2002).

5.1.2. New technology, new problems

Although DNA microarray technology has revolutionized the study of gene expression, it is prone to a number of methodological and interpretive pitfalls (Kazan *et al.*, 2001; Donson *et al.*, 2002) and caution must be applied when drawing conclusions from microarray data. Microarray data not only incorporates the inherent deviations that come with biological research, but also compounds this with the technical limitations that are inevitable when developing new micro-technology. Generally, the smaller the scale of an experiment the higher the variation imposed on it and microarrays have many sources of experimental error (table 5.1). The sources of experimental error listed arise from a mix of chemistry, photo-physics and engineering. Reducing experimental errors from data derived from microarrays can be achieved by normalizing the data. However, although many useful normalization strategies have been developed, no single method is thorough enough to have become standard (Finkelstein *et al.*, 2001). When choosing or devising normalization methods it must be remembered that microarray data is often not distributed normally (Finkelstein *et al.*, 2001).

The design of a microarray chip can vastly reduce experimental errors and thus the task of normalization. Since background noise is variable within the microchip due to it being highly sensitive to surface chemistry and washing methods (Schuchhardt *et al.*, 2000), oligo/cDNA arrangement should be random and controls ought to be present throughout the microchip. Controls could be a set of housekeeping genes whose transcriptional activity should not react to the experimental condition.

Table 5.1. Some of the major source of fluctuations in microarray experiments. Adapted from Schuchhardt et al., 2000

Source of Flucuation	Description
mRNA preparation	Different tissues have different degree of RNA degradation
cDNA synthesis	RT-PCR will produce cDNA species of varying lengths and thus probe specificities
Labelling	Labelling may fluctuate depending on nucleotide composition and dye
Pin Geometry	Pins have different characteristics and surface properties and therefore transporter different amounts of DNA
Target Fixation	The fraction of DNA that is chemically linked to the slide surface from the droplet is unknown
Hybridization parameters	The efficiency of the hybridisation reaction is influenced by a number of experimental parameters.
Slide inhomogeneities	The DNA maybe distributed unequally over the slide or the hybridisation may perform differently in different parts of the slide
Non-specific hybridization	A common source of error in molecular biology that cannot be completely excluded
Non-specific background and over-shining	Non-specific radiation and signals from neighbouring spots
Image analysis	Inaccurate scanning, saturation effects and variations in spot shape

Wu *et al.* (2001) has complied a list from data available from the AFGC (*Arabidopsis* Functional Genomic Consortium, http://afgc,stanford.edu), of thirty DNA elements with the most stable expression patterns, and conversely a top thirty of the most variable. Alternatively, spiking controls can be utilized to supplement normalization. These spiking controls comprise non-homologous genes from a genetically distant organism that are included on the microchip array. Then *in vitro* transcribed RNAs of clones to these genes are added (spiked) to each test sample prior to dye labelling. The theory being that in the absence of experimental error, spiking controls should produce a ratio of 1. Spiking controls are a remedy for differences in dye incorporation that is probably due to their large molecular size. Normalization by spiking controls is dependent on accurate measurement and aliquoting of RNA, which when working with such small quantities can prove challenging.

Designing bespoke microchips has the advantages of enabling the oligos contained on the microchip to cater more specifically to an area of research. However, as noted by Finkelstein *et al.*, (2002), if a bespoke array is too focused new phenomena may go undetected. Therefore a microarray chip ideally would also include "detective genes" which are known to monitor pathways and metabolic processes outside the experimental focus of the array (Finkelstein *et al.*, 2002).

Once the microarray chip has been designed a protocol must be devised. A comprehensive list of protocols, research group websites and company websites is available from The *Arabidopsis* Information Resource (TAIR, http://www.*Arabidopsis*.org/info/expression/index.jsp, Huala *et al.*, 2001). As with all experiments the method of obtaining the data can affect the results and with adaptations to protocols evolving so rapidly, methods must be stated sufficiently to allow scrutiny. With this in mind Brazma *et al.* (2001) suggested a guideline for the minimum information about a microarray experiment in an effort to form some standards that would enable easier comparison and analysis.

There is no doubt that microarray experiments will evolve and improve not only with regard to the design and applications to biological research (Finkelstein *et al.*, 2002; Wullschledger and Difazio, 2003), but also in the accuracy of the results obtained from the data they produce (Yuen *et al.*, 2002; Yang and Speed, 2002).

5.1.3. Regulation of gene expression by FR light

As described in the general introduction, plants monitor light using a variety of photoreceptors. The phytochrome family consists of five members, which react to levels of red (R) and far-red (FR) light from the environment (Whitelam *et al.*, 1998). The R/FR ratio provides signals

that inform the plant of the appropriate developmental strategy. De-etiolation of seedlings by FR light is known to be instigated exclusively by PHYA (Nagatani *et al.*, 1993). Microarray experiments in different mutant backgrounds have been used to investigate the contribution of various loci that have been shown to be affect phytochrome signalling (Wang *et al.*, 2002; Ma *et al.*, 2003). Of particular relevance to this chapter is a microarray experiment by Tepperman *et al.* (2001), which used a high-density oligonucleotide Affymetrix microarray chip that contained probe sets to approximately 8,200 genes to assess the regulation of various genes by PHYA during de-etiolation under FR light. The effect of PHYA was explored by comparing WT and *phyA-101* (phyA null mutant) seedlings when grown in the dark for 4 and 5 days and also when after 4 days of etiolated growth they were irradiated for 1, 3, 6, 12, 18 and 24 h with continuous FR (Tepperman *et al.*, 2001). Using the common criteria of a 2-fold increase or decrease in gene expression to characterise up-regulated and down-regulated genes by PHYA signalling, 10% of the genes on the array were identified as being regulated by FR, with 67% showing up-regulation and 33% down-regulation (Tepperman *et al.*, 2001). The data was also analysed with regards to the functional classification of the gene and also whether a response was early (up to 1 h) or late (3-24 h). This gave a unique insight into how seedlings respond genomically to control photomorphogenesis during FR-mediated de-etiolation. Investigation of the supplementary data supplied by Tepperman *et al.* (2001) revealed that two sugar transporters responded to the FR treatment, one of which was AtSUC1 and the other At4g36670, a putative *mannitol* transporter (according to annotations in the NCBI database) that is uncharacterised (identified in this study using the accession code given by Tepperman *et al.*, 2001). Interestingly the two sugar transporters showed opposite transcriptional regulation by the photoreceptor, phytochrome A, during de-etiolation under FR light (Tepperman *et al.*, 2001). Both genes were classified as late regulated, but while *AtSUC1* was induced, the mannitol transporter, At4g36670, was classified as a late repressed gene.

In this chapter the effect of a FR light treatment on the expression of *Arabidopsis* transporters is described using an oligonucleotide AMT array representing all of the genes coding for *Arabidopsis* transporters. The microarray used in the previous analysis (Tepperman *et al.*, 2001) had a much smaller complement of transporters than that used here and the experiment described in this chapter should provide a more representative picture of changes in transporter gene expression in seedlings adapting to their light environment.

5.2. Results

5.2.1. Design of the *Arabidopsis* Membrane Transporter (AMT) array

The oligonucleotide-based microarray was designed and produced as part of a project involving seven laboratories, all focusing on different aspects of plant membrane transport processes (Maathuis *et al.*, 2003). Each group in the consortium contributed a list of *Arabidopsis* transporter genes, including those that were of relevance to their research. Work described here identifies a list of mRNA sequences for all known and putative *Arabidopsis* sugar transporters; other members of this laboratory and the consortium were responsible for other transporter families. The list was compiled using the *Arabidopsis* Membrane Protein Library (AMPL) produced by John Ward (http://www.cbs.umn.edu/*Arabidopsis*/; Ward, 2001) and updated by searching for homologues of all known plant sugar transporters in M*at*DB (http://mips.gsf.de/proj/thal/, Schoof, *et al.*, 2002) and EMBI at the EBI (http://srs.ebi.ac.uk/). The list of *Arabidopsis* sugar transporters is given in table 5.2. The *Arabidopsis* Membrane Transporter (AMT) array comprises a total of 1250 oligonucleotide (50-mers) representing 1153 genes of which 57 are controls (Maathuis *et al.*, 2003). The oligonucleotides were designed at MWG (Ebersberg, Germany) on the basis of confirmed or predicted mRNA sequences. All probes were spotted in replicate on each array. The use of 50-mers rather than cDNAs allowed the design of gene-specific probes for nearly all genes represented on the array. In only eight cases were gene sequences too similar to ensure specific hybridisation signals, including two putative, unnamed sugar transporters At2g16130 and At2g16120 (92.7% homology) (Maathuis *et al.*, 2003). Most genes have been assigned to labelled transporter families based either on functional characterisation or, more frequently, on the basis of sequence homology with characterised transporters. Genes in the group labelled "other transporters" are homologous with members of transporter families characterised in non-plant systems and genes in the group labelled "putative transporters" were represented on the array since their high number of predicted membrane spanning domains indicated that these encode integral membrane proteins potentially mediating transport functions (Maathuis *et al.*, 2003). Finally, it should be noted that although most genes were represented by one probe on the AMT array, 54 ABC transporters were represented by two or three oligonucleotide probes (Maathuis *et al.*, 2003).

Table 5.2 List of *Arabidopsis* sugar transporters. Some sugar transporters are putatively identified as their function was predicted based on sequence similarity to known sugar transporters. The far right column shows the families in which they are characterised according to the *Arabidopsis* Membrane Protein Library. Key: SPF, Sucrose Permease Family; PSTF, Putative Sugar Transporter Family; STLPF, Sugar Transporter Like Protein Family; STF, Sugar Transporter Family.

MIPS Code	Arabidopsis ESTs	mRNA matches	PROSITE Motifs	Family (AMPL)
SUC - SUCROSE SYMPORTER FAMILY				
At1g22710 ***SUC2***	AI100290; N65067; H36128; T42333; H36415; T76707; R64756; N65734; AV518914; AV533487; AV533123; AV545384; AV547645; AV560279; AV560168; AV564693; AV784150; AV787413; AV786791; AV790811; AV792069; AV793317; AV793671; AV793757; AV794453;	AY048256, AY050986, AY091774, AY113946, BT000684		SPF
At1g09960 ***SUC4***		AF175321, AY072092, BT004418		SPF
At1g66570 ***SUC7***				SPF
At1g71880 ***SUC1***	AI996403; AA395728; AV439701; AV441803; AV521964; AV522557; AV552257; AV785703; AV790369; AV792989; AV794551;	AY049275	426-436 Prokaryotic membrane lipoprotein lipid attachment site	SPF
At1g71890 ***SUC5***	AV556349;			SPF
At2g02860 ***SUC3***	F14112; AV539384; AV543570;	AJ289165	57-67 Prokaryotic membrane lipoprotein lipid attachment site, 448-458 Prokaryotic membrane lipoprotein lipid attachment site	SPF
At2g14670 ***SUC8***				SPF
AT5g06170 ***SUC9***				SPF
AT5g43610 ***SUC6***				SPF

STF1 - SUGAR TRANSPORTER FAMILY 1				
At1g75220	AI992471; H76587; AV439711; AV526551; AV560962;	AF412060, AY124845	105-121 Sugar transport proteins signatures, 147-172 Sugar transport proteins signatures	PSTF
At1g19450	AV538385; AV550650; AV555051; AV560291; AV563014; AV562335; AV792147;	AY059848, AY093274	389-399 Prokaryotic membrane lipoprotein lipid attachment site, 106-122 Sugar transport proteins signatures, 148-173 Sugar transport proteins signatures	PSTF
At1g54750				PSTF
At1g54730	AI996511; AV539996;	AY054504	7-23 Sugar transport proteins signatures	PSTF
At1g54740	R65549;			PSTF
At3g05150	AU228397; AU237355;		330-340 Prokaryotic membrane lipoprotein lipid attachment site, 85-101 Sugar transport proteins signatures	PSTF
At5g18840		AY064144, BT000608		PSTF
At2g48020	AI999333; AV522458; AV525755; AV783856;	AY120715, BT000053		PSTF
At4g04750	AU230309;		35-45 Prokaryotic membrane lipoprotein lipid attachment site, 42-52 Prokaryotic membrane lipoprotein lipid attachment site, 318-328 Prokaryotic membrane lipoprotein lipid attachment site, 114-139 Sugar transport proteins signatures	PSTF
At1g08900 ***AtSUGTL2***		AY072458, AF386931	124-149 Sugar transport proteins signatures	PSTF
At4g04760			30-40 Prokaryotic membrane lipoprotein lipid attachment site, 37-47 Prokaryotic membrane lipoprotein lipid attachment site, 314-324 Prokaryotic membrane lipoprotein lipid attachment site, 109-134 Sugar transport proteins signatures	PSTF
At1g08890 ***AtSUGTL4***	AI999682; AV522229; AV560352; AV565106;		33-43 Prokaryotic membrane lipoprotein lipid attachment site, 183-208 Sugar transport proteins signatures	PSTF
At1g08930 ***AtSUGTL1***	T22578; N37585; T43808; AA395424; AV784302; AV791575;	D89051	83-126 Bacterial regulatory proteins, araC family signature and profile, 157-182 Sugar transport proteins signatures	PSTF
At1g08920 ***AtSUGTL3***	AI997735; W43122; AV557816; AV788757;	AF367260, AY133547	49-64 Phosphopantetheine attachment site, 159-169 Prokaryotic membrane lipoprotein lipid attachment site, 89-105 Sugar transport proteins signatures, 319-334 Sugar transport proteins signatures	PSTF
At3g05400	N38010; AU226281; AU235554; AV560822;	AY063856, AY091216		PSTF

AtSUGTL5	AV561241; AV563791; AV565018; AV788665;			
At3g05165		AY048207, AY077661		PSTF
At3g05160				PSTF
At5g27360	AA042276; AU230307; AU239043; AV562877; AV788580;	AY026255		PSTF
At5g27350	H36312; F14224; AV519573; AV526613; AV527547; AV536931; AV545199; AV553899; AV558613; AV785205;	AY026254, AF410282, AY143858		PSTF
STF2 - SUGAR TRANSPORTER FAMILY 2				
At1g20840 ***AtSUGTRPR***	AA651168; AA712259; R65557; T75686; T04824; H76831; AV528162; AV543558; AV546664; AV548888; AV554138; AV564253;		61-77 Sugar transport proteins signatures, 103-128 Sugar transport proteins signatures	STLPF
At4g35300 ***AtPHS1*** (F23E12.140)	AI997242; AV518219; AV520494; AV524916; AV526144; AV526825;	AY094465		STLPF
At3g51490			63-79 Sugar transport proteins signatures, 105-130 Sugar transport proteins signatures	STLPF
At2g35740			332-348 Sugar transport proteins signatures, 128-153 Sugar transport proteins signatures	PSTF
At4g16480	AU227953; AU236946; AV524951;		346-356 Prokaryotic membrane lipoprotein lipid attachment site, 333-349 Sugar transport proteins signatures, 129-154 Sugar transport proteins signatures	PSTF
At1g30220		AY074333, AY123031	177-190 Lipocalin signature, 331-347 Sugar transport proteins signatures, 130-155 Sugar transport proteins signatures	PSTF
At2g43330	AA585906; AI099661; AI993234; T75731; AV532568; AV566390;	AY063901, AY096505	133-158 Sugar transport proteins signatures	PSTF
STF3 - SUGAR TRANSPORTER FAMILY 3				
At1g79410	AV793073;	AY125523		STF
At1g16370	AU228939; AU237843;			STF
At1g79360	AU226012; AU235342;			STF
At1g16390	AV537940; AV550068; AV562782; AV781434;	AY078972, BT000863	122-132 Prokaryotic membrane lipoprotein lipid attachment site	STF

At1g73220	AV561523; AV565117; AV790418;	BT002455	397-407 Prokaryotic membrane lipoprotein lipid attachment site	STF
At3g20660			125-135 Prokaryotic membrane lipoprotein lipid attachment site, 385-400 Sugar transport proteins signatures, 193-218 Sugar transport proteins signatures	STF
At3g13050		AY054262, BT004522	357-372 Sugar transport proteins signatures	STF
STF4 - SUGAR TRANSPORTER FAMILY 4				
At1g79820	F14093; F14007; AV531971; AV532038; AV794509;	AY080624	214-229 Sugar transport proteins signatures, 23-48 Sugar transport proteins signatures	PSTF
At1g67300	AI993759; N96130; AV522420; AV786729;		110-127 Sugar transport proteins signatures, 345-361 Sugar transport proteins signatures	PSTF
At1g05030			81-106 Sugar transport proteins signatures	PSTF
At5g16150 ***pGlcT***	AA713112; T46284;	AY058152, AY091052, AY117359		PSTF
STP - HEXOSE **S**UGAR **T**RANS**P**ORTER FAMILY				
At1g11260 ***STP1***	AI100271; AI995613; H37045; H37050; R65119; T42183; T42226; R65439; R90498; R90677; H36936; N96990; AA042710; Z47057; AV440317; AV518501; AV518543; AV525850; AV537114; AV539265; AV540986; AV541233; AV543372; AV547497; AV551671; AV552071; AV788620; AV790618; AV791202;	AY054249, AY059781, AY133845	358-368 Prokaryotic membrane lipoprotein lipid attachment site, 338-354 Sugar transport proteins signatures, 142-167 Sugar transport proteins signatures	PSTF
At1g07340 ***STP2***			141-166, 336-352 Sugar transport proteins signatures	PSTF
At5g61520 ***STP3***	AI997045; AV535065;	ATAJ2399, BT000560, AY128303	333-349 Sugar transport proteins signatures, 138-163 Sugar transport proteins signatures	PSTF
At3g19930 ***STP4***	AA597981; AI998516; AA395572; AV440378; AV540065; AV542252; AV544189; AV547906; AV552668; AV791740;	AF428342, AF367352, AY133592	376-383 ATP/GTP-binding site motif A (P-loop), 337-353 Sugar transport proteins signatures, 141-166 Sugar transport proteins signatures	PSTF
At1g34580 ***STP5***	AU230677; AU239376; AV557150;	AK118511	24-34 Prokaryotic membrane lipoprotein lipid attachment site, 207-217 Prokaryotic membrane lipoprotein lipid attachment site, 142-167 Sugar transport proteins signatures	PSTF
At3g05960 ***STP6***			139-164 Sugar transport proteins signatures	PSTF
At4g02050	AI995708; AU227163; AU236285;		340-355 Sugar transport proteins signatures, 145-170 Sugar transport	PSTF

STP7			proteins signatures	
At5g26250 ***STP8***				PSTF
At1g50310 ***STP9***			340-356 Sugar transport proteins signatures, 144-169 Sugar transport proteins signatures	PSTF
At3g19940 ***STP10***			339-355 Sugar transport proteins signatures, 144-169 Sugar transport proteins signatures	PSTF
At5g23270 ***STP11***			211-221 Prokaryotic membrane lipoprotein lipid attachment site, 340-356 Sugar transport proteins signatures, 144-169 Sugar transport proteins signatures	PSTF
At4g21480 ***STP12***			336-352 Sugar transport proteins signatures, 142-167 Sugar transport proteins signatures	PSTF
At5g26340 ***STP13***	AA651163; R65136; H36990; AV439925; AV441340; AV442368; AV442013; AV526563; AV782745; AV784109; AV789913; AV793709;	AF250340, AY045591, AY052692, AY143909	510-517 ATP/GTP-binding site motif A (P-loop), 339-355 Sugar transport proteins signatures	PSTF
At1g77210 ***STP14***	AU231302; AU239941; AV441077; AV442769;	AK119057	341-357 Sugar transport proteins signatures, 145-170 Sugar transport proteins signatures	PSTF
PMTF – PUTATIVE MANNITOL TRANSPORTER FAMILY				
At4g36670	AI997793; AU230198; AU238947;	AK118092	Annotated as putative mannitol transporter in NCBI database 74-90 Sugar transport proteins signatures	PSTF
At2g18480			Annotated as putative mannitol transporter in NCBI database 75-82 ATP/GTP-binding site motif A (P-loop), 79-95 Sugar transport proteins signatures, 121-146 Sugar transport proteins signatures	PSTF
At3g18830	AV534200; AV545725; AV549998; AV565268; AV784947;	AY065183	Annotated as putative mannitol transporter in NCBI database	PSTF
At2g16120			Annotated as putative mannitol transporter in NCBI database 83-99 Sugar transport proteins signatures, 125-150 Sugar transport proteins signatures	PSTF
At2g16130			Annotated as putative mannitol transporter in NCBI database 83-99 Sugar transport proteins signatures, 125-150 Sugar transport proteins signatures	PSTF

STF5 - SUGAR TRANSPORTER FAMILY 5				
At3g03090	AA713083; AI999720;		200-216 Sugar transport proteins signatures	PSTF
At5g17010	AU236320;		112-128 Sugar transport proteins signatures, 325-340 Sugar transport proteins signatures, 154-179 Sugar transport proteins signatures	PSTF
At5g59250	Z48412; Z48411; AV548032; AV782266; AV791971; AV792766;		181-191 Prokaryotic membrane lipoprotein lipid attachment site, 436-451 Sugar transport proteins signatures	PSTF
At2g20780	F14099;		133-149 Sugar transport proteins signatures, 175-200 Sugar transport proteins signatures	PSTF

5.2.2. Phylogentic analysis of the sugar transporters

A phylogenetic analysis of the sugar transporters represented on the AMT array was carried out to determine how related they were to each other as this might supply clues as to the roles of some of the uncharacterised sugar transporters. The amino acid sequence of the 70 sugar transporters was compiled, and a phylogenetic tree was constructed with bootstrap values for 500 replicates, which is the maximum parsimony (figure 5.1). The phylogenetic tree shows eight separate clusters, which may suggest eight separate families (figure 5.1). The AMPL (http://www.cbs.umn.edu/*Arabidopsis*/) suggests that there are four families of sugar transporters. Figure 5.2 shows a copy of the phylogenetic tree from figure 5.1, but illustrates the four families that these sugar transporters belong to according the AMPL. The phylogenetic analysis carried out in this study indicates that the family of STP hexose transporters that is incorporated into a large family of 51 putative sugar transporters in the AMPL database is a distinct family and also shows that 6 genes which have been suggested to be putative mannitol transporters (NCBI database) and which were also included in the putative sugar transporter family of the AMPL database, also form a separate family (figure 5.2).

5.2.3. Analysis of expression of *Arabidopsis* transporters genes in response to FR light

Total RNA was extracted from two batches of seedlings; the first were grown continuously in the dark for 84 h and the second were grown in the dark for 72 h then put under FR light illumination for 12 h. After the RNA was purified and quantified, 100 µg was used to produce the labelled cDNA, which was hybridised to the microarray chip. The microarray chip was scanned at high resolution and is shown in figure 5.3. After careful calibration of the grid used to circle each spot, the microarray chip was analysed using Scanalyze software (Version 2.35, B Eisen, Stanford, USA) to produce an Excel spreadsheet with comprehensive data regarding each spot, including the position, spot intensity, background and most importantly its MRAT. The MRAT refers to the median of the set of background corrected single pixel ratios for all pixels within the spot (ScanAlyze User Manual, 1998-9, Stanford University). Unlike a mean value a median gives a better representation of a group of data that is not normally distributed. Since the pixels that make up a spot may include extreme values that may arise from an artefact it is advisable to use the median.

Initially a mean of the replicates for each gene was produced. Each gene had two replicates of the same oligo, except the ABC genes that contained two replicates of three different oligos for each gene. Then the background to signal ratio was assessed and those genes that showed signal

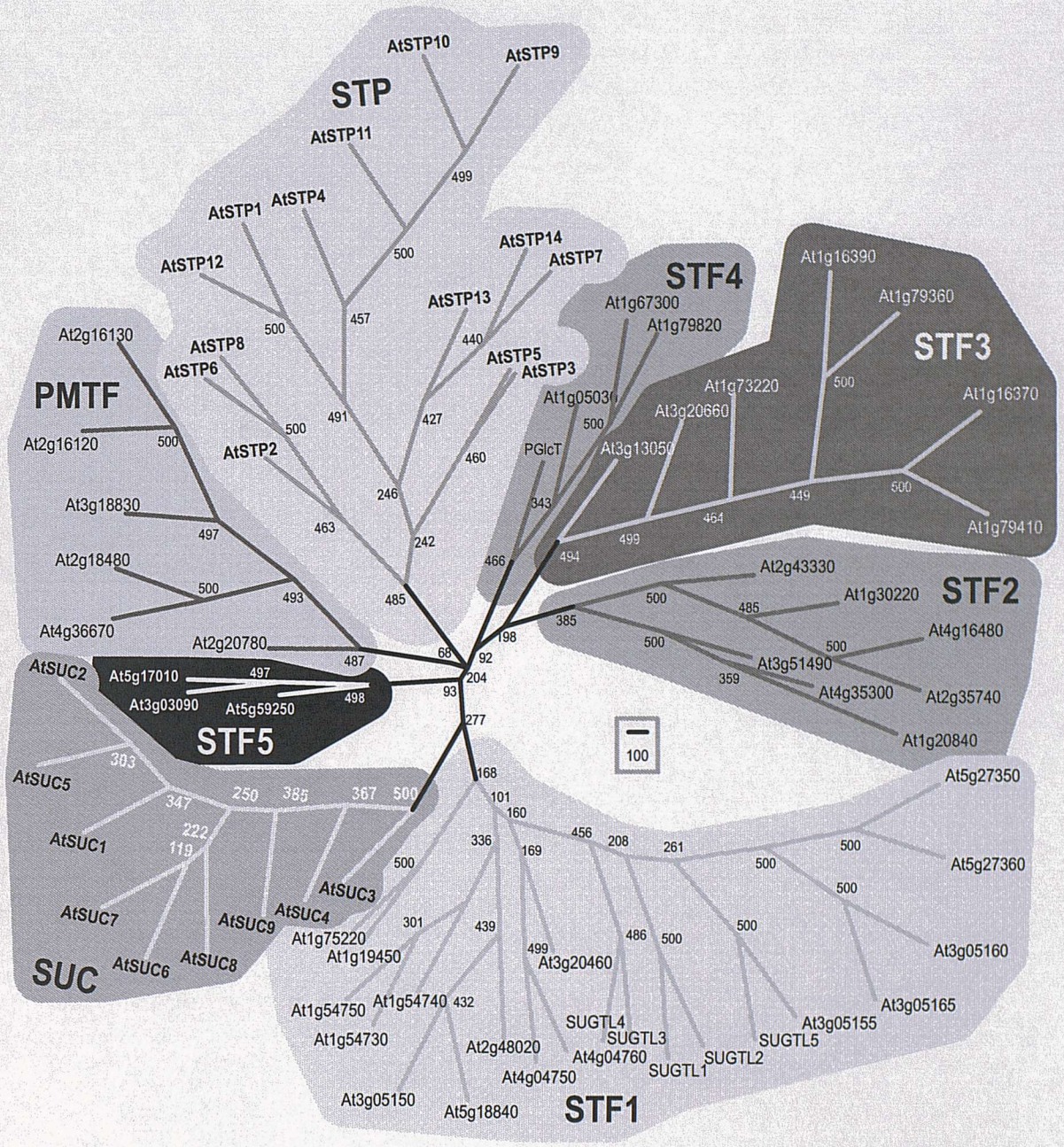


Figure 5.1. Phylogenetic tree analysis of all proposed *Arabidopsis* sugar transporters. A list of characterized and putative *Arabidopsis* sugar transporters was compiled using a combination of websites (MIPS (http://www.mips.biochem.mpg.de/proj/thal/db/index.html), TAIR BLAST (http://www.arabidopsis.org/Blast/), *Arabidopsis* Membrane Protein Library (http://www.cbs.umn.edu/arabidopsis/), EMBL (http://srs.ebi.ac.uk/)). Bootstrap values shown at internal nodes indicate their occurrence in 500 replicate analyses. MIPS codes are given and in some cases sugar transporters names are stated where possible (see table 5.1 for MIPS codes to named sugar transporters. The putative hexose symporter family (STP) and the putative sucrose symporter family (SUC) include some well characterised sugar transporters. All other sugar transporter families (STF1-5) and the putative mannitol transporter family (PMTF) are novel and contrast to those given in the AMPL.

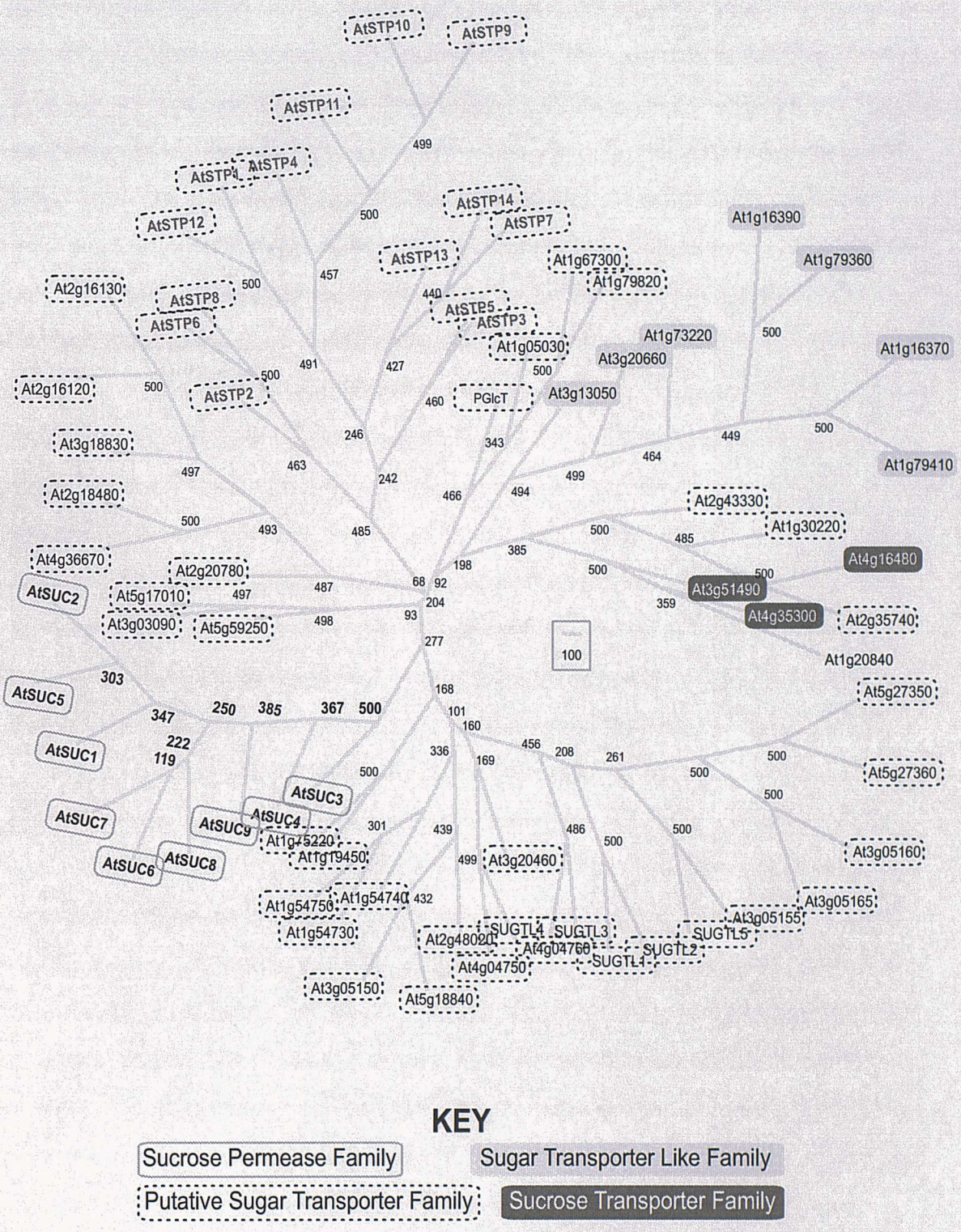


Figure 5.2. Phylogenetic tree analysis of the new classification of sugar transporter families. The phylogenetic tree is identical to that shown in figure 5.1, however indicated in this diagram are the original families that each sugar transporter belongs to according to the *Arabidopsis* Membrane Protein Library (http://www.cbs.umn.edu/arabidopsis/).

intensities of less than twice that of the background were deemed as unreliable. This was necessary because the hybridised microarray chip showed a red colour cast that varied across the microarray chip; this was thought to be a result of incomplete washing of the chip, which is supposed to remove any unattached dyes after hybridisation. Table 5.3 shows the mean and median for the spot intensity, spot background and the ratio of the two (spot I/B ratio) for each dye which represents the level of noise. Comparison of the median spot I/B ratio showed that Cy5 had much more background interference; furthermore, despite the fact that the median Cy5 spot intensity was ~2 fold-higher than that of Cy3, the Cy5 background was 10-fold higher than that of the Cy3 dye. The higher spot background in the Cy5 dye has caused a large number of genes to incur very low spot I/B ratios as indicated by the very low mean spot I/B ratio in comparison to that of the Cy3 dye. Even though each value of each spot was determined taking into account its background (a remedy to the "normal" spatial variability of the background), in this extreme case such interference with the results cannot be ruled out. This stringent approach meant that the possibility of the high background causing false positives or negatives was kept to a minimum. A disappointing consequence was that 45% of the genes were deemed too unreliable to consider in the analysis. Undoubtedly this compromised the global aim of the experiment and result in the loss of potentially important results. However, the intention was to repeat the experiment to provide a more reliable dataset for analysis.

The spiking controls are genes added to the microarray that do not feature within the experimental genes. A known and equal amount of *in vitro* RNA is added (spiked) to each of the experimental RNA samples prior to labelled cDNA synthesis. Thus, in theory, the Cy3/Cy5 ratio and therefore the MRAT value for each of the spiking control spots should be 1. Due to the low spot I/B of the Cy5 dye, 4 out of 8 spiking control genes were dismissed. Of the 4 that remained, the MRAT for these spiking controls ranged from 0.79 – 4.44 with a mean of 2.6. This mean value should be used to calibrate the MRATs for the rest of the genes to give an accurate representation of its relative increase or decrease *in vivo*. It was decided that due to the discrepancies associated with the high Cy5 background that had affected the spiking controls and also the wide deviation in their MRATs, that applying this calibration would not be worthwhile until a second set of data from a repeat experiment was available. In any case, using the spiking controls to calibrate the data would not alter the order of the transporters affected by FR light. However, it is recognised that such a calibration would alter which genes were above or below the thresholds set to denote up and down-regulated genes.

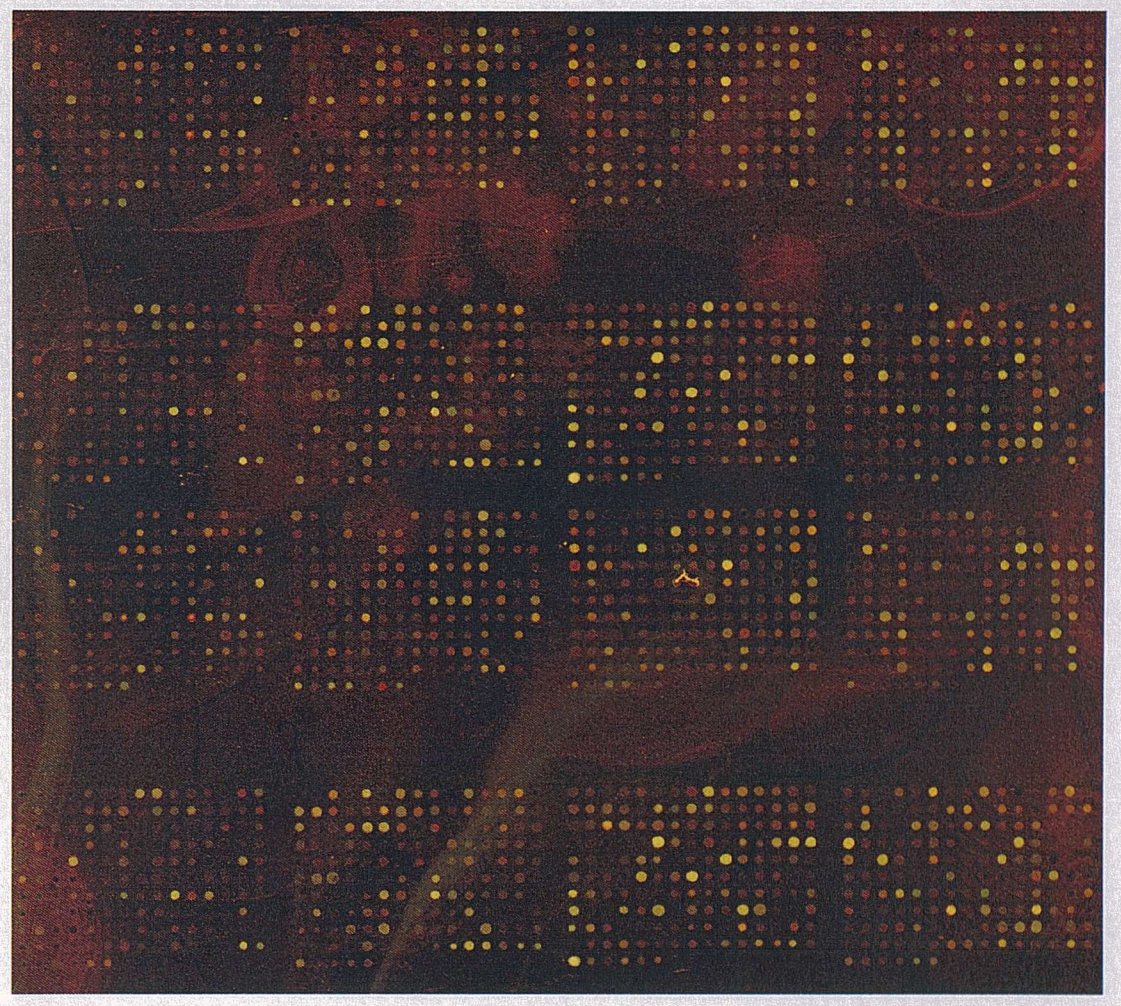


Figure 5.3. Scan of the microarray post hybridization with the labeled cDNA. 100 µg total RNA from etiolated and far-red light treated *Arabidopsis* seedlings was isolated and the derived cDNA was labeled with separate dyes. The labeled cDNAs were hybridized to the microarray and visualised using an Axon microchip scanner. Bar = 1 mm.

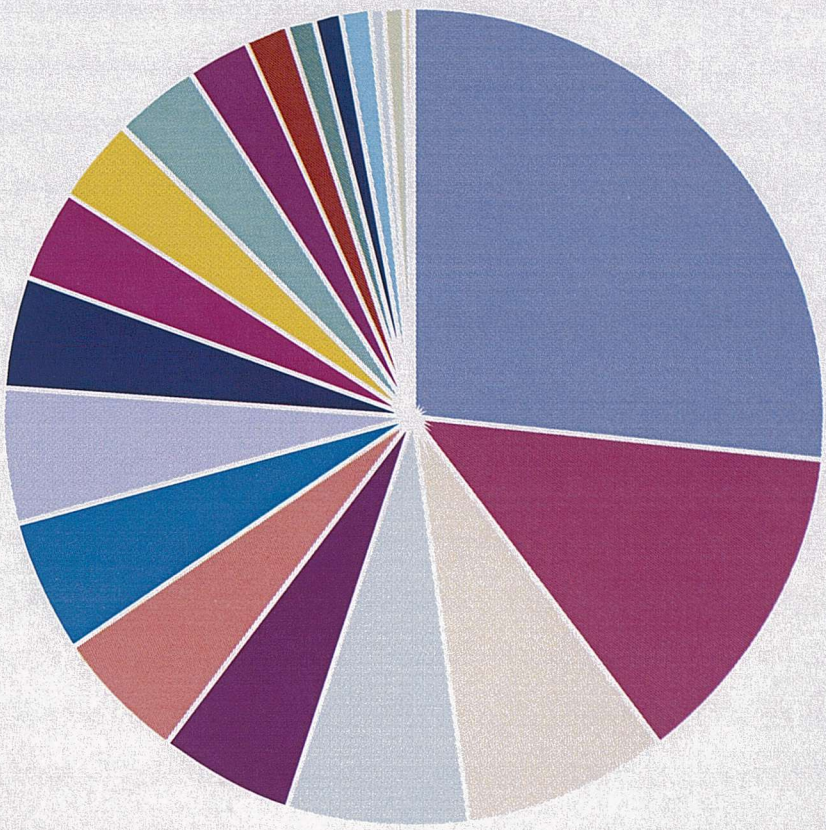
Table 5.3. Table showing the spot intensity and background values for each dye. The ratio of the intensity and background of each spot were calculated. Comprising all spots the medians and means of the mean spot intensity, mean spot background and ratio of spot intensity/background were calculated for each of the channels (Cy3, green dye; Cy5, red dye).

	Cy3 Intensity	Cy3 Background	Cy5 Intensity	Cy5 Background
Median	323	41	821	460
Median I/B Ratio	**8**		**2**	
Mean	1305	64	2151	502
Mean I/B Ratio	**20**		**4**	

The oligos on the microarray chip also included a number of constitutive controls, chosen due to their characterized responses to a range of environmental factors. Concentrating on the effect of light, Ma *et al.* (2001) produced a table of genes that have been reported in the literature and their responses to various light conditions. Using this table it is possible to compare values obtained from the microarray data featured in this chapter. Ma *et al.* (2001) showed that WT seedlings grown in FR light produced a large induction, 19.6-fold, in the *Lhc1B2* gene that codes for photosystem II chlorophyll *a/b* binding protein and a slight induction, 3.6-fold, of the plastocyanin gene. In comparison, the microarray data obtained here showed much less of an induction, 3.9-fold for *Lhb1B2*, but a similar induction of 3.6-fold, for the plastocyanin gene. However the accession codes were different and following database searches of EMBL at the EBI (www.ebi.ac.uk) and sequence alignments using clustalW (data not shown) it appears that whereas *Lhb1B2* referred to by Ma *et al.*, (2001) is likely to be the same gene (95.6% identity) the plastocyanin gene may be a different one (73.6% identity). Thus, while the differing results obtained for the *Lhb1B2* can be compared the same cannot be said for the plastocyanin gene.

The thresholds set to categorise genes were based on a 2-fold increase or decrease relative to the control conditions (etiolated seedlings grown entirely in darkness). For example, genes with a MRAT of ≤ 0.5 were considered as down-regulated by FR light and those genes with a MRAT of ≥ 2 were considered as up-regulated by FR light.

Of the 1096 genes included on the microarray chip (excluding spiking controls), 250 of the genes were classified as up-regulated. That is 23% of all of genes on the microarray or 41% of all the genes that produced a sufficient I/B ratio. The contribution of transporter families in the FR up-regulated genes were analysed and are shown in figure 5.4. The largest group accounting for almost a quarter of all transporters up-regulated have yet to be classified and characterized. Second on the list are members of the ATP binding cassette (ABC) superfamily, a large and diverse group of proteins, most of which are primary pumps that use ATP hydrolysis to drive transport (Theodoulou *et al.*, 2000). Third on the list is the family of amino acid transporters which are important in protein biosynthesis, an expected requirement of seedlings undergoing photomorphogenesis (Ortiz-Lopez *et al.,* 2000). Fourth are the family which will be expored later, sugar transporters and invertases family that are responsible for the movement of sugars to fuel growth. Metals have many varied roles in plants including cofactors for enzymes, transcription factors and proteins and of course metal ion homeostasis and are 8th largest family of transporters to be up-regulated in *Arabidopsis* seedlings during de-etiolation by FR light (for a review on metal transporters in plants refer to Williams *et al.*,



Putative Transporters	26.6%	Water channel	3.2%
ABC transporters	13.1%	Antiporters	2.5%
Amino Acid transporters	8.2%	Glutamate receptors	1.8%
Sugar transporters & Invertases	7.1%	Peptide transporters	1.1%
In MATE family	5.3%	K transporters	1.1%
Misc	5.3%	Other anion transporters	1.1%
P-type pump	5.3%	Sulphate transporters	0.7%
Metal transporters	5.3%	Auxin transporters	0.7%
Channels	4.3%	Stress induced	0.4%
V-type pumps	3.5%	Nitrate transporters	0.0%
Phosphate transporters	3.5%		

Figure 5.4. The percentage of transporter gene families that show up-regulation in *Arabidopsis* seedlings de-etiolating by FR light. Lists were compiled as extensively as possible to contain all transporters genes in the *Arabidopsis* genome. Characterized and putative genes were assigned to families according to known and predicted function where possible. Using microarray data from a bespoke *Arabidopsis* transporter chip, the percentage of gene families in the list of genes that were classified (≥2-fold increase in gene expression) as showing up-regulation in de-etiolating *Arabidopsis* seedlings by FR light were calculated. Of the 604 that produced sufficient spot intensity over background noise 250 were shown to be up-regulated. The pie chart above shows how these genes were distributed between the gene families.

2000b). Of note are two of the Nramp heavy metal transporter genes which are shown to be up-regulated; *AtNramp1* (At1g80830) two-fold and *AtNramp4* (At5g67330) three-fold. Expression of *AtNramp4* has been found in both aerial and roots of *Arabidopsis* seedling whereas *AtNramp1* while is shown to be expressed in aerial *Arabidopsis* seedlings tissue its expression is especially abundant in roots (Thomine *et al.*, 2000). The most responsive metal transporter *YSL7* (At1g65730) showed a four-fold increase in *Arabidopsis* seedlings during de-etiolation by FR light, which currently is uncharacterized.

Figure 5.5 shows a graph which represents the percentage of gene in each transporter family that were classified ($\geq$2-fold increase in gene expression) as up-regulated in *Arabidopsis* seedlings during de-etiolation by FR light and thereby the involvement of the transporter families in *Arabidopsis* seedlings under FR-light de-etiolation taking in account the size of the transporter family. Using this approach the peptide transporter family appeared to be the most responsive, as a third of the family that comprises 9 transporters are up-regulated (figure 5.5). Second are the family of putative transporters that have yet to be characterized. In years to come when these putative transporters have been studied it will be interesting discover their contribution to the de-etiolation by FR light of *Arabidopsis* seedlings. Third, fourth and fifth are families of pumps; P-type pumps, ABC transporters and V-type pumps (respectively). Pump converts the energy currency of ATP into proton gradients that drive many solute transporters and thus their high degree of involvement is logical in *Arabidopsis* seedlings undergoing such drastic developmental changes. Just over a quarter of genes in the sugar transporter and invertase family are up-regulated in *Arabidopsis* seedlings under FR-light de-etiolation. For the affect of individual sugar transporter genes please refer to table 5.5.

Table 5.4 shows the transporter genes that were most affected by FR light. Three P-type ATPases, *AHA2*, *AHA9* and *ECA2*, were in the top 10 most up-regulated genes. The results presented here indicate that AHA2 and AHA9 could have important roles in seedlings undergoing de-etiolation. AtECA2 has not been characterised and as yet, there are no indications of its role or localisation. Insertional mutants of these P-type ATPases may prove useful in investigating the role of these genes during the de-etiolation process. An auxin transporter and a Ca^{2+}-H^{+} antiporter are also strongly induced by FR light. The importance of auxin in mediating photomorphogenetic responses has become increasingly apparent (Halliday and Fankhauser, 2003). In addition, it is well established that calcium plays a role in light-signalling processes (Bowler *et al.*, 1994) and regulation of calcium transport may be important for this. Also on the list is an amino acid transporter, which could be utilized to re-allocate resources required during photomorphogenesis. An ABC transporter and a

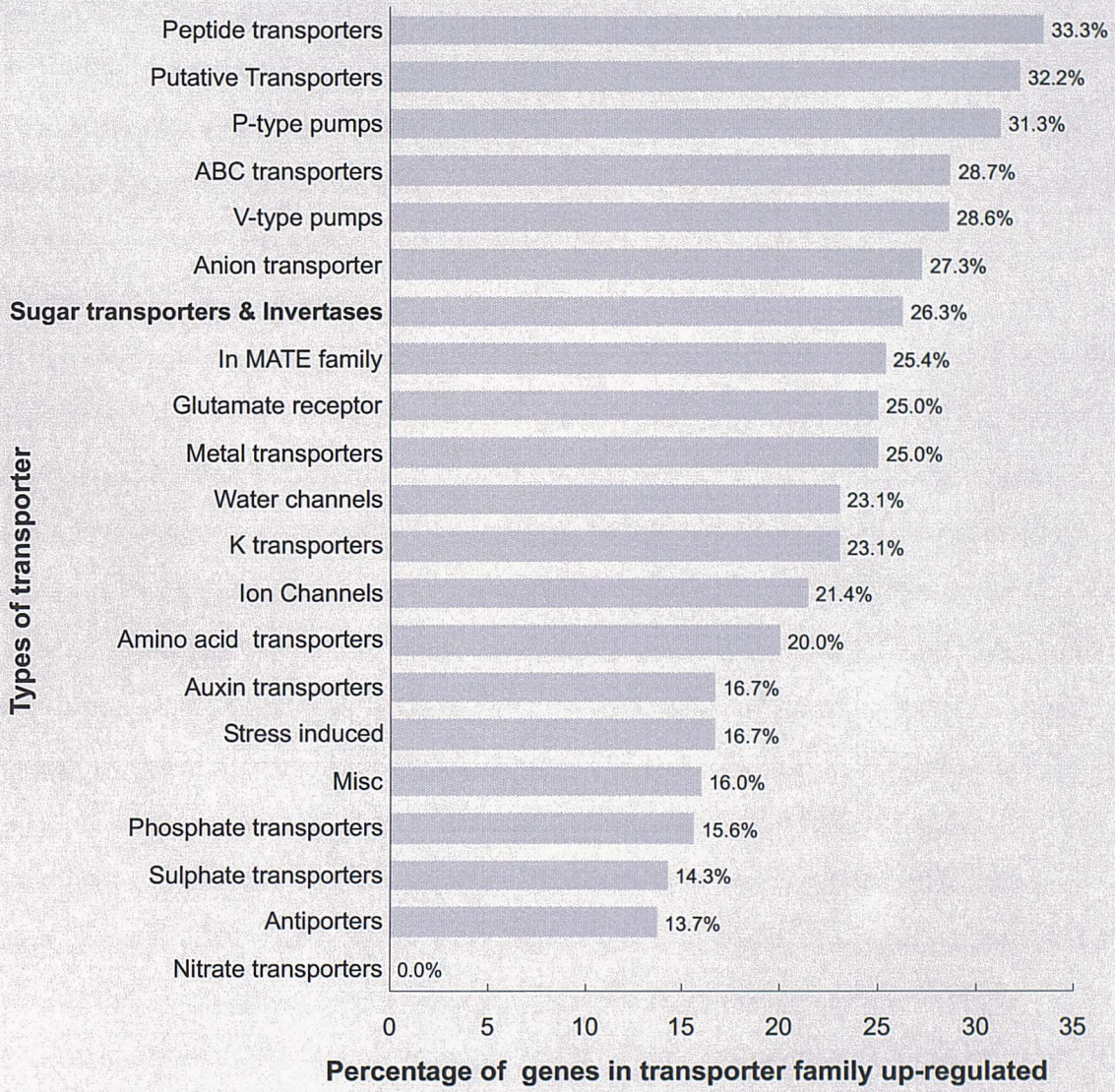


Figure 5.5. The Percentage of genes in each transporter family up-regulated in *Arabidopsis* seedlings de-etiolating by FR light. Lists were compiled as extensively as possible to contain all transporters genes in the *Arabidopsis* genome. Characterized and putative genes were assigned to families according to known and predicted function where possible. Using microarray data from a bespoke *Arabidopsis* transporter chip, the number of genes in each gene family that were classified (≥2-fold increase in gene expression) as showing up-regulation in de-etiolating *Arabidopsis* seedlings by FR light were calculated as a percentage of the number of genes within that family and are shown above in order from most to least affected.

PPase are also induced by FR light, but the function of these genes is not known. The down-regulation of certain genes (see table 5.4) may indicate that their transport properties are no longer required in the new growth strategy in response to FR light. In the present study few genes were down-regulated by FR more than two-fold and only one, *STP4*, more than three-fold (table 5.4).

5.2.4. Effect of FR Light on Sugar Transporter Gene Expression in *Arabidopsis*

The microarray data shows that the sugar transporter genes were affected in a variety of ways (table 5.5). The sugar transporter genes with the highest MRAT values, which represent the highest up-regulation, unfortunately had very low spot I/B for both dyes and thus these values cannot be trusted. However, the results of those sugar transporter genes in any subsequent repeat experiments will be eagerly inspected.

The sugar transporter that shows the highest induction of gene expression and that has been characterized is *AtSUC5*. Previously, it has been reported that RT-PCR detected *AtSUC5* expression in all tissues of *Arabidopsis* (Ludwig *et al.*, 2000). Conversely, in work that contributed to chapter 3, *AtSUC5* expression could not be found in seedlings. Although, it must be noted that the seedlings used to investigate how sucrose supplementation affected *AtSUC5* expression were grown in continuous white light while the seedlings used in this chapter were given only 12 h FR light. It may be that AtSUC5 has a more specific transient role during the de-etiolation process. Another sugar transporter gene, *AtSTP9,* which encodes a hexose transporter also previously reported not to be present in seedlings (Schneidereit *et al.*, 2003) was also up regulated (2-fold) in this study. Localization at a cellular level using a specific antibody revealed that, in contrast to the early accumulation of *AtSTP9* transcripts in young pollen, the AtSTP9 protein is only found at low levels in mature pollen, but is highly abundant in germinating pollen tubes (Schneidereit *et al.*, 2003).

The results of the microarray show that *AtSTP1* is up regulated 2.4-fold. AtSTP1 is a relatively well characterised hexose transporter that has been suggested to be the major protein of glucose transport in *Arabidopsis* seedlings (Sherson *et al.*, 2000). Its role was suggested to be in the uptake of hexose sugars derived from sucrose hydrolysed in the apoplast after transport from the cotyledons to the root (Sherson *et al.*, 2000). One hypothesis for the increase in *AtSTP1* gene expression might be the requirement of increased levels of hexose sugars at the roots in response to a light-dependent increase in root growth and development.

In chapter 4, *AtSUC2* expression was shown to occur throughout the vascular system of the seedling and was remarkably attenuated by the supplementation of sucrose. However, no distinct

Table 5.4. Top 10 and top 8 transporter genes most up/down-regulated transporter genes by far-red light respectively.

Gene Name	MIPS code	Gene family / function	Channel 1 Intensity / background ratio	Channel 2 Intensity / background ratio	Median spot ratio
AHA9	At1g80660	P-type pump	167.1	15.5	20.61
CAX7	At5g17860	Ca-H antiporter	39.0	56.7	12.84
ECA2/ACA5	At4g00900	P-type pump	12.6	9.4	9.88
Amino acid transporter	At4g16370	Amino acid transporter	25.0	3.4	8.45
AVP1	At1g15690	PPase	5.8	2.9	6.65
AtAUXR2	At2g21050	Auxin transporter	2.9	2.7	6.48
FPS1	At5g47770	Constitutive	48.1	9.0	5.93
hypothetical protein	At1g47530	In MATE family	24.4	8.3	5.61
AtMRP3	At3g13080	ABC	56.4	16.2	5.57
AHA2	At4g30190	P-type pump	30.4	2.7	5.41
AtATH11	At5g61730	ABC	100.3	5.3	0.48
putative protein	At3g02600	PAP2 family	39.4	3.5	0.47
NRT2.6	At5g14570	Nitrate transporter	175.4	4.3	0.42
NHD2	At1g49810	Na-H antiporter	113.0	2.1	0.40
mitochondrial phosphate transporter	At3g48850	Mitochondrial phosphate translocator	354.5	9.6	0.38
hypothetical protein	At1g18010	Misc	121.1	2.6	0.37
Amino acid transporter	At3g13620	Amino acid transporter	455.1	15.2	0.37
STP4	At3g19930	Sugar transporter	251.9	3.3	0.17

Table 5.5. Transcriptional response of all *Arabidopsis* sugar transporters to a FR light treatment. The expression of all sugar transporters on the AMT microarray was analyzed and is shown in order from the most up-regulated to the most down-regulated. Up-regulated genes were deemed to be those with a media spot ratio of ≥2 and down-regulated genes ≤0.5. Those in between are classified as un-affected during de-etiolation by far-red light. Lines in light grey represent genes that did not produce a sufficient intensity / background ratio (≥2) to be considered as reliable results.

Gene name	EMBL Acc	MIPS code	Channel 1 Intensity / Background Ratio	Channel 2 Intensity / Background Ratio	Median Spot Ratio
FR UP-REGULATED					
STP32		At2g16130	1.1	1.4	41.1
STP33		At4g36670	2.4	1.1	15.1
STP31		At2g16120	1.4	0.9	6.6
STP35		At1g19450	1.2	1.0	6.4
SUGTL1	AJ249967	At1g08930	6.3	1.1	6.1
STP30		At2g48020	10.6	1.5	5.4
STP40		**At2g43330**	**8.4**	**3.8**	**5.4**
STP10		**At3g19940**	**9.2**	**3.0**	**3.4**
SUC5	**AJ252133**	**At1g71890**	**5.6**	**2.2**	**3.3**
STP11		**At5g23270**	**28.8**	**5.9**	**3.0**
STP47		**At3g05160**	**7.8**	**2.6**	**2.9**
STP13		**At5g26340**	**12.5**	**3.6**	**2.9**
STP42		**At3g18830**	**3.1**	**1.1**	**2.7**
Sugar1		**At3g20660**	**27.1**	**4.5**	**2.7**
SUC9		**At5g43610**	**10.4**	**2.0**	**2.7**
STP14		**At1g77210**	**111.7**	**11.0**	**2.7**
STP34		At1g34580	2.6	1.0	2.7
STP48		At1g79820	4.4	1.2	2.6
STP39		At2g18480	5.2	2.0	2.6
PGlcT		**At5g16150**	**8.9**	**2.4**	**2.4**
STP6		At3g05960	13.1	1.7	2.4
SUGTL4		**At1g08890**	**83.4**	**15.9**	**2.4**
STP1	**X55350**	**At1g11260**	**53.6**	**9.1**	**2.4**
STP44		At1g30220	21.6	1.3	2.4
STP24		**At5g27360**	**204.3**	**38.6**	**2.3**
STP37		**At4g16480**	**78.2**	**4.3**	**2.3**
Sugar transporter		At1g79410	2.8	0.8	2.3
SUGTL2	**AJ249968**	**At1g08920**	**6.5**	**2.5**	**2.2**
SUC2	**X75382**	**At1g22710**	**21.6**	**3.2**	**2.2**
Sugar transporter		At1g16390	3.9	1.4	2.2
Sugar transporter		At1g73220	4.0	1.1	2.1
STP9		**At1g50310**	**54.1**	**10.9**	**2.0**
Sugar4		**At3g51490**	**98.3**	**11.3**	**2.0**
STP12		**At4g21480**	**10.8**	**2.5**	**2.0**

Table 5.5. Cont.

FR UN-RESPONSIVE					
SUC1	**X75365**	**At1g71880**	**9.8**	**2.4**	**1.8**
STP28		At4g04760	2.3	1.2	1.8
SUC8		**At5g06170**	**10.3**	**3.2**	**1.7**
STP27		**At4g04750**	**12.1**	**2.1**	**1.7**
STP29		At1g75220	4.5	1.2	1.7
Sugar6		**At1g07290**	**22.6**	**3.6**	**1.6**
SUGTL3	AJ249969	At1g08900	6.0	1.3	1.6
STP26		**At1g67300**	**90.0**	**5.5**	**1.6**
STP20		**At3g20460**	**107.2**	**7.3**	**1.6**
STP46		At3g05150	6.1	1.6	1.6
SUC4	**AJ289166**	**At1g09960**	**18.1**	**2.6**	**1.6**
Sugar3		At1g20840	6.9	1.1	1.6
STP41		At5g59250	16.1	1.6	1.6
SUC3	AJ289165	At2g02860	5.5	1.2	1.5
STP36		At3g03090	6.1	1.4	1.5
STP23		At5g27350	5.4	1.1	1.3
SUC6		At2g14670	12.9	1.9	1.3
SUC7		**At1g66570**	**16.9**	**3.2**	**1.3**
Sugar transporter		**At1g79360**	**37.8**	**5.3**	**1.3**
Sugar transporter		**At1g16370**	**24.0**	**2.7**	**1.2**
STP8		**At5g26250**	**9.0**	**2.7**	**1.2**
STP43		At5g17010	3.8	1.6	1.2
STP38		**At2g20780**	**41.7**	**4.9**	**1.1**
STP25		**At5g18840**	**17.6**	**2.0**	**1.0**
STP2		At1g07340	30.8	3.3	1.0
STP3		**At5g61520**	**32.9**	**3.0**	**1.0**
Sugar2		**At4g35300**	**48.0**	**2.7**	**0.9**
Sugar5		**At2g13650**	**146.6**	**12.6**	**0.9**
STP7		**At4g02050**	**56.2**	**6.4**	**0.9**
SUGTL5		**At3g05400**	**15.7**	**2.0**	**0.9**
STP21		At1g05030	3.6	1.3	0.8
STP45		**At1g54730**	**45.6**	**5.5**	**0.8**
STP22		**At2g35740**	**20.1**	**3.9**	**0.7**
FR DOWN-REGULATED					
STP4	**X66857**	**At3g19930**	**251.9**	**3.3**	**0.2**

differences were found between seedlings grown in the light, dark or when etiolated seedlings were given 24 h exposure to WL. In contrast, this microarray experiment showed that *AtSUC2* expression was induced two-fold when seedlings are irradiated with FR light. The role of AtSUC2 in mature *Arabidopsis* in sucrose phloem loading is well accepted and other roles such as retrieval of phloem leakage and unloading have been suggested (Truernit and Sauer, 1995). The increase in *AtSUC2* might be in response to the required mobilization of sucrose from the starch reserves stored in the cotyledons to fuel photomorphogenesis.

Like *AtSTP1, AtSTP4* has been shown to be expressed in the root of seedlings both here (figure 4.1) and by Truernit *et al.* (1996). *AtSTP4* was the first hexose transporter that was reported to show an induction in gene expression after wounding (Truernit *et al.*, 1996). Based on its presence in classic sink tissues, the authors proposed a role for STP4 as a general sink-specific hexose transporter that also responds to the increased carbohydrate demand of cells responding to environmental stress. Unlike *AtSTP1, AtSTP4* expression is strongly down-regulated in response to FR light (table 5.5). Surprisingly, the data from this experiment shows that *AtSTP4* is the only sugar transporter to show this response. For all of the results described above it should be emphasised that conclusions are based on only a single experiment. It will be essential to increase the number of replicates for the data in order to draw firmer conclusions. Unfortunately, it was not possible to repeat this experiment because of time constraints. In addition, information obtained from the array will need to be verified using an alternative method such as RT-PCR using gene-specific primers.

5.2.5. Identification of putative *cis*-regulatory elements mediating the response to FR light

It is logical to presume that genes that appear to be regulated by a shared factor may contain identical elements within their promoter regions. Such elements are referred to as *cis*-regulatory elements (CREs). As microarray experiments enable a genome wide transcription profile of genes responsive to a regulatory treatment, in this case FR light, the data produced is ideal for predicting putative CREs.

The upstream regions of the genes most affected during the FR light treatment were analysed using the web based program Bioprospector, which employs an algorithm developed to find sequence motifs from a set of DNA sequences (Liu *et al.*, 2001). Two sets of DNA sequences were collected; one incorporating the upstream regions of the 10 must up-regulated genes and the other containing the same for the 8 genes that were shown to be down-regulated. A region of approximately 4 kb upstream of the transcription start site was obtained from the MIPs website for each gene in the

list. This list was then formatted and entered into the Bioprospector program. The top 5 putative CREs for the most up- and down-regulated genes and their occurrence in each promoter region can be seen in tables 5.6 and 5.7, respectively.

None of the CREs predicted from the up-regulated genes are present in all genes and only two out of the top 5 putative CREs are identical in all genes from which they were predicted. All CREs predicted from the most down-regulated genes are degenerative, however the three CREs occur in all of the promoter regions with two of these occurring more than once for a single gene. However, those with two CREs in their promoter region did not show the strongest down regulation. The promoter region of the *AHA9* gene that showed the greatest up-regulation only contained the CRE rated fifth, which appears in only 60% of the gene promoter regions of the top 10 most up-regulated genes.

The results described here would need further bioinformatics analysis before testing experimentally. Advances in bioinformatics and the understanding of promoter motifs has prompted researchers to produce a range of web-based programs designed to predict putative CREs; for example Bioprospector, SPEXS, Alibaba, MotifSampler. Bioinformatisists are now starting to produce more advanced online programs to analyse promoter regions for regulatory elements designed with microarray data in mind (Roven and Bussemaker, 2003; Shah *et al.*, 2003). There are also a number of databases dedicated to the analysis of promoter elements in plants such as AGRIS, PlantProm, AtProbe, PlantCARE, AtcisDB and PLACE (Higo *et al.*, 1999). These could be used to look for the predicted light-regulated elements that have been characterised to date (Argüello-Astorga and Herrera-Estrella, 1998).

Table 5.6. Positions of putative *cis*-regulatory elements in the promoter sequences upstream of the ten most up-regulated genes by far-red light. For each transporter gene approximately 4 Kbp upstream of the transcription start site of the coding sequence was obtained from the MIPs website and entered into the Bioprospector program (http://bioprospector.stanford.edu/, Liu *et al.*, 2001). Listed below are the top five most promising putative *cis*-regulatory elements.

Transporter	Positions of the putative cis-regulatory elements Key: Starting alignment position sequence of the aligned segments (r53 means 53 from the end of sequence in reverse direction, f47 is 47 from beginning of sequence in forward direction).				
	GAACC$^{T}_{C}$T$^{A}_{G}$	GGT$^{T}_{C}$TAG	TGAATCTA	GAACCAAT	GGTTCT$^{A}_{T}$A
AHA9					f172
CAX7	r1463	f1508, r3280		f2324, r2764	
ECA2/ACA5	r3824, f221	f3538, r1167	r1567	r3539, r3600, r2421	
Aminoacid transporter	f104, r4042		f335, f3903, f4008	f1012	
AVP1	r3339	f2446, f2414	f4363		f1683
AtAUXR2	f3203		r2125	r300	f937
FPS1	f554	r4082	r2174, f837	r1810	r1406
hypothetical protein	r3303, r3675, r1985		f773	r3014	r3408, r3776
AtMRP3	r753, f3961	r1955, f3861			r1459, r994, r1468
AHA2					

Table 5.7. Positions of putative *cis*-regulatory elements in the promoter sequences upstream of the eight down-regulated genes by far-red light. For each transporter gene approximately 4 Kbp upstream of the transcription start site of the coding sequence was obtained from the MIPs website and entered into the Bioprospector program (http://bioprospector.stanford.edu/, Liu *et al.*, 2001). Listed below are the top five most promising putative *cis*-regulatory elements.

Transporter	Positions of the putative cis-regulatory elements Key: Starting alignment position sequence of the aligned segments (r53 means 53 from the end of sequence in reverse direction, f47 is 47 from beginning of sequence in forward direction).				
	T/G A G G/T T A/C C G	T G C C C A/G T T G C G	T G C C C A/G T G C	C G A/T T T A/C C G	A G T/G T C C G G/T A
AtATH11	r3501	r1239	r1239	f1825	r3502
putative protein	r3461	f240, f2948	f240, f2948	f1173	r3462
NRT2.6	r2442, r4072	r3203	r3203, r3189	f2409	r2443
NHD2	r2893	r2197		r4269	r2894
mitochondrial phosphate transporter	r912	r365		r990, f2629	f1150
hypothetical protein	r1859	r2576	r2576	r2201	r1860
Amino acid transporter	r774	f1313	f1313	f3811	r775
STP4	r55	f1684	f1684		r56

5.3. Discussion

This chapter shows the first phylogenetic analysis of all characterized and putative sugar transporters in *Arabidopsis* (figure 5.1) and amends previous families of sugar transporters suggested (Ward, 2001; www.cbs.umn.edu/arabidopsis). The phylogenetic analysis shows that the SUC family of sucrose transporter and the STP family of hexose transporters are separate from the other 6 families of sugar transporters. Interestingly, the SUC and STP families are so far unique in that they contain transporters that have been characterized. Apart from the putative mannitol transporters family, which previously was grouped in with sugar transporters in the STP and STF1 families (figure 5.2), it is not known why the families of STF1-STF5 are distinct. Since phylogenetic analysis assigns proteins to families according to their amino acid sequence, it may be that families STF1-5 have functionally distinct roles that have yet to be discovered.

Also novel is the design and use of a comprehensive *Arabidopsis* transporter microarray chip. Transporters are responsible for the movement and allocation of solutes that is essential in the growth and development of a seedling undergoing FR-mediated photomorphogenesis. By using this bespoke microarray chip it was possible to analysis the types of transporters that were most active in this change of growth strategy (figure 5.4 and 5.5). Curiously, most of the transporter genes that were up-regulated were putative transporters that had not been assigned a possible function. Obviously their role is important in the adaptation of *Arabidoposis* seedlings to FR-mediated de-etiolation and in the future is it hoped that their function will be discovered to complete the picture. Surprisingly just over a quarter of sugar transporter that had a sufficient signal:background ratio ($\geq$2:1) were shown to be up-regulated and only 1 out of the 71 sugar transporter was classified as down-regulated (table 5.5). It is quite possible that without the limitations imposed on the data analysis by the low Cy5 (red dye) signal:background ratio a higher proportion of sugar transporters might have been shown to be up-regulated. The microarray data was also used to predict *cis*-regulatory promoter domains that were responsive to FR-mediated phytochrome signalling, this is something that was not done from the results of a similar microarray experiment (Tepperman *et al.*, 2001). Since a specific light treatment was used that was known to be monitored by a single photoreceptor, phytochrome A, the chances of finding common promoter elements would be greater than when studying a more complex developmental adaptation of a plant to its environment.

5.3.1. Phylogenetic analysis of sugar transporters in *Arabidopsis*

The phylogenetic analysis indicates that *Arabidopsis* contains a number of sub-families of sugar transporters that have yet to be investigated. Moreover, only a minority of transporters have been studied in detail. Sugar transporter family 4 contains the putative plastid glucose transporter, *pGlcT*, which is unique in that it was the first and so far only sugar transporter to be localised to an organelle (Weber *et al.*, 2000). It could be that the other sugar transporters within that family may also have roles in organelles. Along with AtSUC3, At4g35300 was noted by Lalonde *et al.* (1999) as showing a possible extended central cytosolic domain, an indication of a possible sugar sensing function. The glucose sensors in yeast RGT2 and SNF3 have extended cytoplasmic C-terminal extensions, which sets them apart from the yeast glucose transporters (Özcan *et al.*, 1996). In spite of this the amino acid sequences of AtSUC3 and At4g35300 are not particularly similar as indicated by the distance between them in the phylogenetic tree (figure 5.1). The putative mannitol transporter, At4g36670, (PMTF; figure 5.1) was classified as a late repressed gene upon FR irradiation (Tepperman *et al.*, 2001). Unfortunately at this point in time no more is known about this putative transporter. Since it has been shown to respond to environmental signals, it would be interesting to explore where it is expressed and what its role is *in vivo*.

The sucrose transporters, *AtSUC2*, and *AtSUC4* have both been shown to be expressed in the vascular tissue (Stadler and Sauer, 1996; Weise *et al.*, 2000), but they are not as closely related as other members of the *SUC* family. However, while these two sucrose transporters have a similar location, their transport characteristics are very different. Sugar transporter family 1 (STF1, figure 5.1) contains a number of putative sugar transporters named SUGTL1-5. The names for these sugar transporter genes were obtained from EMBL where the sequences were released between October 1999 and January 2000 (EMBL accessions AJ249967, AJ249968, AJ249969, AJ249970, AJ249971). As yet, there is no published information on these putative sugar transporters. No functional role can be assigned to members of sugar transporter families 3 and 6 either. Prosite motifs provided by the MIPS website provide clues as to the possible functions of some of the putative sugar transporters. Details of these motifs together with gene locus codes, EST accessions, mRNA accessions and gene families, designated from both the phylogenetic analysis shown here and by the AMPL website, are listed for each sugar transporter gene in Table 5.2.

Within the STP and SUC families there exists a variety of transport characteristics and roles; some tissue specific and others found throughout the plant. Thus it would seem that only subtle differences in the amino acid composition are required to produce such diversity within a family. It will

certainly be interesting to determine the roles of the more divergent members of the *Arabidopsis* sugar transporter families. Furthermore, when the absence of a single sucrose transporter such as AtSUC2 (shown in chapter 3) can produce such detrimental effects to the growth and development of a plant due to its ubiquitous role, are there enough roles left to warrant another 69 sugar transporters? With the many projects that offer a comprehensive library of insertional mutants, plants unable to produce functional proteins of these sugar transporters might provide clues (for details of such projects refer to the introduction in chapter 3). Preliminary work characterizing a number of sugar transporter insertional mutants obtained from the SAIL lines (Syngenta, Cambridge, UK) was undertaken in this laboratory (chapter 3.2.6). One of these plants included is a putative *suc5* mutant and as *AtSUC5* expression was induced 3.3-fold after 12 h FR light it might be interesting to investigate if a phenotype is observed when grown in these specific light conditions.

5.3.2. Analysis of microarray data

The use of microarrays to investigate gene expression enables the response of genes to be analysed in various ways. As well as simply studying which genes are most highly induced or repressed, the response of gene families with similar roles can be investigated. Additionally, the utilization of microarray data in analysing gene promoters can also be explored and developed.

Attempts to obtain a replicate set of data for the FR light treatment were unfortunately not successful. The importance of replicate DNA microarray experiments have been well publicized (Finkelstein *et al.*, 2002; Lee *et al.*, 2000) and is especially relevant in this chapter as the microarray data shows a particularly low Cy5 spot I/B ratio. While the results from the spiking controls and the low percentage of down-regulated genes indicate that the ratios required decreasing, the only comparable control, *Lhb1B2*, produced an induction that was five times lower than others have reported (Ma *et al.*, 2001). However, important differences in the light treatment given to the seedlings should be noted; the experiment described in this chapter investigated the effect of FR light in 3.5-d-old seedlings whereas Ma *et al.* (2001) compared 6-d-old seedlings grown in continuous darkness and FR light.

It appears that other research groups are also experiencing similar problems with dye background levels, as suggestions aimed at overcoming this experimental error have been published (Martinez *et al.*, 2003; Mills and Gordon, 2001). With more time it would be interesting to apply the suggestions made in these articles to find out if the data acquisition could be improved. A repeat

experiment with a better spot intensity to background ratio and/or a cleaner background might not only provide more reliable data, but also enable more genes to be studied.

Using an Affymetrix microarray chip that contains a diverse range of 8200 genes showed that 10% of those genes respond to FR light in a phyA-dependent manner (Tepperman *et al.*, 2001). Wang *et al.*, (2002) used an *Arabidopsis* 9.2K EST microarray to investigate the genomic effects of FR light in the Col, Ler, No-0 and RLD ecotypes and found that 18, 18, 16, and 16% of the 6126 genes displayed a 2-fold or more differential expression, respectively. In the experiment shown here 23.5% of the genes on the bespoke *Arabidopsis* transporter microarray were deemed to be responsive to FR. Furthermore, of those genes that were responsive, 250 were up-regulated and only 8 were down-regulated. Obviously the number of genes in each group is dependent on the thresholds set to categorise their response and thus, as used for previous microarray experiments (Donson *et al.*, 2002, table 2), a 2-fold difference was sought to indicate both up-regulation (MRAT ≥2) and down-regulation (MRAT ≤0.5). Particular to this microarray experiment is the large number of data for genes that had to be disregarded due to high spot background noise for one of the dyes. Unfortunately, only 55% of the genes on the microarray chip had a sufficient spot intensity / background ratio (≥2 I/B) to be included in the analysis.

5.3.3. Analysis of *Arabidopsis* transporter genes regulated by FR-mediated photomorphogeneisis

A striking result was the occurrence of three P-type ATPase genes in the top ten most up-regulated genes (table 5.4). Forty-six P-type ATPase genes have been identified in *Arabidopsis*, the largest number yet identified in any organism (Baxter *et al.*, 2003). P-type ATPases (Family 3.A.3 - The P-Type ATPase Superfamily) are found in all three branches of life and are used to translocate a diverse set of ions, including H+, Na+/K+, H+/K+, Ca2+, heavy metals, and possibly lipids (Axelsen and Palmgren, 1998). *AHA9* is expressed in anthers (Houlne and Boutry, 1994) while *AHA2* is expressed predominantly, but not exclusively, in the root epidermis (Baxter *et al.*, 2003). Phylogenetic analysis shows that *AHA2* shares a high degree of homology to *AHA1,* but is not similar to *AHA9*, the most up-regulated gene. P-type ATPases are known to generate ion gradients used by many secondary transporters, such as amino acid and sugar transporters. Thus these proteins are a point of regulation for transporter proteins that maybe utilized when a plant is adapting to environmental signals such as FR light.

The sugar transporter that was most induced by FR light and that has also appeared in the literature was *AtSUC5* from the sucrose transporter family (*SUC*, see figure 5.1). However *AtSUC5* has been shown not only to transporter sucrose but also biotin (vitamin H); thus its role in FR induced photomorphogenesis may be to transport this substrate (Ludwig *et al.*, 2000). Plant lines that have mutations in genes required for biotin synthesis have been shown to arrest at various developmental stages (Patton *et al.*, 1998; Shellhammer and Meinke, 1990). Further work is necessary to investigate this.

Only one sugar transporter, *AtSTP4*, was shown to be significantly down-regulated. The work in chapter 3 shows that the expression of *AtSTP4* is localised to the roots where it is especially prevalent at the root tips. This agrees with the proposal by Truernit *et al.* (1996) that AtSTP4 transports hexose sugars to sink tissues such as anthers and root tips and that it is wound inducible. Down-regulation of this gene should be tested using reporter-gene technology or RT-PCR before drawing any firm conclusions. The role of AtSTP1 in seedlings has been studied in much more detail (Sherson *et al.*, 2000). The dramatically reduced transport of glucose by *stp1* mutant plants when fed up to 1 mM glucose solution in comparison to WT was interpreted as an indication that AtSTP1 is the major monosaccharide transporter in *Arabidopsis* seedlings and that they do not operate low-affinity hexose transporters (Sherson *et al.*, 2000). It would be interesting to perform similar studies not only with a *stp4* mutant but also with a *stp1/stp4* double mutant to see the effect on glucose transport. Since both AtSTP1 and AtSTP4 are high-affinity hexose transporters expressed in the roots it is not clear why they have such divergent expression patterns under FR.

Light affects the expression of many genes (Tobin and Silverthorne, 1985). Studying the genomic effects of FR irradiation has the advantage that only one photoreceptor is known to be responsible for mediating the initial signal controlling photomorphogenesis (Whitelam and Devlin, 1997). Various studies have corroborated the theory that PHYA resides in the cytosol in etiolated seedlings, but upon perceiving FR-light it translocates into the nucleus (Nagy *et al.*, 2000). The constituents of the pathways that initiate the PHYA signal have been the focus of studies involving mutant screens. Two particularly elegant studies using microarray analysis have been undertaken to investigate the genomic impact of PHYA signalling intermediates (Wang *et al.*, 2002; Ma *et al.*, 2003). Microarray analysis of 16 different PHYA signalling intermediate knockout lines (with the four appropriate WT lines) enabled Wang *et al.* (2002) to propose that FHY1, FAR1 and FHY3 act upstream, near the start of the phyA signalling network, whereas FIN29, SPA1 and REP1 acts further down the network, in a more specific capacity, affecting a small set of genes. Analysis of the

supplementary material supplied by Ma *et al.* (2003) has shown that while *AtSUC2* expression is induced 11-fold higher in 6 d light-grown seedlings compared to etiolated seedlings, this is reduced to 3.3 and 1.7 in the *cop1-1* and *det1-1* mutants respectively. This indicates that these light-signalling components are part of the pathway that regulates *AtSUC2* expression and possibly other sugar transporters that are up-regulated. COP1 has been proposed to be part of an ubiquitin ligase which in darkness interacts with and leads to the degradation of HY5, a basic Leu zipper type transcription factor (Ang *et al.*, 1998; Osterlund *et al.*, 1999). HY5 binds to the G-box motif of multiple light-inducible promoters (Chattopadhyay *et al.*, 1998). The function of DET1 seems to be more universal due to its involvement with histone acetyl transferases and the H2B histone (Benvenuto *et al.,* 2002; Schroeder *et al.,* 2002). Histone acetyl transferases regulate chromatin remodelling responsible for the unfolding and packing of chromatin necessary for the expression or repression of genes (Reichmann, 2000). DET1 encodes a nuclear localized protein (Pepper and Chory, 1997), which binds to a protein, homologous to a human protein that binds to DNA is response to UV damage (Schroeder *et al.,* 2002). In humans UV-Damaged DNA Binding Protein 2 (DDB2) interacts with histone acetyl transferases (Datta *et al.,* 2001). A null mutation in the *Arabidopsis* DDB1 homologue produces no obvious phenotype, however, it does enhance the phenotype of the *det1* allele as double mutants (*ddba1det1*) show retarded growth of the light-grown phenotype when grown in darkness and stamen development in the flowers also appears under-developed compared to wildtype (Schroeder *et al.,* 2002). More direct evidence of the role of DET1 in chromatin remodelling comes from its binding to nonacetylated amino terminal tails of the core histone H2B (Benvenuto *et al.,* 2002). Thus, it is becoming increasingly apparent that DET1 is responsible for mediating transcriptional regulation of many genes that control photomorphogenesis and thereby plays a pivotal role in light signalling.

A TAIR blast search against the *Arabidopsis* 3000bp upstream dataset (3000bp of sequence preceding the 5' end of each transcription unit) with the sequence of the G-box motif (Chattopadhyay *et al.*, 1998) did not reveal its presence in any sugar transporters. However, other studies are finding light-responsive motifs within the promoters of sugar transporters. Using data from microarray analysis from *Arabidopsis,* Rossel *et al.* (2002) proposed various putative motifs that either induce or repress gene expression in response to high light. One of the motifs suggested to repress gene expression was found to be present in the promoter region of the hexose transporter, *AtSTP1* (At1g11260).

With diligent updating of the various website databases with the genome-wide data obtained from microarray experiments it is hoped that the blank spaces in the pathways between the

photoreceptors and the genes they affect will gradually be filled in. However, with light-signalling pathways showing extensive cross-talk (Ma *et al.*, 2003) and increasing evidence for strong interactions with hormone-signalling pathways, a realistic understanding of the mechanism by which all factors involves in light-signalling dictates photomorphogenesis may take some time.

Chapter 6.

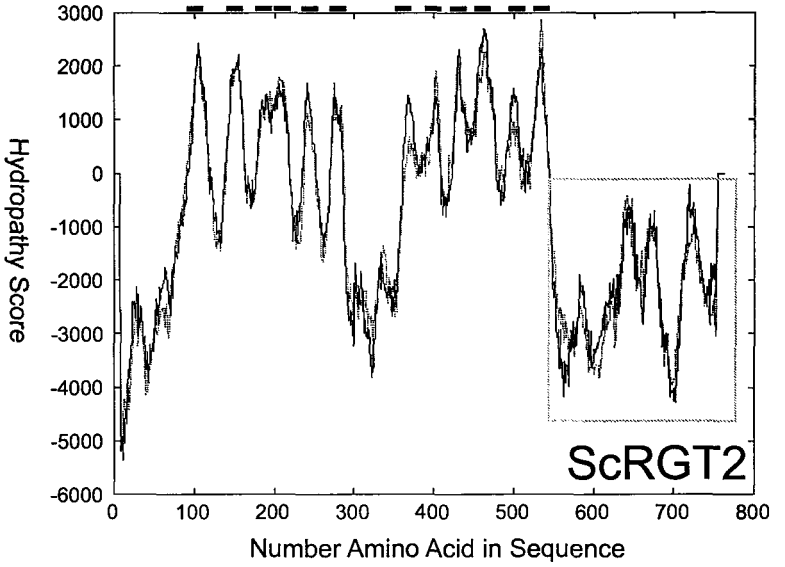
Cloning and Analysis of Putative *Arabidopsis* Sugar Sensors/Transporters

6.1. Introduction.

The application of genomics in biological research has enabled the genes of different organism to be compared. Yeast (*Saccharomyces cerevisiae)* is a unicellular eukaryotic organism which lends itself to the study of metabolic processes without the complication of cellular communication and differentiation. Therefore, yeast can be used as a model to investigate genes involved in metabolic processes in other eukaryotic cells, such as plant cells. However, the major transport sugar in plants is sucrose while *S. cerevisiae* mainly uses glucose. Many components of sugar metabolism show similarities such as the predicted structure of sugar transporters and the role of hexokinase (HXK) (Smeekins and Rook, 1997). Interestingly, the HXK found in mammalian systems, glucokinase is also thought to have a role in glucose sensing (Matschinsky *et al.,* 1993).

In yeast, as in plant cells, HXK appears to have a dual role (Trumbly, 1992; Jang and Sheen, 1994; Cortès *et al.,* 2003). HXK is an enzyme that phosphorylates glucose in the first step of sugar metabolism by glycolysis. Additionally, it has also been shown to initiate the signal for the repression of glucose down-regulated genes (Jang and Sheen, 1994). Indeed, transgenic *Arabidopsis* over- or under-expressing AtHXK produce a hyper- or hypo-sensitive response to glucose (Jang *et al.,* 1997).

However, in yeast, HXK is not the only sugar sensor present as two membrane bound proteins that are similar in their structure to glucose transporters have also been shown to affect the glucose sensitivity of certain genes. *ScRGT2* and *ScSNF3,* which both show a high degree of similarity to mammalian and yeast glucose transporters (Liang and Gaber, 1996), have been shown to be responsible for altering the expression of hexose transporter (HXT) genes in response to glucose levels, thus implicating them as glucose sensors (Özcan *et al.,* 1996). Further analysis using yeast complementation revealed that although they show a high degree of similarity to glucose transporters, neither restored glucose transport in yeast defunct in hexose transporter genes even when over-expressed, indicating that these glucose sensors do not transport glucose (Özcan *et al.,* 1998). Unlike yeast glucose transporters, ScRGT2 and ScSNF3 have extended cytoplasmic C-terminal extensions (Özcan *et al.*, 1996) (figure 6.1). The C-terminal extensions are dissimilar except for a stretch of 25 amino acids, 16 of which are identical among repeats (Özcan *et al.*, 1998).



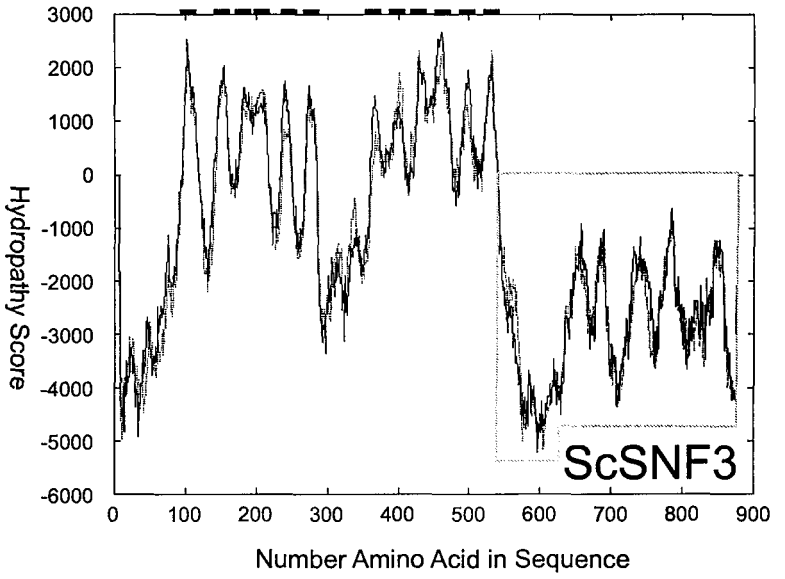


Figure 6.1. Hydropathy plots of two *Saccharomyces cerevisiae* glucose sensors. Amino acid sequences obtained from EMBL were used with TMPred server to predict regions of transmembrane domains and the C-termini glucose sensing domain highlighted by a grey box.

ScRGT2 has two of these sequences whereas ScSNF3 has only one (Özcan *et al.*, 1998). Deletion of the ScRGT2 C-termini tail prevents its ability to sense high levels of glucose and induce ScHXT1 expression; similarly, deletions of the ScSNF3 terminal tail prevents its ability to sense low levels of glucose and induce ScHXT2 expression (Özcan *et al.*, 1998). The transfer of the ScSNF3 tail to ScHXT1 and ScHXT2 converts these glucose transporters into glucose sensors, suggesting that the requirements for the sensing function are present entirely in the C-termini (Özcan *et al.*, 1998). Interestingly, two *Arabidopsis* sugar transporters AtPHS1 (putative hexose sensor, At4g35300) and AtSUT2/AtSUC3 (*Arabidopsis* sucrose transporter/sensor, At2g02860) also have extended cytosolic extensions (Lalonde *et al.*, 1999).

This chapter describes the analysis of their predicted structure and the cloning of the two sugar transporters/sensors with aim of confirming the predicted RNA and amino acid sequences and studying their transport kinetics and putative sensing function.

6.2. Results

6.2.1. Sequence Analysis of Putative Sugar Sensors.

As suggested by Lalonde *et al.* (1999) both AtSUC3 and AtPHS1 stand out from other sugar transporters in their respective families by showing extended cytosolic loops between the 6^{th} and 7^{th} membrane domain (figures 6.2 and 6.3). Unlike AtSUC3 which seems to be unique in its family AtPHS1 (At4g35300, STF2, figure 5.1) is not alone in showing this feature in its family as another 2 putative sugar transporters/sensors show this characteristic, AtSUGTRPR (At1g20840) and At3g51490.

Amino acid sequence determines the function of the protein. Thus it could be expected that the glucose sensing domain from the yeast sensor might bear some resemblance to the extended cytosolic loop domain of a putative *Arabidopsis* hexose sensor. Using the hydropathy plots the location of these extended cytosolic loop domains along the amino acid sequence for these proteins were estimated (AtPHS1, 150-525; ScSNF3, 550-end; ScRGT2, 550-end) and were aligned using a software program called DIALIGN. DIALIGN is an alignment program that is especially efficient where sequences are not globally related but share local similarities as in this case (Morgenstern, 1999). As might be expected the alignment showed significant homology between the two yeast glucose sensors, especially at the start of the sequence although very little similarity towards the end (figure 6.4). Two regions of sequence at the beginning (white text, black boxes) shows three amino acids identical to both the yeast sensor C-termini tails. The first and most promising of these regions of homology is only nine amino acids away from the last transmembrane which raises doubt about this site being involved in sugar sensing due to the spatial limitation of being so close to the membrane.

An amino acid alignment was also produced for the three putative sugar transporters/sensors that contain extended central cytosolic loops (figure 6.5) which were assigned to the same family by phylogenetic analysis (figure 5.1). The several regions of homology in the area of the extended central cytosolic loop suggest that these putative sugar transporters/sensors contain a common function. A BLAST search shows that the sequence LPESPRWLVSKGRM is unique to these three putative sugar transporters/sensors and thus may be a required component for their function. It was noticed that part of this sequence, PESPRWL, consistently appeared in other putative sugar transporters. To investigate if this sequence was conserved in all sugar transporters another BLAST search was performed. It was shown that 33 putative sugar transporters contained this sequence including all of the family of six putative mannitol transporters (PMTF, figure 5.1) and the AtSUGTLs

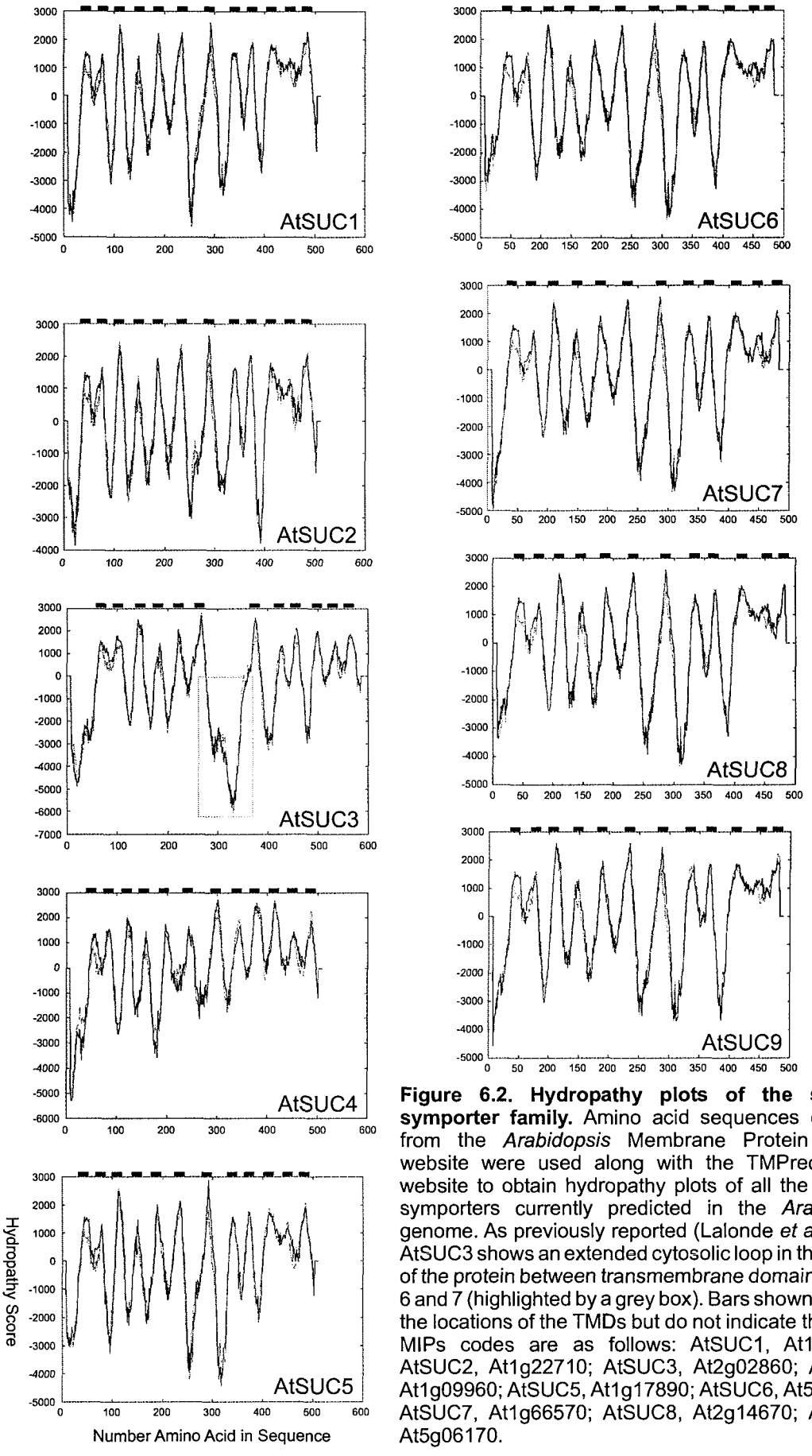


Figure 6.2. Hydropathy plots of the sucrose symporter family. Amino acid sequences obtained from the *Arabidopsis* Membrane Protein Library website were used along with the TMPred server website to obtain hydropathy plots of all the sucrose symporters currently predicted in the *Arabidopsis* genome. As previously reported (Lalonde *et al.*, 1999) AtSUC3 shows an extended cytosolic loop in the middle of the protein between transmembrane domains (TMD) 6 and 7 (highlighted by a grey box). Bars shown suggest the locations of the TMDs but do not indicate their size. MIPs codes are as follows: AtSUC1, At1g71880; AtSUC2, At1g22710; AtSUC3, At2g02860; AtSUC4, At1g09960; AtSUC5, At1g17890; AtSUC6, At5g43610; AtSUC7, At1g66570; AtSUC8, At2g14670; AtSUC9, At5g06170.

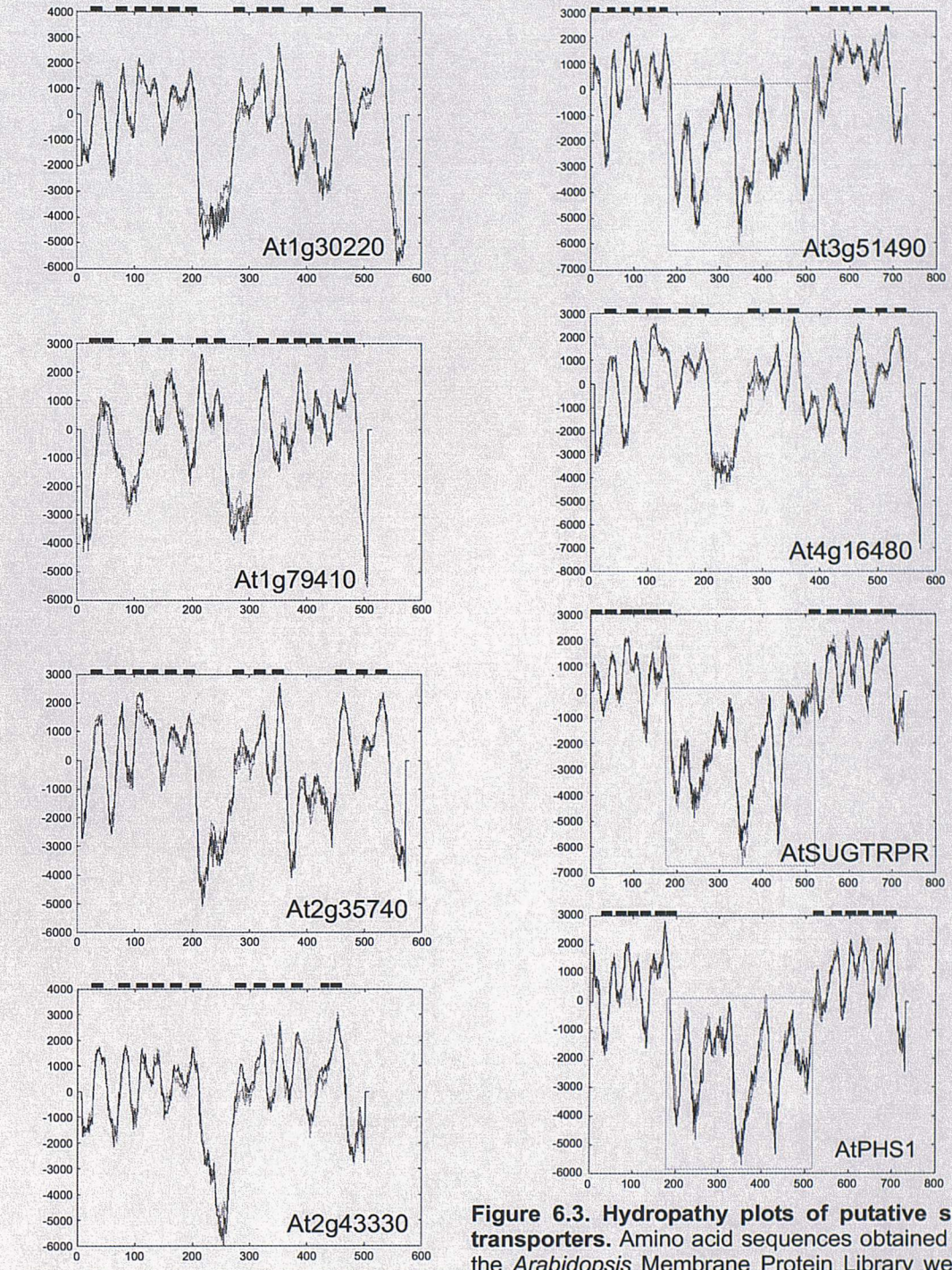


Figure 6.3. Hydropathy plots of putative sugar transporters. Amino acid sequences obtained from the *Arabidopsis* Membrane Protein Library website were used along with the TMPred server website to obtain hydropathy plots. Apart from AtSTP1 which is a characterized hexose transporter of the STP family the other transport proteins are putative and constitute the sugar transporter family 2 (figure 5.1 & table 5.2.). As previously reported (Lalonde *et al.*, 1999) AtPHS1 (F23E12.140) shows an extended cytosolic loop in the middle of the protein between transmembrane domains (TMD) 6 and 7 (highlighted by a grey box). This unusual feature is also seen in AtSUGTRPR and At3g51490. Bars shown suggest the locations of the TMDs but do not indicate their size. MIPs codes not shown are as follows: AtSUGTRPR, At1g20840; AtPHS1, At4g35300; AtSTP1, At1g11260.

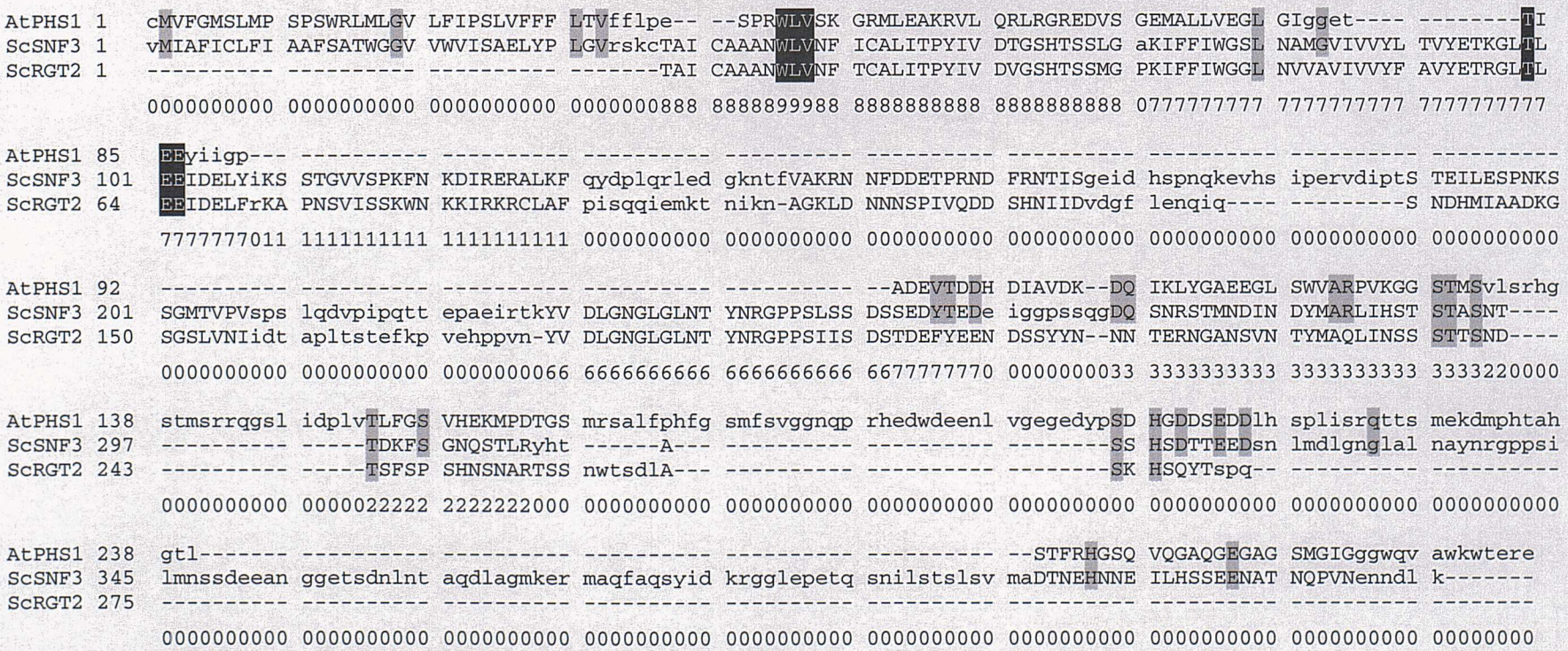


Figure 6.4. Alignment of the cytosolic loop of a putative hexose sensor/transporter to the C-terminal sensing region of two Yeast glucose sensors. AtPHS1 (At4g35300, F23E12.140) has been proposed to be a putative hexose sensor due to its extended cytosolic loop between transmembrane domain 6 and 7 (figure 6.2) which were reminiscent of the extended C-termini of the Yeast (*Saccharomyces cerevisiae)* glucose sensors ScSNF3 and ScRGT2 (Lalonde *et al.,* 1999). The cytosolic loop and C-termini domains were estimated using the hydroptahy plots (figure 6.2, 6.1) and the following amino acids were aligned; AtPHS1, 150-525; ScSNF3, 550-end; ScRGT2, 550-end. The software package DIALIGN (Morgenstern, 1999) was used as it is especially efficient where sequences are not globally related but share only local similarities, as is the case with many protein families. Please note that only upper-case letters are considered to be aligned. Numbers below the alignment reflects the degree of local similarity among sequences.

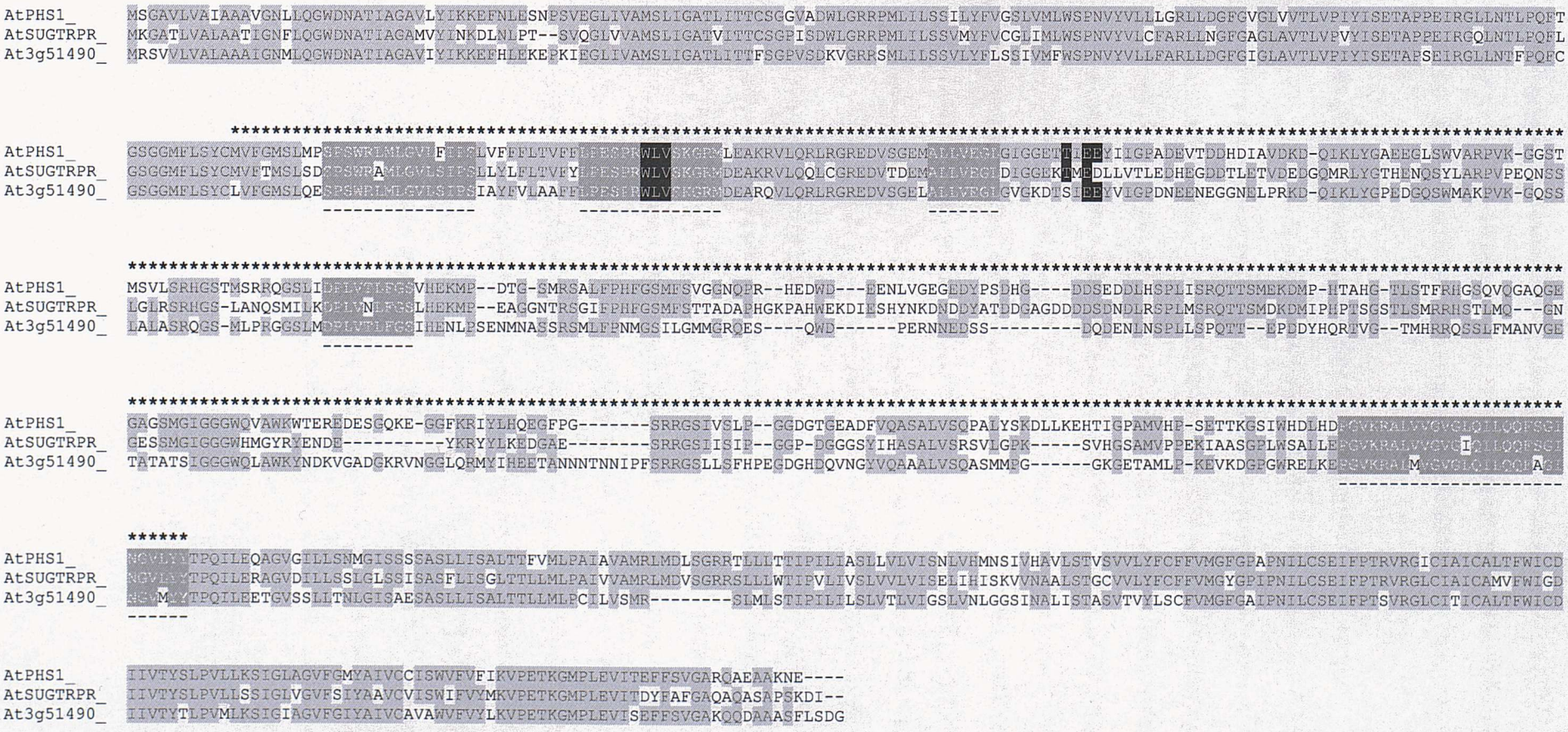


Figure 6.5. Alignment of three putative sugar transporters/sensors which contain extended central cytosolic loops. Putative sugar transporters/sensors AtPHS1 (At4g35300, F23E12.140), AtSUGTRPR (At1g20840) and At3g51490 all occur in the same family according to phylogenetic analysis (figure 5.1) and all contain extended central cytosolic loops which were postulated to be a sign of a sensing function (Lalonde *et al.,* 1999). Using ClustalW the amino acid sequences of the three putative sugar transporters/sensors were aligned. The row of stars shows the extended central cytosolic loop for AtPHS1 as determined by hydropathy plot (figure 6.3). Grey squares with black text show regions of homology. Dark grey squares with underlined white text hightlight regions of consistent homology between these three usual transporters/sensors that correspond to the extended central cytosolic loop of AtPHS1 (amino acids 150-525). Black squares show regions of similarity between the extended central cytosolic loops of AtPHS1 and the C-termini sensor regions of yeast glucose sensors (figure 6.4).

that are incorporated in the largest group of sugar transporters (STF1, figure 5.1). Interestingly, none of the sucrose transporter family, SUC or hexose transporter family, STP contained this sequence. At this moment it is not possible to draw any inferences from these findings.

6.2.2. PCR amplification and Cloning of *AtSUC3* and *AtPHS1*

Initially primers were designed for *AtSUC3* and *AtPHS1*. The primers were designed to anneal to regions just upstream of the 5' start of the coding region and a little downstream of the 3' end of the coding region to enable cloning of the entire coding region of the gene (for primer sequences please refer to table 2.1). Primers with similar properties (melting temperature, nucleotide length, G/C ratio) were found that were specific to their target as assessed by a BLAST search of the whole *Arabidopsis* genome (data not shown). RNA Transcripts for *AtSUC3* appeared to be present in many tissues of *Arabidopsis* (figure 6.6A). Although, semi-quantitative PCR was not possible from this experiment (the control primers did not work for some of the reactions) the experiment did indicated that transcript were most prevalent in the *Arabidopsis* stem cDNA preparation and therefore this prep was used to bulk amplify the cDNA (figure 6.6B). Before the PCR product was cloned it was first sequenced to ensure that the primers where amplifying the correct cDNA. This was confirmed (figure 6.7). The cDNA was then cloned using the pGEM-T Easy Vector (Promega, Hamsphire, UK) and transformed into *Escherichia coli* competent cells by heat shock. Using the blue/white screen for transformed colonies positive colonies were isolated, cultured and their plasmid was isolated and digested with *Eco*R1 to release the insert. The results of the digested plasmid were run on a gel which confirmed the presence of the insert within the plasmid (figure 6.6C).

RNA transcripts for *AtPHS1* were also found in *Arabidopsis* stem however amplification was weak and variable in comparison to that of *AtSUC3* even when using an increased reaction volume and amount of template (figure 6.8). However the single bands of product produced from the PCR reactions suggest that the primers are specific to the gene of interest as confirmed by a BLAST search of the sequencing result (figure 6.9).

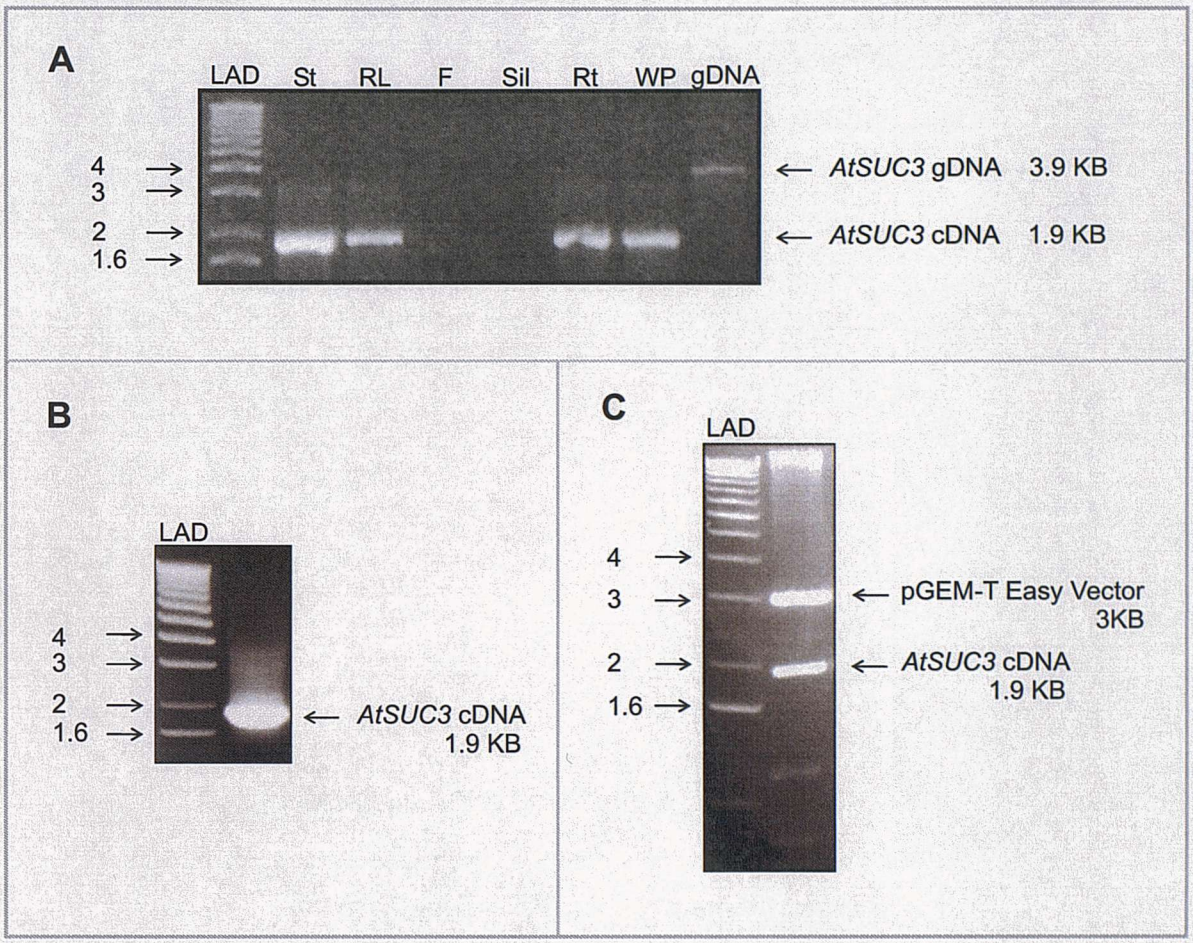


Figure 6.6. The PCR amplification and cloning of *AtSUC3* (At2g02860).

A: Tissue specific expression of AtSUC3. Each 10 µl PCR consisted of 500 ng of *Arabidopsis* stem cDNA, 500 nM of each *AtSUC3* specific forward and reverse primers, 200 µM of each dNTP and 0.1 units of AGS Gold Taq (Hybaid, Middlesex, UK) polymerase in 1X Klentaq buffer. The PCR thermocycle was as follows; 94°C for 1 min, 94°C for 30 s (melting step), 60°C for 30 s (annealing step) then 72°C for 2 min (extension step), 72°C for 5 min. The melting, annealing and extension steps were cycled 30X. LAD, 1 KB DNA Ladder (Hybaid, Middlesex, UK); St, Stem; RL, Rosette Leaf; F, Flowers; Sil, Silique; Rt, Root; WP, Whole Plant (aerial).

B; Bulk PCR from Stem cDNA. Each 20 µl PCR consisted of 500 ng of *Arabidopsis* stem cDNA, 500 nM of each *AtSUC3* specific forward and reverse primers, 200 µM of each dNTP and 0.1 units of Klentaq proof-reading polymerase (Hybaid, Middlesex, UK) in 1X Klentaq buffer. The PCR thermocycle was as follows; 94°C for 1 min, 94°C for 30 s (melting step), 60°C for 30 s (annealing step) then 72°C for 2 min (extension step), 72°C for 5 min. The melting, annealing and extension steps were cycled 40X

C; The plasmids from positive colonies for *AtSUC3* clones were isolated and cut by restriction digest with *EcoR1* and run on a 1% TAE agarose gel to confirm the presence of the insert.

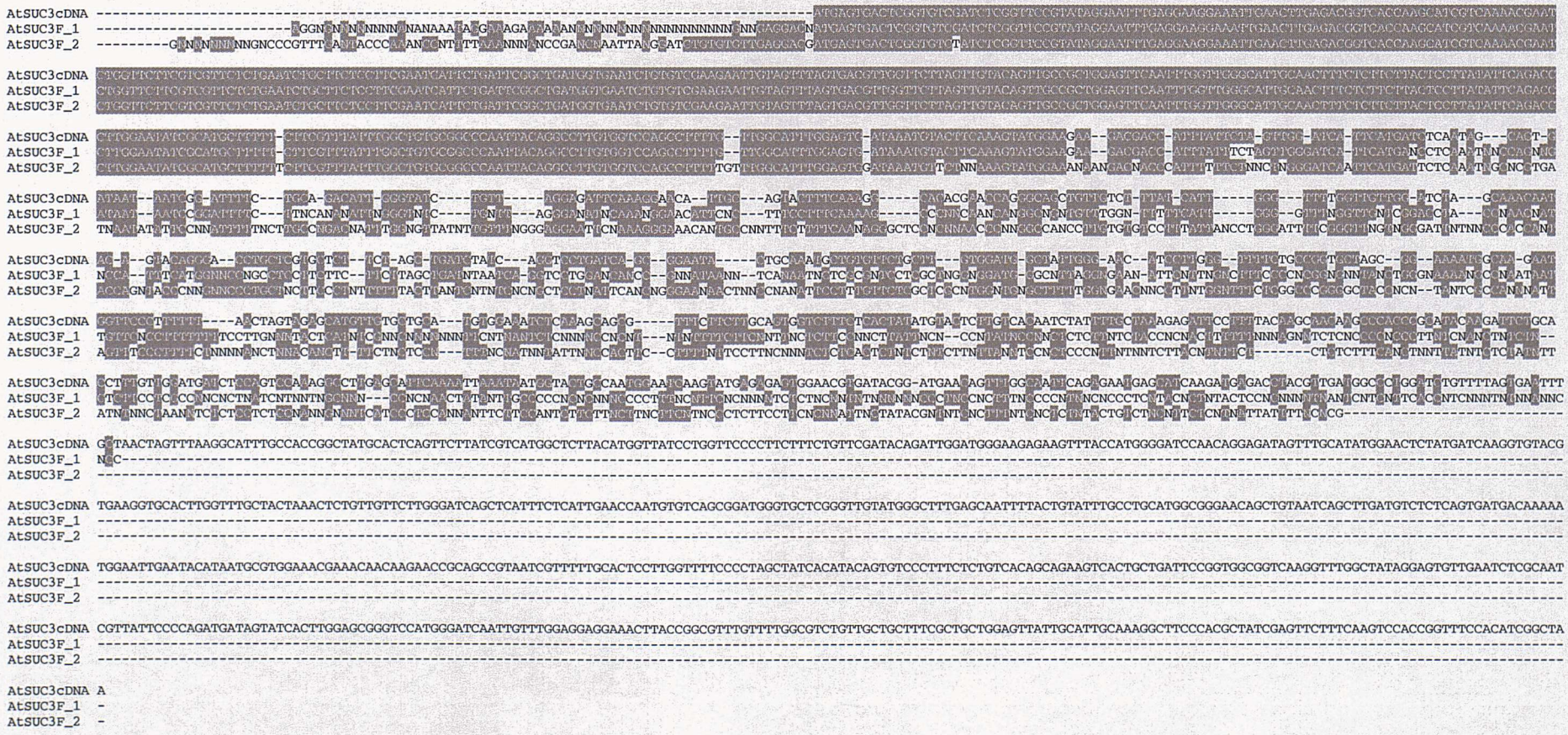


Figure 6.7. Sequencing of *AtSUC3* (also *AtSUT2*) PCR product. To check the specificity of the *AtSUC3* primers cDNA products were sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, UK) processed on the ABI PRISM® 377 DNA sequencer (PE Applied Biosystems, UK). N refers to un-identified nucleotides. AtSUC3cDNA refers to the predicted sequence obtained from the MIPS database, M*At*DB and AtSUC3F_1 and AtSUC3F_2 are the sequences obtained from the PCR product using the forward gene specific primer (for PCR primer details refer to table 2.1).

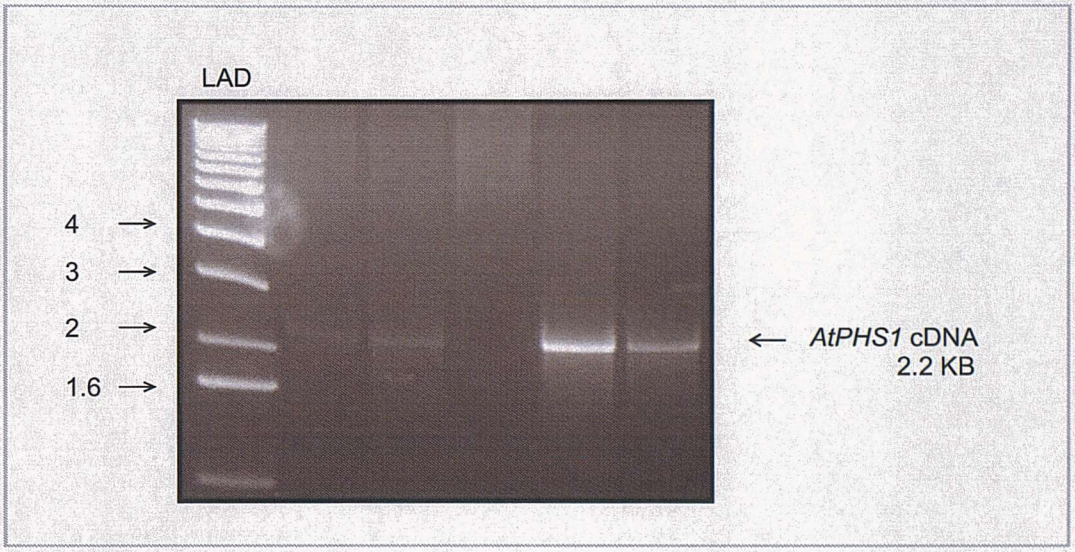


Figure 6.8. The variable PCR amplification of *AtPHS1* (At4g35300, *F23E12.140*) cDNA. Each 20 µl PCR consisted of 500 ng of *Arabidopsis* stem cDNA, 500 nM of each *AtPHS1* specific forward and reverse primers, 200 µM of each dNTP and 0.1 units of Klentaq proof-reading polymerase in 1X Klentaq buffer (Hybaid, Middlesex, UK). The PCR thermocycle was as follows; 94°C for 1 min, 94°C for 30 s (melting step), 60°C for 60 s (annealing step) then 72°C for .2.5 min (extension step), 72°C for 5 min. The melting, annealing and extension steps were cycled 35X. LAD, 1 KB DNA Ladder (Hybaid, Middlesex, UK).

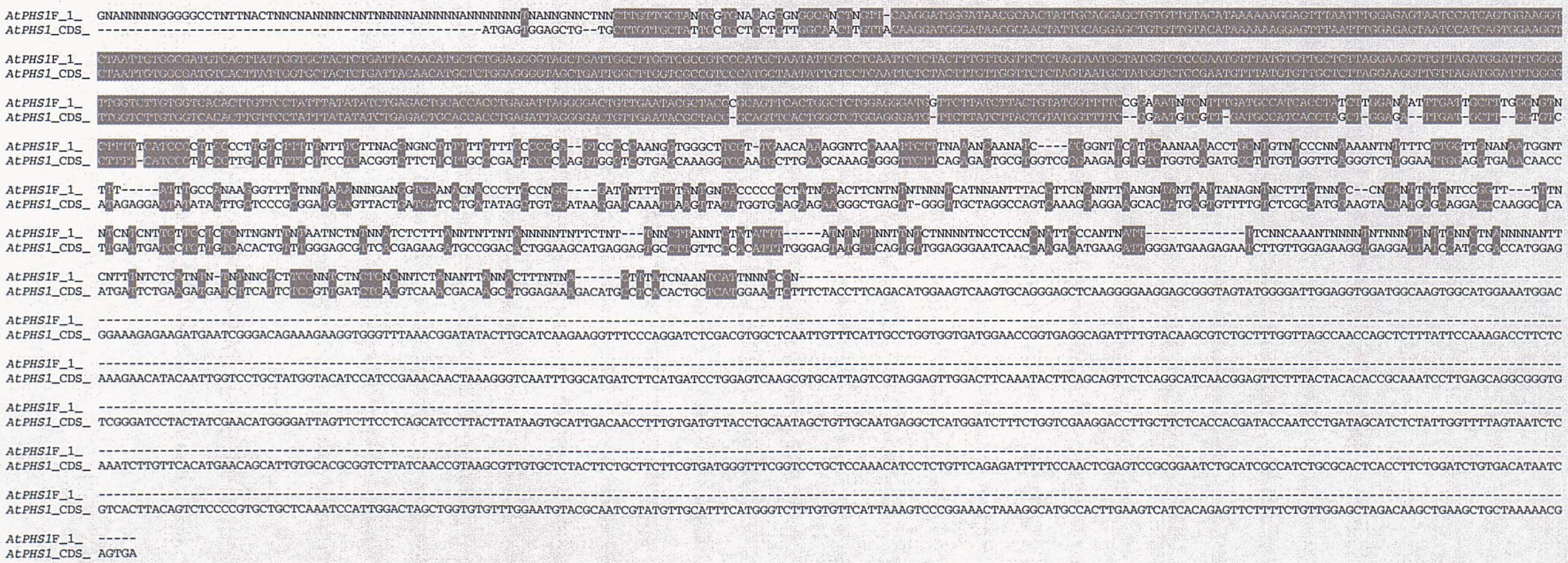


Figure 6.9. Sequencing of *AtPHS1* (also *F23E12.140*) PCR product. To check the specificity of the *AtPHS1* primers cDNA products were sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, UK) processed on the ABI PRISM® 377 DNA sequencer (PE Applied Biosystems, UK). N refers to un-identified nucleotides. AtPHS_cDNA refers to the predicted sequence obtained from the MIPS database, M*At*DB and AtPHS_1 is the sequences obtained from the PCR product using the forward gene specific primer (for PCR primer details refer to table 2.1).

6.3. Discussion

6.3.1. The Role of AtSUC3

It is unfortunate that the work presented in this chapter could not be continued to a satisfactory conclusion. The project developed well in other investigations (chapters) which warranted more attention and also publications characterising AtSUC3 (Barker *et al.*, 2000) indicated that other researchers were more advanced in studying this putative sugar transporter.

Barker *et al.* (2000) showed a predicted topology with 12 transmembrane domains with a large cytosolic loop this in agreement with the hydrophobicity plot shown in this chapter (figure 6.2). The predicted topology diagram by Barker *et al.* (2000) also showed two central conserved boxes within the cytosolic loop; one at the start and the other at the end. These are conserved in SUC3 from both *Arabidopsis* and Tomato and are thought to be an indication of a common function. Tomato *LcSUC3* RNA transcripts were shown be present in all tissues but most abundant in leaf tissue and more specifically in source leaf tissue than sink leaf tissue (Barker *et al.*, 2000). The results in this chapter showed SUC3 most abundant in stem *Arabidopsis* tissue but also present to a lesser degree in leaf and root tissue. Unlike Barker *et al.* (2000) transcripts were not detected in flower and siliques tissues. These differences in the localisation of SUC3 may be due to the different plant species studied and/or different developmental stages of the plants. Using immunocytochemical localisation *Lc*SUC3 was shown to be situated in the sieve elements of the phloem in Tomato (Barker *et al.*, 2000). Differences in the sites of sucrose transporters have been demonstrated before; SUC2 is located in sieve elements of the phloem in plants of the Solanaceae family such as tomato, tobacco and potato (Kühn *et al.*, 1997) whereas in *Arabidopsis* which is part of the Brassicaceae family the protein resides in the companion cells of the phloem (Stadler and Sauer, 1996). Later Meyer *et al.*, (2000) showed that rather than AtSUC3 being located in the companion cells of the phloem as with AtSUC2, AtSUC3 was located outside of the phloem adjacent to it in the large parenchymatic cells between the mesophyll and the phloem suggesting a role in sucrose funnelling from the mesophyll towards the phloem.

One of the most interesting results by Barker *et al.* (2000) is the negative functional complementation of mutant yeast strain by LeSUC3 which indicated that this protein although structurally very similar to sucrose transporter does not transport sucrose. This is a similar scenario to glucose sensors in yeast which do not transporter glucose even though they are structurally similar to yeast glucose transporters (Özcan *et al.* 1996). However, it was later shown that AtSUC3 does

transport sucrose with a K_m of 1.9 mM (Meyer *et al.*, 2000) making it a high affinity sucrose transporter akin to AtSUC1, AtSUC2 (K_m 0.45 mM and 0.53 mM respectively, Sauer and Stoltz, 1994) and AtSUC5 (K_m 1 mM, Ludwig *et al.*, 2000) as opposed to the low affinity sucrose transporter AtSUC4 (K_m 11.6 mM, Weise *et al.*, 2000). Mutagenesis on the cytosolic loop of AtSUC3 resulted in the deletion of 55 amino acids (295-349) which resulted in hardly any difference in sucrose transport properties (Meyer *et al.*, 2000) indicating that the cytosolic loop does not have a role in the transport of sucrose. The transport of sucrose by AtSUC3 does not rule out a role in sucrose sensing as other proteins have been shown to have dual roles such as hexokinase which includes separate domains for glucose phosphorylation and sensing (Entian and Fröhlich, 1984). However, if AtSUC3 does not have a sensing function and neither do any of the other sucrose transporters, surely this would hamper the efficiency of a sugar sensing system. One caveat of a plant cell that has a membrane bound hexose sensor and no sucrose sensor is that only sucrose that has been broken down by invertase prior to transportation into the cell will be monitored whereas sucrose that is transported into the cell will not be detected. It may be that a sucrose sensing function operates by a different mechanism to glucose sensing in yeast. The reported interaction between AtSUC2/AtSUT1 and AtSUC3/AtSUT2 raises the question of protein-protein interactions as a means of modifying sugar transport in response to cellular sugar status (Reinders *et al.*, 2002). This protein interaction may be the role of the extended cytosolic loop domain which would explain why deletions within this domain did not affect sucrose transport (Meyer *et al.*, 2000).

It was unfortunate that an *AtSUC3* mutant could not be found in the SAIL T-DNA collection as studies using such a plant might help us detect its role in the plant and more intriguingly with respect to its putative sucrose sensing function. One possibility would be to compare the expression of genes affected by the sugar status of the plant in a wildtype and *suc3* knockout mutant. However since preventing the function of a sucrose transporter will affect the sugar status in the plant difference between a wildtype and *suc3* knock-out mutant can not be immediately attributed to a sensing function. Assuming that sugar transporter and sensing are separate functions, one approach would be to combine this approach with site directed mutagenesis of *AtSUC3* in an effort to analyse the sensing function while retaining default transport.

Recently it would appear that T-DNA mutants have been produced that may well disrupt this gene. Using the SIGnAL "T-DNA Express" *Arabidopsis* Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) 7 mutants are registered in the database as being located within the gene of *AtSUC3* (At2g02860). These comprises three GABI-Kat mutants, 1 in an intron, another within an

exon and another in the 5' untranslated region, a SALK and a SAIL mutant also in the 5' untranslated region and 2 SALK and 2 GABI-Kat mutants in the promoter region. With more time some of these mutants could have been obtained and studied.

6.3.2. The Role of AtPHS1

It was regrettable that progress with *AtPHS1* (At4g35300) was slower than that of AtSUC3. The low yield of amplified cDNA could be interpreted as a sign that *AtPHS1* transcription is very low. Alternatively it may be a sign that the role of this sugar transporter/sensor is apparent only under certain conditions. Unlike AtSUC3, there have been no published studies involving *AtPHS1* so only speculative proposals for its function can be made. Since *AtPHS1* is predicted to be a hexose transporter/sensor it was possible to compare the putative sensing region, the extended central cytosolic loop to the sensing regions of two yeast glucose sensors (figure 6.4). Since these regions are possibly concerned with the same function which is universal (i.e. a glucose molecule is the same in all organisms) similarities might be apparent. While two areas were highlighted as showing similarities, the small areas of homology cast in doubt whether this is a sign of shared function. However, the alignment of the three putative sugar transporters/sensors showed several region of consistent homology within the extended central cytosolic loop suggesting a conserved function (figure 6.5). And one of those regions of consistent homology contained an amino acid triplet also found in the C-terminal sensing domain of yeast glucose sensors (figure 6.4).

It is intriguing that *AtPHS1* is not alone within its family as two other putative transporters also contain an extended cytosolic loop (figure 6.3). If these genes do encode proteins with a sensing function it may be that they have different locations within *Arabidopsis* which allows for tissue specificity in controlling sugar status. A relatively quick and easy way to investigate this would be to design specific primers and perform PCR on tissue specific cDNA to ascertain in which tissue they are expressed. This could be followed up more accurately by *in situ* hybridisation or, more appropriately for investigating the function of the protein, immunocytochemical localization. Using the SIGnAL "T-DNA Express" *Arabidopsis* Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) it was found that T-DNA mutants in all these putative transporters/sensors can be obtained from various T-DNA collections. It would be interesting to investigate the role of these putative transporters/sensors by studying the phenotype of plants that can not transcribe these genes.

Chapter 7.

General Discussion

7.1. Discussion of major findings

This thesis reports on a variety of different approaches to the study of the role and regulation of sugar transporters in plants: reporter gene analysis, reverse genetics, microarray technology, sequence analysis and cloning.

Using a reverse genetics approach which utilized insertional mutants from the SLAT collection two sucrose transporters were studied in detail and produced very different results. Initially, phenotypes of the *suc1-1* mutants were indistinguishable from the wildtype control plants. AtSUC1 expression has been shown to occur in floral tissue (Stadler *et al.,* 1999). With this in mind, experiments were designed to investigate the time to flowering (figure 3.11) in which both *suc1-1* het and hom showed a delay compared to wildtype. Since the heterozygous *suc1-1* mutants also showed this phenotype this subtle difference may be a result of the different genetic backgrounds between the wildtype and *suc1-1* mutants. *AtSUC1* promoter driven GUS staining shows *AtSUC1* expression only in the pollen grains of flowers and is absent from the anthers and floral buds (Stadler *et al.,* 1999). Experiments in this study showed that the pollen germination of *suc1-1* hom is retarded *in vitro* (figure 3.13). This further strengthens the proposal by Stadler *et al.* (1999) that pollen tube growth is one of the roles of AtSUC1. AtSUC1 expression is also seen in pollen grains released from the anthers and growing pollen tubes penetrating the walls of the papillae (Stadler *et al.,* 1999). Furthermore, in the same paper it was shown that the presence of sucrose and not either glucose or fructose was essential in the germination of *Arabidopsis* pollen, an indication that AtSUC1 is responsible for the import of sucrose as a carbon source for pollen metabolism. Since *suc1-1* homozygous plants are able to produce quantities of viable seed akin to wildtype it would appear that AtSUC1 is not the only sucrose transporter that performs this role.

The expression of SUC2/SUT1 has been found in the source leafs of a variety of plant species; *Arabidopsis* (Truernit and Sauer, 1995; Stadler and Sauer, 1996), carrot (Shakya and Sturm, 1998), *Plantago major* (Stadler *et al.,* 1995), potato (Riesmeier, *et al.,* 1993), rice (Hirose *et al.,* 1997) and tobacco (Bürkle *et al.,* 1998). In all species, studies have indicated that the role of this sucrose transporter is in phloem loading but its location is species dependant. SUT1/SUC2 is located in sieve

elements of the phloem in plants of the Solanaceae family such as tomato, tobacco and potato (Kühn *et al.,* 1997) whereas in *Arabidopsis* which is part of the Brassicaceae family SUT1/SUC2 resides in the companion cells of the phloem (Stadler and Sauer, 1996). *AtSUC2* promoter-driven GUS staining in the vascular tissue of the cotyledons (figure 4.2) and the phenotype of retarded growth and development of the *suc2-4* homozygous mutants (figure 3.14) is in agreement with these previous reports and supports existing evidence that the role of SUT1/SUC2 is in the phloem loading of source leaves.

There is little evidence for regarding the affect of sugars on the transcriptional regulation of sugar transporters. The 30-50% reduction in sucrose symporter activity of plasma membrane vesicles from leaves that were fed 100 mM sucrose prompted Chiou and Bush (1998) to propose that sucrose is a signal molecule in assimilate partitioning. This shows that sucrose symporter activity responds to sucrose levels. What remains to be elucidated is which sucrose transporter genes are affected by sugar status and how. Both RNA and protein levels for SUT1/SUC2 has been shown to be down-regulated by sucrose supplementation and results in this thesis show that this response is very sensitive as down-regulation of AtSUC2 is seen at 5 mM sucrose supplementation (figure 4.10) (*OsSUT1,* 100 mM, Matsukura *et al.,* 2000; BvSUT1, 100 mM, Vaughn *et al.,* 2002). However, since this is the concentration of sucrose supplementation in the media and not necessarily the concentration of sucrose that is causing the down-regulation in the cotyledons of the *Arabidopsis* seedlings it is not possible to comment on the physiological significance of this finding. There is plentiful evidence supporting the role of SUC2/SUT1 in phloem loading (Riesmeier, *et al.,* 1993; Gahrtz *et al.,* 1994; Lemoine *et al.,* 1996; Burkle *et al.,* 1998; Schulz *et al.,* 1998; Shakya and Sturm, 1998; Gottwald *et al.,* 2000; Wright *et al.,* 2003). With this in mind it seems surprising that the genomic response to an increase in sucrose is a decrease in expression. Logically it would make sense that in response to an increase in sucrose in source tissues the major sucrose phloem loader increases in abundance to pack the phloem which would aid bulk flow from source to sink tissues. Maybe this scenario is too simplistic and further investigations will discover the reasons for the sucrose-mediated down-regulation of SUC2/SUT1 shown in this thesis.

7.2. Future work.

Much of the work in this thesis has produced some interesting preliminary findings. It was unfortunate that efforts towards the end of the project to obtain a repeat data set for the microarray experiment were unfruitful. This repeat was especially important not only because of the inherent

variability in microarray data but additionally because in the initial experiments an exceptionally low signal to background ratio caused by a variable smearing of the chip which hampered the acquisition of reliable data. Using the data obtained from the microarray experiments it was expected that interesting findings would be investigated more closely using northern blot or RT-PCR analysis. The microarray data showed that AtSUC2 was up-regulated 2-fold during de-etiolation by FR light (chapter 5) even though no increase of AtSUC2 was seen upon de-etiolation by white light for 24 hrs (figure 4.8). The AtSUC2-GUS mutants would enable this microarray finding to be investigated by assaying transcriptional activity before and after 12 h FR de-etiolation using the GUS quantification system. Predicting the *cis*-regulatory elements utilized the microarray data and relied on the correlation of promoter region for the transporter genes most up/down regulated sharing regions of homology. However, due to the efforts made to reduce rogue data genes that maybe should have been included in the list of the genes most up/down regulated might have been excluded due to their exceedingly low signal to background ratio, thus weakening the homology between the promoter sequences of gene in the lists. This goes some way to explain why no common *cis*-regulatory elements were found in all transporter genes that were most up/down regulated. Therefore with further repeats of the microarray experiment and a reliable data set the *cis*-regulatory elements prediction should also be repeated in an effort to improve the discovery of putative *cis*-regulatory elements responsive to FR de-etiolation.

The role of AtSUC1 at first appeared more subtle or perhaps transient as there was no dramatic phenotypic difference between the *suc1-1* knockout mutant and wild-type plants. Specific analysis showed poor in vitro pollen germination and growth in the *suc1-1* mutant (Francini and Williams, unpublished). With more time I would have liked to further this analysis by performing RT-PCR for sucrose transporter genes AtSUC2-9 using RNA extracted from flowers to investigate if other sucrose transporters were compensating in the absence of AtSUC1 during the sugar loading of pollen.

AtSTP4 also showed down-regulation by sucrose but to a lesser degree and also less sensitive (25% reduction at 30 mM). Although this was also shown using a RT-PCR approach preliminary GUS assays failed to show this sucrose-mediated down-regulation. This discrepancy may be attributable to the longevity of the GUS reporter product resulting in a less sensitive assay for such small responses.

7.3. Tissue specific distribution and *in planta* role of sugar transporters

The sequencing of the *Arabidopsis* genome has shown that there are many putative sugar transporter genes. The phylogenetic analysis (figure 5.1) indicates that there are 8 major families, only 3 of which contain members that have been characterized. Already with only 12 of the 71 putative sugar transporters being investigated sugar transporters have been found in most tissues in *Arabidopsis*. This suggests the roles of sugar transporters in *Arabidopsis* overlap causing functional redundancy. The reason for this may be that different sugar transporters possess different transport properties allowing moderation of sugar transport and thus tight regulation. In potato SUC2/SUT1 and SUC4/SUT4 both reside in the sieve elements but have very different affinities for sucrose. With SUC2/SUT1 a *Km* of 0.77 mM is part of the high affinity/low capacity system of sucrose transporters while SUC4/SUT4 with its *Km* of 11.6 mM and *Vmax* of 9.8 is thought to be the opposite, a low affinity/high capacity sucrose transporter (Sauer and Stolz, 1994; Weise *et al.*, 2000). Also different sugar transporters are likely to be regulated in response to difference signals. This enables a system that is responsive to the environmental constraints while allowing subtle changes to carbon partitioning. The response of sugar transporters to light and sugar will be discussed in the next section.

Interestingly, out of the 12 sugar transporters that have so far been studied 5 have been shown to have roles in pollen (STP2,4,6,9 and SUC1, table 7.1). This may represent the important role of sugar import into pollen to fuel pollen germination and fertilization. However sugars are not only sources of carbon but also can serve in creating a water potential gradient to encourage cell expansion, a possible role of SUC1 in the growth of the pollen tube (Williams *et al.*, 2000a). In contrast to the many sugar transporters devoted to pollen it would appear that one of the most important roles in the mobilization of sugars, phloem loading, is undertaken predominately by one sucrose transporter, SUC2/SUT1. As shown here (figure 3.14) and also in the literature, knocking out this gene or attenuating its translation and thus preventing a functional protein has a dramatic effect on the phenotype of the plant. Due to the sink tissues not receiving sufficient sucrose, growth and development is retarded, also the excess sucrose present in the source leaves is converted into forms of carbon storage causing in enlarged starch grains (Schulz *et al.*, 1998; Gottwald *et al.*, 2000). This dramatic phenotype indicates that the loss of SUC2/SUT1 can not be compensated for by other sucrose transporters. However these mutants are able to complete their life cycle which suggests that a limited supply of sugars are able to reach the sink tissues, which as proposed earlier may be due to the contribution of other sugar transporters.

Table 7.1. The *in planta* locations of sugar transporters in *Arabidopsis*. STPs are a family of 14 monosaccharide sugar transporters, SUCs are a family of 9 sucrose transporters and STF1 refers to the Sugar Transporter Family 1 which consists of 21 putative sugar transporters (figure 5.1).

Sugar Transporter	Location	Reference
STPs		
STP1	guard cells, seedlings	Stadler *et al.*, 2003 Sherson *et al.*, 2000
STP2	pollen	Truernit *et al.*, 1999
STP3	green leaves, floral tissue	Büttner *et al.*, 2000
STP4	roots, pollen, stress	Truernit *et al.*, 1996
STP5	?	
STP6	pollen	Scholz-Starke *et al.*, 2003
STP7	?	
STP8	?	
STP9	pollen	Schneidereit *et al.*, 2003
STP10-14	?	
SUCs		
SUC1	anthers, pollen tubes	Stadler *et al.*, 1999; figure 3.13
SUC2/SUT1	companion cells of the phloem	Stadler and Sauer, 1996
SUC3SUT2	carpel cell layer adjacent to vascular tissue	Meyer *et al.*, 2000
SUC4/SUT4	sink leaves	Weise *et al.*, 2000
SUC5	all tissues flowers and silique	Ludwig *et al.*, 2000 figure 3.8
SUC6-SUC9	?	
STF1		
pGLcT	putative chloroplast hexose transporter	Weber *et al.*, 2000

One member of the SUC family does have an unusual characteristic; AtSUC5 has been shown to transport biotin (vitamin H) as well as sucrose (Ludwig *et al.*, 2000). Its location is still not clear since RT-PCR experiments in chapter 3 showed that expression was confined to the flowers and siliques while previous studies also using RT-PCR have found expression in all tissues (Ludwig *et al.*, 2000). Also expression was not found in seedlings whereas the microarray data in chapter 5 showed a 3.3-fold increase in expression in seedlings following a FR light treatment. To clarify the issue of *AtSUC5* gene expression localization, reporter gene technology could be used and it is hoped that the isolation of a *suc5* mutant will facilitate the understanding of this transporter.

SUC3/SUT2 is unique in the sucrose transporter family as it contains an extended central cytosolic loop which mirrors similarities in yeast glucose sensors. Yeast sensors do not transport glucose and so when SUC3/SUT2 was initially found to be unable to transport sucrose by yeast-mutant complementation a sensing function was assumed. However, further analysis confirmed that sucrose transport was apparent for SUC3/SUT2 (Özcan *et al.*, 1998; Barker *et al.*, 2000; Meyer *et al.*, 2000). A sensing function for SUC3/SUT2 has not been ruled out and interestingly studies have shown that the unique (among sucrose transporters) extended central cytosolic loop does not participate in transport (Meyer *et al.*, 2000). The role of this extended central cytosolic loop may be in the process of protein-protein interaction reported between SUC3/SUT2 and SUC2/SUT1 in the enucleate sieve elements (Reinders *et al.*, 2002). It is likely that experiments are underway to investigate this theory most probably by using site-directed mutagenesis to alter to extended central cytosolic loop and assess if and how these interactions are affected. In contrast there has been no mention of AtPHS1 (At4g35300) in the literature or any of the other members that the sugar transporter family 2 (STF2, figure 5.1) which also contain extended central cytosolic loops (AtSUGTRPR (At1g20840) and At3g51490, figure 6.3). A promising way to start the investigation into these unique sugar transporters would be to study, initially, the site of their expression using tissue specific RT-PCR. This could be furthered by obtaining insertional mutants of these genes to investigate their function within the plant.

With continuing investigation into the role of individual sugar transporters in *Arabidopsis* it is hoped that role of each sugar transporter will be discovered and enable us to better understand carbon partitioning in plants.

7.4. The regulation of sugar transporters

As described previously sugar transporters have important roles in the adaptation of plants to their environment; a vital property for a sedentary organism. There is surprisingly little known about the regulation of sugar transporters, however due to the development and use of full genome microarray experiments much data regarding this area is being produced. Unfortunately much of this information is not being utilized as researchers are naturally concentrating on the genes that relate more specifically to their topic area. As yet there is not a satisfactory system for results of such experiment to be comprehensively amalgamated. Such as system would enable a researcher to pick their gene of interest and, using results of this gene from many microarray experiments, view the regulation of this gene with regard to various factors that have been investigated.

7.4.1. Light-mediated transcriptional regulation of sugar transporters

A plant responds to light by adapting its structure a process known as photomorphogenesis. This change in structure brings with it a change in the sugar requirements of different tissues to fuel this adaptation. While the GUS assays in this study did not show convincing evidence of light-mediated regulation of AtSTP4 and AtSUC2 other reports have shown up-regulation of SUC2/SUT1 by light in a variety of different species; leaves of tomato, leaves of carrot, maize seedlings and rice embryos (Kühn *et al.,* 1997; Shakya and Sturm, 1998; Aoki *et al.,* 1999; Matsukura *et al.,* 2000). In contradiction of these findings SUC2/SUT1 expression has also been shown to be higher in etiolated seed and shoots of seedlings compared to those light grown (Hirose *et al.,* 1997). Similarly the *Arabidopsis* guard cell hexose transporter, *AtSTP1,* also shows dark induction which is a surprising result since guard cell chloroplasts are packed with starch at the end of the photoperiod (Stadler *et al.,* 1999). The microarray experiment showed that many sugar transporters were affected during the adaptation of de-etiolated seedlings to FR light which is monitored exclusively by the photoreceptor, phytochrome A (table 5.5). Investigation of the supplementary data of a similar study revealed that two sugar transporters responded to the FR treatment, one of which was *AtSUC1* and the other At4g36670, a putative mannitol transporter (according to annotations in the NCBI database) that is uncharacterised (identified in this study using the accession code given by Tepperman *et al.* (2001). Interestingly the two sugar transporters showed opposite transcriptional regulation by phytochrome A during de-etiolation under FR light (Tepperman *et al.,* 2001). Both genes were classified as late regulated but while *AtSUC1* was induced, the mannitol transporter, At4g36670, was classified as a late repressed gene. In the microarray experiment shown in this study *AtSUC1* is just below the threshold to be classified as FR up-regulated and even though it shows a slight increase (1.8-fold) it is

deemed to be un-affected (table 5.5). Interestingly the mannitol transporter shown to be repressed by Tepperman *et al.* (2001) was the second highest up-regulated sugar transporter gene with 15-fold increase according to the microarray experiment shown in this thesis (table 5.5). However like many genes that data had to be ignored due to its low signal to background ratio of 1.1:1. Care must be taken when inferring light-mediated regulation as light and sugar synthesis are interconnected. When studying light regulation it is difficult to decipher whether a response is due directly to light signalling or indirectly via sugar sensing/signalling. Recently, a novel method to investigate complex interactions of carbon and light signalling (blue, red, far-red) in the regulation of genes involved in nitrogen assimilation has been reported using Boolean circuits to assign genes into categories which allows intricate analysis of which factors regulate and how (Thum *et al.*, 2003).

7.4.2. Transcriptional regulation of sugar transporters by sugars

Given the significance of the role of sugar transporters in controlling cellular sugar levels surprisingly little is know about how their regulation is affected by their substrate. Chiou and Bush (1998) showed sucrose transporter activity to decrease to 35-50% in plasma membrane vesicles isolated from leaves which had been fed 100 mM sucrose (compared to water controls). Since a similar response was not observed by feeding glucose, their results imply sucrose levels of monitored possibly by sucrose transporters or alternatively invertases. Vaughn *et al.* (2002) postulated that a sucrose sensor in the phloem responds to increased sucrose in the phloem by down-regulating transcription of the sucrose transporter gene; due to the rapid transporter protein turnover and mRNA degradation, the phloem loading capacity would be reduced. While the study by Chiou and Bush (1998) shows a connection between sugar levels and sugar transporter transcription the use of plasma membrane vesicles from leaves does not allow the response of individual sugar transporters to be studied. As seen in this study (figure 4.2) and by Truernit and Sauer (1995) GUS histochemical staining is not sufficiently responsive to show subtle differences in *SUC2/SUT1* transcriptional regulation. However using the more sensitive method of GUS quantification by fluorometric assay it was shown that both *AtSTP4* and *AtSUC2/SUT1* are down-regulated in seedlings by sucrose supplementation of the media (figures 4.9 and 4.10). The finding that just 5 mM sucrose supplementation of the media decreases *AtSUC2* expression to 25% of the control (no sucrose) is an interesting result that prompts further experiments. Down regulation of SUC2/SUT1 in citrus leaf discs was observed when an exogenous sugar source of 25 mM sucrose was applied. However, as this was the lowest concentration it is not possible to ascertain the threshold for this response (Li *et al.*, 2003). In contrast to the sugar-mediated down-regulation of sugar transporters a hexose transporter

in grape is up-regulated by glucose (Atanassova *et al.*, 2003). Its presence in many sink tissue suggests a role in the influx of sugars into cells that is able to respond to sugar availability.

7.4.3. Post-transcriptional regulation

Transcriptional regulation is by no means the only way to affect trans-membrane sugar transport. Furthermore transcriptional regulation does not always reflect protein levels (Franklin *et al.*, 2003b). Regulation can also occur at the stage of translation, by phosphorylation of the protein (Ranson-Hodgkins *et al.*, 2003) and potentially by protein-protein interactions (Reinders *et al.*, 2002). Using the split ubiquitin system, Reinders *et al.* (2002) showed that two out of three sucrose transporters that are found in enucleate sieve elements (SUC2/SUT1, SUC3/SUT2, SUC4/SUT4) have the potential to form homooligomers. Also all three sucrose transporters have the potential to interact with each other. Protein-protein interactions have been reported for the mammalian glucose transporters which function in a configuration of four homooligomers that cooperate during transport (Hamill *et al.*, 1999). Another possible mode of transporter regulation, shown by mammalian glucose transporters, is membrane trafficking. GLUT4, a mammalian facilitative glucose transporter moves from intracellular storage areas to the plasma membrane to increase cellular glucose uptake (Simpson *et al.*, 2001). Protein levels of the phloem sucrose transporter, SUC2/SUT1, have been to be responsive to sugar (sucrose) levels suggesting that protein turnover regulation is active in regulating sugar transporters (Vaughn *et al.*, 2002).

7.4.4. Cis-acting factors in the regulation of sugar transporters

Sugar transporter transcription has been shown to be responsive to light, sugars, mechanical stress/phytopathogen attack and hormones (reviewed in Delrot *et al.*, 2000). The abundance of information to be found on bioinformatics websites such as MIPS (Schoof *et al.*, 2002) and TAIR (Huala *et al.*, 2001), together with microarray data means that there is a wealth of data which can be used for promoter analysis. Conserved sequences or motifs within the promoter regions might indicate common transcription factors for the genes with a similar function such as phloem loading (SUC2/SUT1 and SUC4/SUT4) or environmental responses such as wounding (STP3 and STP4). There are various internet databases regarding plant *cis*-regulatory elements (see chapter 5), but by far the most advanced and promising is the *Arabidopsis* Gene Regulatory Information Server (AGRIS, http://arabidopsis.med.ohio-state.edu/) which contains two databases (Davuluri *et al.*, 2003). At*cis*DB consists of the promoter sequences of 27,975 annotated *Arabidopsis* genes with a description of putative *cis*-regulatory elements and AtTFDB contains information on approximately 1,500 transcription factors. The seven sugar transporters featured in the AtTFDB are grouped together in

the *Arabidopsis* C2H2 zinc finger protein family suggesting that all these sugar transporters interact with this transcription factor. Furthermore, all seven of these sugar transporters group together in the phylogenetic tree (chapter 5, figure 5.1) within the same branch labelled as sugar transporter family 1 (STF1) (table 5.2). STF1 contains the SUGTL sugar transporters, two of which are featured in the AtTFDB and one of the two is featured in the AtcisDB (table 7.2).

Twenty-three sugar transporters from six different branches of the phylogenetic tree are present in the At*cis*DB (table 7.2). It would be interesting to find out if the branches of the phylogenetic tree show different *cis*-regulatory elements. Mutant screens in both light (Quail, 2000) and sugar responses (Rook and Bevan, 2003) are enabling the identification of transcription factors involved in the signal pathways.

In addition, the sequences of *cis*-regulatory elements catalogued in the PLACE website (Higo *et al.*, 1999; http://www.dna.affrc.go.jp/htdocs/PLACE) could be used to search the promoter regions of sugar transporters. Not only could this illuminate new regulatory factors in specific sugar transporters, but it also might reveal cross talk in the actions of transcription factors that would help explain some of the pleiotropic effects seen in some mutants. Preliminary TAIR blast homology searches with *cis*-regulatory elements involved in light against the DNA database of loci ~3 Kbp upstream sequences were performed using the TAIR BLAST search facility but sugar transporter genes were not found. The interpretation of this initial search result is that sugar transporter genes do not appear to have any of the most common light-regulatory elements in their promoters. This raises the question of whether observed light-mediated regulatory effects are mediated through direct light-signalling cascades or rather through indirect effects, perhaps from morphological changes or light-driven changes in source-sink relationships.

7.5. Sugars and Light Signalling

The microarray experiments looked at the effect of far-red light signals on sugar transporter expression. Synergistically, sugars have also been shown to affect phytochrome signalling (Dijkwel *et al.*, 1997; Short, 1999). Previously, seedlings containing a plastocyanin promoter-luciferase fusion gene (*PC-LUC*) had been used to show that the gene expression of plastocyanin (a copper containing protein involved in photosynthesis), is repressed by sucrose in *Arabidopsis* seedlings and this provided a link between metabolic repression and light signalling (Dijkwel *et al.*, 1997). Dijkwel *et al.* (1997) used the *PC-LUC* transgenic seedlings used to screen for mutants that showed **s**ucrose **un**coupled photomorphogenesis and isolated several *sun* mutants. Previously it was reported that

Table 7.2. Sugar transporters that feature within in the *Arabidopsis* Gene Regulatory Information Server (AGRIS). Information obtained from the web-based database at http://arabidopsis.med.ohio-state.edu/ (for more information refer to Davuluri *et al.*, 2003). * name of BAC.

AGRIS AtTFDB	AGRIS AtcisDB	Gene Names	Sugar Transporter Family
	At1g22710	*SUC2*	SUC
	At1g66570	*SUC7*	SUC
	At1g71890	*SUC1*	SUC
	At2g14670	*SUC8*	SUC
	At5g06170	*SUC9*	SUC
At1g54720			STF1
At3g05150	At3g05150		STF1
	At5g18840		STF1
	At2g48020		STF1
At1g08900		*SUGTL2*	STF1
At1g08890			STF1
	At4g04750		STF1
	At4g04760		STF1
At3g05400	At3g05400	*SUGTL5*	STF1
At3g05155	At3g05155.		STF1
At3g05160	At3g05160		STF1
	At5g27360		STF1
	At5g27350		STF1
	At4g35300	*AtPHS1 (F23E12.140)**	STF2
	At1g71880		STF4
	At5g16150	*pGlcT*	STF4
	At4g36670		STF5
	At2g18480		STF5
	At3g18830		STF5
	At2g16120		STF5
	At2g16130		STF5
	At5g17010		STF6

sucrose repressed the inhibition of hypocotyl elongation under FR light (Whitelam *et al.*, 1993), but when the response was analysed in *sun7*, one of the more severe *sun* mutants, the effect was much reduced (Dijkwel *et al.*, 1997). This infers that the gene product of *SUN7* functions in the sucrose-dependent repression of the inhibition of hypocotyl elongation. Another mutant, *sun6,* indicated that its protein has a role in the block of greening, not in the inhibition of hypocotyl elongation. (Dijkwel *et al.*, 1997). This confirmed previous results, which suggested that the block of greening and the inhibition of hypocotyl elongation were controlled by separate branches of phytochrome A signalling. Moreover, since both greening and hypocotyl elongation have implications in the requirement for sugars, this indicates that the signal pathways that cause changes in sugar synthesis and allocation diverge at an early stage. However, this is not evidence that these pathways stay distinct; common transcription factors might operate in both pathways. This divergence could merely be a signal amplification step required to initiate the large change in genomic expression.

In the light of data which showed that members of the phytochrome family affect the accumulation of other phytochromes (Hirschfeld *et al.*, 1998), Short (1999) used an *Arabidopsis* mutant over-expressing a *PHYB* transgene to investigate further the role of sugars in phytochrome signalling. In the presence of sucrose, *PHYB* over-expression repressed the PHYA FR inhibition of hypocotyl elongation, which added to the relationship between sugars and light, signalling the possibility of interplay between the photoreceptors, such as phytochrome and sugars (Short, 1999).

One of the photomorphogenetic mutants, *det3,* lacks a subunit C of a V-ATPase (Sze *et al.*, 1999). As a result *det3* mutants display a phenotype when grown in the dark similar to that of a WT plant grown in the light, showing cotyledon development and inhibition hypocotyl elongation (Cabrera y Poch *et al.*, 2003). This shows that sugar transport and to some degree allocation in response to a FR treatment in de-etiolating seedlings, which is signalled exclusively through phytochrome A, can be regulated without interfering with sugar transporter expression. In accordance with this, microarray analysis of seedling de-etiolating under FR light showed that three P-type ATPases were shown to be in the top 10 most up-regulated genes (table 5.4). This suggests that these genes were in demand to fuel the trafficking of molecules for photomorphogeneis by the initiation of the proton gradient required by transporters.

7.6. Monitoring sugar status – sugar sensing.

As well as their importance in metabolism sugars are also thought to have a role in signalling (Sheen *et al.,* 1999). To better understand this process it would be advantageous if the process of

sugar sensing was understood. For an introduction to this rapidly growing topic please refer to section 1.5.

Koch (1996) reviewed the effect of sugars on genomic expression and proposed the feast or famine response. The "famine" genes induced by low levels of sugars are associated with photosynthesis and remobilisation of stored carbohydrates whereas the "feast" genes which are induced upon high sugar levels are involved in the storage of sugars, pigment synthesis and respiration. A growing area of plant biology is investigating the mechanisms by which plants monitor sugar levels (Thum *et al.*, 2003; reviewed in Rolland *et al.*, 2002). There has been much evidence in support of hexokinase having a role in sugar sensing (Jang *et al.*, 1997). However, hexokinase independent pathways also appear to operate, as non-metabolizable glucose analogs, such as 3-O-methylglucose and 6-deoxyglucose, have been shown to induce responses produced by sucrose (Roitsch, 1999). Similarly, the sucrose repression of the inhibition of hypocotyl extension, which can also be elicited by mannose and 2-deoxyglucose, suggests that interaction between sugars and light signalling pathways do not require hexokinase (Short, 1999). As with phytochrome signalling, researchers in sugar sensing have also used mutant screens to isolate lines that are relatively insensitive or oversensitive to the levels of certain sugars (Rook and Bevan, 2003). Crossing photomorphogenetic mutants with sugar sensing mutants, together with microarray analysis might aid in isolating genes that overlap in these two signalling pathways.

To regulate sugar levels sugar sensing must be communicated with the rest of the cell and possibly further (e.g. other cells in the tissue and other tissues). The Ser/Thr protein kinase, SNF1 has been implicated as one of the major components of sugar signalling in yeast (Carlson, 1999). Antisense repression of SnRK (an orthologue of SNF1) in potato prevents transcriptional activation of a sucrose inducible sucrose synthase gene, indicating a role in sucrose mediated up-regulation of genes (Purcell *et al.,* 1998). 14-3-3 proteins have been shown to have various roles in signal transduction by binding to phosphorylated substrates thereby affecting protein function, protein-protein interactions and protein degradation (Finnie *et al.,* 1999). SnRK has been shown to regulate the binding of 14-3-3 proteins to sucrose phosphate synthase (Bhalerao *et al.,* 1999; Sugden *et al.,* 1999); such interactions might also be responsible for the regulation of sugar transporters. Sugars have been shown to affect the levels of the signalling messenger, Ca^{2+} (Furuichi *et al.*, 2001), which acts as a secondary messenger in many of the diverse signal transduction pathways in plants. Also *StCDPK1,* which encodes a calcium-dependent protein kinase from *Solanum tuberosum* (potato), is induced by high sucrose concentration (Raíces *et al.*, 2003). In support of these findings are the

results of the microarray analysis of seedling de-etiolating under FR light which showed that a Ca^{2+}/H^{+} antiporter was the second most up-regulated gene (table 5.4), probably due to its role in the signalling required to drive photomorphogenesis.

7.7. Hormone Connections

Unlike yeast which is a unicellular organism plants possess a variety of different tissues with specialized functions, therefore more of a long distance strategy is required for sugar signalling in plants. Similar to animals plants have a range of hormones that co-ordinate development. Hormones may be responsible for co-ordinating sugar transporter expression that aids growth and development or possibly hormones might be directed by sugar levels.

Tepperman *et al.* (2001) showed that 3.3% of the genes responsive to FR light were related to hormone pathways. While this might seem a small percentage, hormones can affect a large number of genes (Hobbie *et al.*, 1994; Goda *et al.*, 2002) and thus produce considerable signal amplification. The microarray experiment described in this study shows the putative auxin transporter, *AtAUXR2,* to be the sixth most responsive transporter in *Arabidopsis* genome in seedling de-etiolating under FR light (table 5.2). Auxin has pleiotropic effects on plant development and also it is known to be involved in mediating phototropic and geotropic responses (Davies, 1995). These auxin effects may be mediated through a variety of signalling intermediates, including the MAP kinase cascade (Hirt, 1997) and possibly calcium (Hobbie *et al.*, 1994). *Arabidopsis* auxin mutants have indicated that auxin interacts with other signalling pathways such as those mediating responses to ethylene, cytokinin, abscisic acid, giberrellins and light (reviewed in Swarup *et al.*, 2002). Interestingly, due to the similar phenotypes of the photomorphogenic mutant *hy5* and the auxin-insensitive *digageotropica (dgt)* mutant, Oyama *et al.* (1997) suggested that *DGT* and *HY5* regulate common signalling pathways in *Arabidopsis.* The protein product of *HY5*, an *Arabidopsis* ATB2 bZIP transcription factor (Oyama *et al.*, 1997) is repressed by sucrose (Rook *et al.*, 1998). Brassinosteriods, another plant hormone family, have been shown to repress de-etiolation as treatment of etiolated seedlings with a brassinosteriod biosynthesis inhibitor induced characteristics of de-etiolation (Nagata *et al.*, 2000). Conversely, brassinosteriod biosynthesis genes have been shown to be repressed by light (Ma *et al.*, 2001). Analysis of the supplementary data from Ogawa *et al.* (2003) shows that *AtSUGTL2* is induced upon the application of gibberellin to seeds.

Arabidopsis mutants isolated by their abnormal response to sugar have been found to be deficient in genes associated with abscisic acid (ABA), ethylene and jasmonate signalling (Leon and

Sheen, 2003). From the analysis of these sugar responsive mutants, Leon and Sheen (2003) have proposed that ethylene acts as an antagonist of glucose repression while ABA promotes it. The production of double mutants has enabled a clearer picture of these interactions to be resolved with evidence indicating that ethylene affects glucose signalling through the ABA pathway to promote germination and seedling development (Ghassemian *et al.*, 2000). The shoot promoting growth effect of ethylene in the light was reported by Smalle *et al.* (1997).

As more studies that investigate the role of hormones in the adaptation of plants to light and sugars are being published, it is becoming increasingly clear that hormones are major factors in the signalling pathways for light and sugar.

7.8. Conclusions

It is becoming increasingly apparent that the sugar transporter genes are subject to some degree of regulation at the transcriptional level and that this is responsive to multiple signalling inputs. Here, I have concentrated on sugar- and light-signalling mechanisms in the control of transcriptional gene expression. The results are not open to simple interpretation but rather suggest complex interactions of these pathways.

In elucidating the *in planta* roles for the various transporter genes, the study of comprehensive insertional mutant collections will be highly valuable although possible redundancy of function between related family members and overlapping expression patterns may require mutant lines containing multiple inserts to be isolated in order to reveal the full story. Information available from transcriptional studies of spatial and temporal expression will be extremely helpful in assessing the potential for such masking of function.

Transcriptional studies are vital in describing the regulation and function characteristics of the transporter genes, but it is becoming increasingly clear that proteomic studies are also necessary to provide a comprehensive picture. Whilst transcriptional regulation can provide a powerful control point for tissue-specific and long-term developmental changes in transporter activity, it may prove that post-translational modifications permit a far more rapid and flexible means of altering transporter function within a complex and dynamic system.

Chapter 8

References

Aharoni,A. and Vorst,O. (2002) DNA microarrays for functional plant genomics. Plant Molecular Biology **48**:99-118.

Altschul,S.F., Gish,W., Miller,W., Myers,E.W., and Lipman,D.J. (1990) Basic local alignment search tool. Journal of Molecular Biology **215**:403-410.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W., and Lipman,D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research **25**:3389-3402.

Ang,L.H., Chattopadhyay,S., Wei,N., Oyama,T., Okada,K., Batschauer,A., and Deng,X.W. (1998) Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of *Arabidopsis* development. Molecular Cell **1**:213-222.

Aoki,N., Hirose,T., Takahashi,S., Ono,K., Ishimari,K., and Ohsugi,R. (1999) Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (*Zea mays L.*). Plant and Cell Physiology **40**:1078.

Aphalo,P.J., Ballare,C.L., and Scopel,A.L. (1999) Plant-plant signaling, the shade-avoidance response and competition. Journal of Experimental Biology **50**:1629-1634.

Arguello-Astorga,G. and Herrera-Estrella,L. (1998) Evolution of light-regulated plant promoters. Annual Review of Plant Physiology and Plant Molecular Biology **49**:525-555.

Atanassova,R., Leterrier,M., Gaillard,C., Agasse,A., Sagot,E., Coutos-Thevenot,P., and Delrot,S. (2003) Sugar-regulated expression of a putative hexose transport gene in grape. Plant Physiology **131**:326-334.

Aukerman,M.J., Hirschfeld,M., Wester,L., Weaver,M., Clack,T., Amasino,R.M., and Sharrock,R.A. (1997) A deletion in the *PHYD* gene of the *Arabidopsis Wassilewskija* ecotype defines a role for phytochrome D in red/far-red light sensing. Plant Cell **9**:1317-1326.

Axelsen,K.B. and Palmgren,M.G. (1998) Evolution of substrate specificities in the P-type ATPase superfamily. Journal of Molecular Evolution **46**:84-101.

Baker,D.A. (1978) Proton co-transport of organic solutes by plant cells. New Phytologist **81**:485-497.

Ballare,C.L. (1999) Keeping up with the neighbours: phytochrome sensing and other signaling mechanisms. Trends in Plant Science **4**:97-102.

Barker,L., Kühn,C., Weise,A., Schulz,A., Gebhardt,C., Hirner,B., Hellmann,H., Schulze,W., Ward,J.M., and Frommer,W.B. (2000) SUT2, a putative sucrose sensor in sieve elements. Plant Cell **12**:1153-1164.

Barth,I., Meyer,S., and Sauer,N. (2003) PmSUC3: Characterization of a SUT2/SUC3-type sucrose transporter from *Plantago major*. Plant Cell **15**:1375-1385.

Baxter,I., Tchieu,J., Sussman,M.R., Boutry,M., Palmgren,M.G., Gribskov,M., Harper,J.F., and Axelsen,K.B. (2003) Genomic comparison of P-type ATPase ion pumps in *Arabidopsis* and rice. Plant Physiology **132**:618-628.

Benvenuto,G., Formiggini,F., Laflamme,P., Malakhov,M. and Bowler,C. (2002) The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. Current Biology **12**:1529–1534.

Bhalerao,R.P., Salchert,K., Bako,L., Okresz,L. Szabados,L., Muranaka,T., Machida,Y., Schell,J. and Koncz,C. (1999) Regulatory interaction of PRL1 WD protein with *Arabidopsis* SNF1-like protein kinases. Proceeding of the National Academy of Sciences USA **96**:5322-5327.

Bick,J.A., Neelam,A., Smith,E., Nelson,S.J., Hall,J.L., and Williams,L.E. (1998) Expression analysis of a sucrose carrier in the germinating seedling of *Ricinus communis*. Plant Molecular Biology **38**:425-435.

Bligny,R. and Douce,R. (2001) NMR and plant metabolism. Current Opinion in Plant Biology **4**:191-196.

Bowler,C., Neuhaus,G., Yamagata,H. and Chua,N.H. (1994) Cyclic GMP and calcium mediate phytochrome phototransduction. Cell **77**:73-81.

Boyes,D.C., Zayed, A.M., Ascenzi,R., McCaskill,A.J., Hoffman,N.E., Davis,K.R., and Görlach,J. (2001) Growth stage–based phenotypic analysis of Arabidopsis: A model for high throughput functional genomics in plants. Plant Cell **13**:1499-1510.

Brazma,A., Hingamp,P., Quackenbush,J., Sherlock,G., Spellman,P., Stoeckert,C., Aach,J., Ansorge,W., Ball,C.A., Causton,H.C., Gaasterland,T., Glenisson,P., Frank C.P. Holstege,F.C.P., Irene F. Kim,I.F., Victor Markowitz,V., Matese,J.C, Parkinson,H., Robinson,A., Sarkans,U., Schulze-Kremer,S, Stewart,J., Taylor,R., Vilo,J., and Vingron,V. (2001) Minimum information about a microarray experiment (MIAME) - toward standards for microarray data. Nature Genetics **29**:365–371.

Briggs,W.R. and Olney,M.A. (2001) Photoreceptors in plant photomorphogenesis to date. Five phytochromes, two cryptochromes, one phototropin, and one superchrome. Plant Physiology **125**:85-88.

Briggs,W.R. and Christie,J.M. (2002) Phototropins 1 and 2: versatile plant blue-light receptors. Trends in Plant Science **7**:204-210.

Buckhout,T.J. and Thimm,O. (2003) Insights into metabolism obtained from microarray analysis. Current Opinion in Plant Biology **6**:288-296.

Burkle,L., Hibberd,J.M., Quick,W.P., Kühn,C., Hirner,B., and Frommer,W.B. (1998) The H^+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. Plant Physiology **118**:59-68.

Bush,D.R. (1989) Proton-coupled sucrose transport in plasmalemma vesicles isolated from sugar-beet (*Beta-Vulgaris l Cv* Great Western) leaves. Plant Physiology **89**:1318-1323.

Bush,D.R. (1990) Electrogenicity, pH-dependence, and stoichiometry of the proton-sucrose symport. Plant Physiology **93**:1590-1596.

Bush,D.R. (1993) Inhibitors of the proton-sucrose symport. Archives of Biochemistry and Biophysics **307**:355-360.

Bush,D.R. (1999) Sugar transporters in plant biology. Current Opinion in Plant Biology **2**:187-191.

Büttner,M. and Sauer N. (2000) Monosaccharide transporters in plants: structure, function and physiology. Biochimica et Biophysica Acta - Biomembranes **1465**:263-274.

Büttner,M., Truernit,E., Baier,K., Scholz-Starke,J., Sontheim,M., Lauterbach,C., Huss,V.A.R., and Sauer,N. (2000) AtSTP3, a green leaf-specific, low affinity monosaccharide-H^+ symporter of *Arabidopsis thaliana*. Plant, Cell and Environment **23**:175-184.

Cabrera y Poch,H.L., Peto,C.A., and Chory, J. (1993) A mutation in the *Arabidopsis DET3* gene uncouples photoregulated leaf development from gene expression and chloroplast biogenesis. Plant Journal **4**:671–682.

Carlson,M. (1999) Glucose repression in yeast. Current Opinion in Microbiology **2**:202-207

Carroll,B.J., Klimyuk,V.I., Thomas,C.M., Bishop,G.J., Harrison,K., Scofield,S.R., and Jones,J.D.G. (1995) Germinal transpositions of the maize element dissociation from T-DNA loci in tomato. Genetics **139**:407-420.

Casal,J.J. (1995) Coupling of phytochrome-B to the control of hypocotyl growth in *Arabidopsis*. Planta **196**:23-29.

Casal,J.J., Sanchez,R.A., and Botto,J.F. (1998) Modes of action of phytochromes. Journal of Experimental Botany **49** :127-138.

Caspari,T., Will,A., Opekarova,M., Sauer,N., and Tanner,W. (1994) Hexose/H^+ symporters in lower and higher-plants. Journal of Experimental Biology **196**:483-491.

Chattopadhyay,S., Ang,L.H., Puente,P., Deng,X.W., and Wei,N. (1998) *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. Plant Cell **10**:673-683.

Cherry,J.R., Hondred,D., Walker,J.M., Keller,J.M., Hershey,H.P., and Vierstra,R.D. (1993) Carboxy-terminal deletion analysis of oat phytochrome-A reveals the presence of separate domains required for structure and biological-activity. Plant Cell **5**:565-575.

Chiou,T.J. and Bush,D.R. (1998) Sucrose is a signal molecule in assimilate partitioning. Proceedings of the National Academy of Sciences of the United States of America **95**:4784-4788.

Clack,T., Mathews,S., and Sharrock,R.A. (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by 5 genes - the sequences and expression of *PHYD* and *PHYE*. Plant Molecular Biology **25**:413-427.

Cortès,S., Gromova,M., Evrard,A., Roby,C., Heyraud,A., Rolin,D.B., Raymond,P., and Brouquisse,R.M. (2003) In plants, 3-o-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. Plant Physiology **131**:824-837.

Datta,A., Bagchi,S., Nag,A., Shiyanov,P., Adami,G.R., Yoon,T. and Raychaudhuri,P. (2001) The p48 subunit of the damaged-DNA binding protein DDB associates with the CBP/p300 family of histone acetyltransferase. Mutation Research **486**:89–97.

Davies, P.J. (1995) The plant hormones: their nature, occurrence and functions. In *Plant Hormones: Physiology, Biochemistry and Molecular Biology* (Davies, P.J., ed.). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1 12.

Davuluri,R.V., Sun,H., Palaniswamy,S.K., Matthews,N., Molina,C., Kurtz,M., and Grotewold,E. (2003) AGRIS: *Arabidopsis* Gene Regulatory Information Server, an information resource of *Arabidopsis cis*-regulatory elements and transcription factors. BMC Bioinformatics **4**:art-25.

Delrot,S., Atanassova,R., and Maurousset,L. (2000) Regulation of sugar, amino acid and peptide plant membrane transporters. Biochimica et Biophysica Acta-Biomembranes **1465**:281-306.

Devlin,P.F., Patel,S.R., and Whitelam,G.C. (1998) Phytochrome E influences internode elongation and flowering time in *Arabidopsis*. Plant Cell **10**:1479-1487.

Devlin,P.F., Robson,P.R.H., Patel,S.R., Goosey,L., Sharrock,R.A., and Whitelam,G.C. (1999) Phytochrome D acts in the shade-avoidance syndrome in *Arabidopsis* by controlling elongation growth and flowering time. Plant Physiology **119**:909-915.

Dewitt,N.D., Hong,B.M., Sussman,M.R., and Harper,J.F. (1996) Targeting of two *Arabidopsis* H^+-ATPase isoforms to the plasma membrane. Plant Physiology **112**:833-844.

Dijkwel,P.P., Huijser,C., Weisbeek,P.J., Chua,N.H., and Smeekens,S.C.M. (1997) Sucrose control of phytochrome A signaling in *Arabidopsis*. Plant Cell **9**:583-595.

Donson,J,. Fang,Y,. Espiritu-Santo,G,. Xing,W,. Salazar,A,. Miyamoto,S,. Armendarez,V, and Volkmuth,W (2002) Comprehensive gene expression analysis by transcript profiling. Plant Molecular Biology **48**:75-97.

Elich,T.D., McDonagh,A.F., Palma,L.A. & Lagarias,J.C. (1989) Phytochrome chromophore biosynthesis. Journal of Biological Chemistry **264**:183-189.

Entian,K.-D. and Frohlich,K.-H. (1984) *Saccharomyces cerevisiae* mutants provide evidence of hexokinase PII as a bifunctional enzyme with catalytic and regulatory domains for triggering carbon catabolic. Journal of Bacteriology **158**:29-35.

Entian,K.D., Hilberg,F., Opitz,H., and Mecke,D. (1985) Cloning of hexokinase structural genes from *Saccharomyces cerevisiae* mutants with regulatory mutations responsible for glucose repression. Molecular and Cellular Biology **5**:3035-3040.

Eschrich,W. (1989) Phloem unloading of photoassimilates. In Transport of Photoassmilates., D.A.Baker and J.A.Milburn, eds Longman Scientific & Technical.).

Eschrich,W. and Fromm,J. (1994) Evidence for 2 pathways of phloem loading. Physiologia Plantarum **90**:699-707.

Fiehn,O. (2002) Metabolomics - the link between genotypes and phenotypes. Plant Molecular Biology **48**:155-171.

Fiehn,O., Kopka,J., Dormann,P., Altmann,T., Trethewey,R.N., and Willmitzer,L. (2000) Metabolite profiling for plant functional genomics. Nature Biotechnology **18**:1157-1161.

Finkelstein,D., Ewing,R., Gollub,J., Sterky,F., Cherry,J.M., and Somerville,S. (2002) Microarray data quality analysis: lessons from the AFGC project. Plant Molecular Biology **48**:119-131.

Finnie,C., Borch,J. and Collinge,D.B. (1999) 14-3-3 proteins: Eukaryotic regulatory proteins with many functions. Plant Molecular Biology **40**: 545-554.

Fisher,D.B. and Cash-Clark,C.E. (2000) Sieve tube unloading and post-phloem transport of fluorescent tracers and proteins injected into sieve tubes via severed aphid stylets. Plant Physiology **123**:125–139.

Fotopoulos,V., Gilbert,M.J., Pittman,J.K., Marvier,A.C., Buchanan,A.J., Sauer,N., Hall,J.L., and Williams,L.E. (2003) The monosaccharide transporter gene, *AtSTP4*, and the cell-wall invertase, *Atbetafruct1*, are induced in *Arabidopsis* during infection with the fungal biotroph *Erysiphe cichoracearum*. Plant Physiology **132**:821-829.

Franklin,K.A., Davis,S.J., Stoddart,W.M., Vierstra,R.D., and Whitelam,G.C. (2003a) Mutant analyses define multiple roles for phytochrome C in *Arabidopsis* photomorphogenesis. Plant Cell **15**:1981-1989.

Franklin,K.A., Linley,P.J., Montgomery,B.L., Lagarias,J.C., Thomas,B, Jackson,S.D. (2003b) Misregulation of tetrapyrrole biosynthesis in transgenic tobacco seedlings expressing mammalian biliverin reductase. Plant Journal **35**:717-728.

Furuichi,T., Mori,I.C., Takahashi,K., and Muto,S. (2001) Sugar-induced increase in cytosolic Ca^{2+} in *Arabidopsis thaliana* whole plants. Plant and Cell Physiology **42**:1149-1155.

Furuya,M. (1993) Molecular properties and biogenesis of phytochromes I and II. Advances in Biophysics **25**:133-167.

Gahrtz,M., Stolz,J., and Sauer,N. (1994) A phloem-specific sucrose-H^+ symporter from *Plantago-major* I supports the model of apoplastic phloem loading. Plant Journal **6**:697-706.

Gamalei,Y.V. (1996) Assimilate export under natural and experimental conditions. Russian Journal of Plant Physiology **43**:282-296.

Ghassemian,M., Nambara,E., Cutler,S., Kawaide,H., Kamiya,Y., and McCourt,P. (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. Plant Cell **12**:1117-1126.

Goda,H., Shimada,Y., Asami,T., Fujioka,S., and Yoshida,S. (2002) Microarray analysis of brassinosteroid-regulated genes in *Arabidopsis*. Plant Physiology **130**:1319-1334.

Goosey,L., Palecanda,L., and Sharrock,R.A. (1997) Differential patterns of expression of the *Arabidopsis PHYB*, *PHYD*, and *PHYE* phytochrome genes. Plant Physiology **115**:959-969.

Goto,N., Kumagai,T., and Koornneef,M. (1991) Flowering responses to light-breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long-day plant. Physiologia Plantarum **83**:209-215.

Gottwald,J.R., Krysan,P.J., Young,J.C., Evert,R.F., and Sussman,M.R. (2000) Genetic evidence for the *in planta* role of phloem-specific plasma membrane sucrose transporters. Proceedings of the National Academy of Sciences of the United States of America **97**:13979-13984.

Graveley,B.R. (2001) Alternative splicing: increasing diversity in the proteomic world. Trends in Genetics **17**:100-107.

Halliday,K.J. and Fankhauser,C. (2003) Phytochrome-hormonal signaling networks. New Phytologist **157**:449-463.

Hamill,S., Cloherty,E.K., and Carruthers, A. (1999) The human erythrocyte sugar transporter presents two sugar import sites. Biochemistry **38**:16974–16983.

Haritatos,E., Medville,R., and Turgeon,R. (2000) Minor vein structure and sugar transport in *Arabidopsis* thaliana. Planta **211**:105-111.

Harms,K., Wohner,R.V., Schulz,B., and Frommer,W.B. (1994) Isolation and characterization of P-type H^+-ATPase genes from potato. Plant Molecular Biology **26**:979-988.

Hellmann,H., Barker,L., Funck,D., and Frommer,W.B. (2000) The regulation of assimilate allocation and transport. Australian Journal Plant Physiology **27**:583-594.

Higo,K., Ugawa,Y., Iwamoto,M., and Korenaga,T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Research. **27**:297-300.

Hirose,T., Imaizumi,N., Scofield,G.N., Furbank,R.T., and Ohsugi,R. (1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa L.*). Plant Cell Physiology **38**:1389-1396.

Hirschfeld,M., Tepperman,J.M., Clack,T., Quail,P.H., and Sharrock,R.A. (1998) Coordination of phytochrome levels in *phyB* mutants of *Arabidopsis* as revealed by apoprotein-specific monoclonal antibodies. Genetics **149**:523-535.

Hirt,H. (1997) Multiple roles of MAP kinases in plant signal transduction. Trends in Plant Science **2**:11-15.

Hobbie,L.J. (1998) Auxin: Molecular genetic approaches in *Arabidopsis*. Plant Physiology and Biochemistry **36**:91-102.

Hofmann,K. and Stoffel,W. (1993) TMbase - A database of membrane spanning proteins segments. Biological Chemisty.Chem.Hoppe-Seyler **37**:166.

Houlne,G. and Boutry,M. (1994) Identification of An *Arabidopsis thaliana* gene encoding a plasma-membrane H^+-ATPase whose expression is restricted to anther tissues. Plant Journal **5**:311-317.

Huala,E., Dickerman,A.W., Garcia-Hernandez,M., Weems,D., Reiser,L., LaFond,F., Hanley,D., Kiphart,D., Zhuang,M.Z., Huang,W., Mueller,L.A., Bhattacharyya,D., Bhaya,D., Sobral,B.W., Beavis,W., Meinke,D.W., Town,C.D., Somerville,C., and Rhee,S.Y. (2001) The *Arabidopsis* Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Research **29**:102-105.

Hulskamp,M., Kopczak,S.D., Horejsi,T.F., Kihl,B.K., and Pruitt,R.E. (1995) Identification of genes required for pollen–stigma recognition in *Arabidopsis thaliana*. Plant Journal **8**:703–714.

Imlau,A., Truernit,E., and Sauer,N. (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11**:309-322.

Jang,J.C., Leon,P., Zhou,L., and Sheen,J. (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell **9**:5-19.

Jang,J.C. and Sheen,J. (1994) Sugar sensing in higher plants. Plant Cell **6**:1665-1679.

Jefferson,R.A. (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter **5**:387-405.

Jefferson,R.A. (1989) The GUS Reporter gene system. Nature **342**:837-838.

Jefferson,R.A., Bevan,M., and Kavanagh,T. (1987a) The use of the *Escherichia coli* β-glucuronidase gene as a gene fusion marker for studies of gene-expression in higher plants. Biochemical Society Transactions **15**:17-18.

Jefferson,R.A., Kavanagh,T.A., and Bevan,M.W. (1987b) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in plants. The EMBO Journal **6**:3901-3907.

Johnson,E., Bradley,M., Harberd,N.P., and Whitelam,G.C. (1994) Photorespnses of light-grown *phyA* mutants of *Arabidopsis*. Phytochrome A is required for the perception of daylength extensions. Plant Physiology **105**:141-149.

Kallarackal,J., Orlich,G., Schobert,C., and Komor,E. (1989) Sucrose transport into the phloem of *Ricinus communis* L. seedlings as measured by the analysis of sieve tube sap. Planta **177**:327-335.

Kasperbauer,M.J. and Kaul,D.L. (1986) Light-mediated bioregulation of tillering and photosynthate partitioning in wheat. Plant Physiology **66**:159-163.

Kasperbauer,M.J., Tso,T.C. and Sorokin.T.P. (1970) Effects of end-of-day red and far-red radiation on free sugars, organic acids and amino acids. Phytochemistry **9**:2091–2095.

Kazan,K., Schenk,P.M., Wilson,I., and Manners,J.M. (2001) DNA microarrays: new tools in the analysis of plant defense responses. Molecular Plant Pathology **2**:177-185.

Keiller,D. and Smith,H. (1989) Control of carbon partitioning by light quality mediated by phytochrome. Plant Science **63**:25-29.

Kempin,S.A., Liljegren,S.J., Block,L.M., Rounsley,S.D., Yanofsky,M.F., and Lam,E. (1997) Targeted disruption in *Arabidopsis*. Nature **389**:802-803.

Kent,W.J. (2002) BLAT - The BLAST-like alignment tool. Genome Research **12**:656-664.

Kiyosue,T., Abe,H., Yamaguchi-Shinozaki,K., and Shinozaki,K. (1998) *ERD6*, a cDNA clone for an early dehydration-induced gene of *Arabidopsis*, encodes a putative sugar transporter. Biochimica et Biophysica Acta-Biomembranes **1370**:187-191.

Koch,K.E. (1996) Carbohydrate-modulated gene expression in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**:509-540.

Koch,K.E., Ying,Z., Wu,Y., and Avigne,W.T. (2000) Multiple paths of sugar-sensing and a sugar/oxygen overlap for genes of sucrose and ethanol metabolism. Journal of Experimental Botany **51**:417–427.

Komor,E., Rotter,M., Tanner,W. (1977) A proton-cotransport system in a higher plant: sucrose transport in *Ricinus communis*. Plant Science Letters **9**:153–162.

Koorneef,M., Rolff,E., Spruitt,C.J.P. (1980) Genetic control of light inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Zeitschrift Fur Pflanzenphysiologie **100**:147-160

Krysan,P.J., Young,J.C., Tax,F., and Sussman,M.R. (1996) Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. Proceedings of the National Academy of Sciences of the United States of America **93**:8145-8150.

Kühn,C., Barker,L., Burkle,L., and Frommer,W.B. (1999) Update on sucrose transport in higher plants. Journal of Experimental Botany **50**:935-953.

Kühn,C., Franceschi,V.R., Schulz,A., Lemoine,R., and Frommer,W.B. (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science **275**:1298-1300.

Lalonde,S., Boles,E., Hellmann,H., Barker,L., Patrick,J.W., Frommer,W.B., and Ward,J.M. (1999) The dual function of sugar carriers. Transport and sugar sensing. Plant Cell **11**:707-726.

Lee,M.L.T., Kuo,F.C., Whitmore,G.A., and Sklar,J. (2000) Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. Proceedings of the National Academy of Sciences of the United States of America **97**:9834-9839.

Lemoine,R. (2000) Sucrose transporters in plants: update on function and structure. Biochimica et Biophysica Acta-Biomembranes **1465**:246-262.

Lemoine,R., Delrot,S., Gallet,O., and Larsson,C. (1989) The sucrose carrier of the plant plasma-membrane 2. Immunological characterization. Biochimica et Biophysica Acta-Biomembranes **978**:65-71.

Leon,P. and Sheen,J. (2003) Sugar and hormone connections. Trends in Plant Science **8**:110-116.

Li,C.Y., Shi,J.X.S., Weiss,D. and Goldschmidt,E.E. (2003) Sugars regulate sucrose transporter gene expression in citrus. Biochemical and Biophysical Research Communications **306**:402-407.

Liang,H. and Gaber,R.F. (1996) A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by SNF3-regulated expression of *HXT6*. Molecular Biology of the Cell **7**:1953-1966

Lin,C.T. (2002) Blue light receptors and signal transduction. Plant Cell **14**:S207-S225.

Liu,X., Brutlag,D.L. and and Liu,J.S. (2001) BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. Pacific Symposium on Biocomputing, 127-138.

Lu,C.A., Ho,T.H., Ho,S.L., and Yu,S.M. (2002) Three novel MYB proteins with one DNA binding repeat mediate sugar and hormone regulation of alpha-amylase gene expression. Plant Cell **14**:1963-1980.

Lucas,W.J., Olesinski,A., Hull,R.J., Haudenshield,J.S., Deom,C.M., Beachy,R.N., and Wolf,S. (1993) Influence of the tobacco mosaic-virus 30-kda movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. Planta **190**:88-96.

Lucas,W.J. and Wolf,S. (1999) Connections between virus movement, macromolecular signaling and assimilate allocation. Current Opinion in Plant Biology **2**:192-197.

Ludwig,A., Stolz,J., and Sauer,N. (2000) Plant sucrose-H^+ symporters mediate the transport of vitamin H. Plant Journal **24**:503-509.

Ma,L.G., Li,J.M., Qu,L.J., Hager,J., Chen,Z.L., Zhao,H.Y., and Deng,X.W. (2001) Light control of *Arabidopsis* development entails coordinated regulation of genome expression and cellular pathways. Plant Cell **13**:2589-2607.

Ma,L.G., Zhao,H.Y., and Deng,X.W. (2003) Analysis of the mutational effects of the *COP/DET/FUS* loci on genome expression profiles reveals their overlapping yet not identical roles in regulating *Arabidopsis* seedling development. Development **130**:969-981.

Maathuis,F.J.M., Filatov,V., Herzyk,P., Krijger,G.C., Axelsen,K.B., Chen,S.X., Green,B.J., Li,Y., Madagan,K.L., Sanchez-Fernandez,R., Forde,B.G., Palmgren,M.G., Rea,P.A., Williams,L.E., Sanders,D., and Amtmann,A. (2003) Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. Plant Journal **35**:675-692.

Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Marger,M.D. and Saier,M.H. (1993) A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. Trends in Biochemical Sciences **18**:13-20.

Martinez,M.J., Aragon,A.D., Rodriguez,A.L., Weber,J.M., Timlin,J.A., Sinclair,M.B., Haaland,D.M., and Werner-Washburne,M. (2003) Identification and removal of contaminating fluorescence from commercial and in-house printed DNA microarrays. Nucleic Acids Research **15**:31.

Matschinsky,F., Liang,Y., Kesavan,P., Wang,L., Froguel,P., Velho,G., Cohen,D., Permutt,M.A., Tanizawa,Y., Jetton,T.L., Niswender,K., and Magnuson,M. (1993) Glucokinase as pancreatic β-cell glucose sensor and diabetes gene. Journal of Clinical Investigation **92**:2092–2098.

Matsukura,C., Saitoh,T., Hirose,T., Ohsugi,R., Perata,P., and Yamaguchi,J. (2000) Sugar uptake and transport in rice embryo. Expression of companion cell- specific sucrose transporter (*OsSUT1*) induced by sugar and light. Plant Physiology **124**:85-93.

McCormac, A.C., Andrea,F., Madan,K.A. Dieter,S., and Matthew,T.J. (2001) Regulation of *HEMA1* expression by phytochrome and a plastid signal during de-etiolation in *Arabidopsis thaliana*. Plant Journal **25**:549-561

McKinney,E.C., Ali,N., Traut,A., Feldmann,K.A., Belostotsky,D.A., Mcdowell,J.M., and Meagher,R.B. (1995) Sequence-based identification of T-DNA insertion mutations in *Arabidopsis* - Actin mutants *act2-1* and *act4-1*. Plant Journal **8**:613-622.

Mele,G. and Hake,S. (2003) Expression profiling of plant development. Genome Biology **4**: 215.

Meyer,S., Lauterbach,C., Niedermeier,M., Barth,I., Sjolund,R.D., and Sauer,N. (2004) Wounding enhances expression of *AtSUC3*, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. Plant Physiology **134**:684-693.

Meyer,S., Melzer,M., Truernit,E., Hummer,C., Besenbeck,R., Stadler,R., and Sauer,N. (2000) *AtSUC3*, a gene encoding a new *Arabidopsis* sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. Plant Journal **24**:869-882.

Mills,J.C. and Gordon,J.I. (2001) A new approach for filtering noise from high-density oligonucleotide microarray datasets. Nucleic Acids Research **29**: 72.

Møller,S.G., Ingles,P.J., and Whitelam,G.C. (2002) The cell biology of phytochrome signaling. New Phytologist **154** :553-590.

Morelli,G. and Ruberti,I. (2002) Light and shade in the photocontrol of *Arabidopsis* growth. Trends in Plant Science **7**:399-404.

Morgenstern,B. (1999) DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics **15**:211-218.

Münch,E. (1930) *Die stoffbewegungen in der pflanze.* Jena, Germany: G. Fischer.

Murashigue,T. and Skoog,F. (1962) A revised medium for rapid growth and bio-essays with tobacco tissue cultures. Physiologia Plantarum **15**:473-497.

Murray,V. (1989) Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Research **17**:8889.

Nagata,N., Min,Y.K., Nakano,T., Asami,T., and Yoshida,S. (2000) Treatment of dark-grown *Arabidopsis thaliana* with a brassinosteroid-biosynthesis inhibitor, brassinazole, induces some characteristics of light-grown plants. Planta **211**:781-790.

Nagatani,A., Chory,J., and Furuya,M. (1991) Phytochrome-B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. Plant and Cell Physiology **32**:1119-1122.

Nagatani,A., Nishizawa,N.K., Mori,S., Kay,S.A., Chua,N.H., and Furuya,M. (1993) Light regulation of hypocotyl elongation and greening in transgenic tobacco seedlings that over-express rice phytochrome-A. Plant and Cell Physiology **34**:825-833.

Nagy,F., Kircher,S., and Schafer,E. (2000) Nucleo-cytoplasmic partitioning of the plant photoreceptors phytochromes. Seminars in Cell & Developmental Biology **11**:505-510.

Neff,M.M., Fankhauser,C., and Chory,J. (2000) Light: an indicator of time and place. Genes & Development **14**:257-271.

Nemeth,K., Salchert,K., Putnoky,P., Bhalerao,R., Koncz-Kalman,Z., Stankovic-Stangeland,B., Bako,L., Mathur,J., Okresz,L., Stabel,S., Geigenberger,P., Stitt,M., Redei,G.P., Schell,J., Koncz,C. (1998) Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in *Arabidopsis*. Genes & Development **12**:3059–3073.

Noiraud,N., Delrot,S., and Lemoine,R. (2000) The sucrose transporter of celery: identification and expression during salt stress. Plant Physiology **122**:1447-1455.

Ochman,H., Gerber,A.S., and Hartl,D.L. (1988) Genetic applications of an inverse polymerase chain-reaction. Genetics **120**:621-623.

Ogawa,M., Hanada,A., Yamauchi,Y., Kuwalhara,A., Kamiya,Y., and Yamaguchi,S. (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. Plant Cell **15**:1591-1604.

O'Keefe,D.P., Tepperman,J.M., Dean,C., Leto,K.J., Erbes,D.L., and Odell,J.T. (1994) Plant expression of a bacterial cytochrome-P450 that catalyzes activation of a sulfonylurea pro-herbicide. Plant Physiology **105**:473-482.

Oparka,K.J. and Roberts,A.G. (2001) Plasmodesmata. A not so open-and-shut case. Plant Physiology **125**:123-126.

Ortiz-Lopez,A., Chang,H-C. and Bush,D.R. (2000) Amino acids transporters in plants. Biochimica et Biophysica Acta - Biomembranes **1465**: 275-280.

Osterlund,M.T., Ang,L.H., and Deng,X.W. (1999) The role of COP1 in repression of *Arabidopsis* photomorphogenic development. Trends in Cell Biology **9**:113-118.

Osterlund,M.T., Wei,N., and Deng,X.W. (2000) The roles of photoreceptor systems and the COP1-targeted destabilization of HY5 in light control of *Arabidopsis* seedling development. Plant Physiology **124**:1520-1524.

Oyama,T., Shimura,Y., and Okada,K. (1997) The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes & Development **11**:2983-2995.

Özcan,S., Dover,J., and Johnston,M. (1998) Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO Journal **17**:2566-2573.

Özcan,S., Dover,J., Rosenwald,A.G., Wolfl,S., and Johnston,M. (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proceedings of the National Academy of Sciences of the United States of America **93**:12428-12432.

Parks,B.M. and Quail,P.H. (1993) *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5**:39-48.

Pate,J.S. and Gunning,B.E.S. (1972) Transfer cells. Annual Review of Plant Physiology **23**:173-196.

Patrick,J.W. (1997) Phloem loading: Sieve element unloading and post-sieve element transport. Annual Review Plant Physiology **48**:191-222.

Patrick,J.W. and Offler,C.E. (1995) Post-sieve element transport of photoassimilates in sink regions. Journal of Experimental Botany **47**:1165-1177.

Patton,D.A., Schetter,A.L., Franzmann,L.H., Nelson,K., Ward,E.R., and Meinke,D.W. (1998) An embryo-defective mutant of *Arabidopsis* disrupted in the final step of biotin synthesis. Plant Physiology **116**:935-946.

Pego,J.V., Kortstee,A.J., Huijser,G., and Smeekens,S.G.M. (2000) Photosynthesis, sugars and the regulation of gene expression. Journal of Experimental Botany **51**:407-416.

Pepper,A.E. and Chory,J. (1997) Extragenic suppressors of the *Arabidopsis det1* mutant identify elements of flowering-time and light-Response regulatory pathways. Genetics **145**:1125-1137.

Purcell,P.C., Smith,A.M., Halford,N.G. (1998) Antisense expression of a sucrose non-fermentating-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tuber and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. Plant Journal **14**:195-202.

Quail,P.H. (2000) Phytochrome-interacting factors. *Seminars in Cell & Developmental Biology* **11**:457-466.

Raices,M., Gargantini,P.R., Chinchilla,D., Crespi,M., Tellez-Inon,M.T., and Ulloa,R.M. (2003) Regulation of CDPK isoforms during tuber development. Plant Molecular Biology **52**:1011-1024.

Ransom-Hodgkins,W.D., Vaughn,M.W., and Bush,D.R. (2003) Protein phosphorylation plays a key role in sucrose-mediated transcriptional regulation of a phloem-specific proton-sucrose symporter. Planta **217**:483-489.

Reed,J.W., Nagpal,P., Poole,D.S., Furuya,M., and Chory,J. (1993) Mutations in the gene for the red far-red light receptor phytochrome-B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell **5**:147-157.

Reinders,A., Schulze,W., Kühn,C., Barker,L., Schulz,A., Ward,J.M., and Frommer,W.B. (2002) Protein-protein interactions between sucrose transporters of different affinities co-localized in the same enucleate sieve element. Plant Cell **14**:1567-1577.

Reinhold,L. and Kaplan,A. (1984) Membrane-transport of sugars and amino-acids. Annual Review of Plant Physiology **35**:45-83.

Riens,B., Lohaus,G., Heineke,D., and Heldt,H.W. (1991) Amino-acid and sucrose content determined in the cytosolic, chloroplastic, and vacuolar compartments and in the phloem sap of spinach leaves. Plant Physiology **97**:227-233.

Riesmeier,J.W., Hirner,B., and Frommer,W.B. (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. Plant Cell **5**:1591-1598.

Riesmeier,J.W., Willmitzer,L., and Frommer,W.B. (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. The EMBO Journal **11**:4705-4713.

Riesmeier,J.W., Willmitzer,L., and Frommer,W.B. (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. The EMBO Journal **13**:1-7.

Roberts,J.K.M. (2002) Proteomics and a future generation of plant molecular biologists. Plant Molecular Biology **48**:143-154.

Roblin,G., Sakr,S., Bonmort,J., and Delrot,S. (1998) Regulation of a plant plasma membrane sucrose transporter by phosphorylation. FEBS Letters **424**:165–168.

Roitsch,T. (1999) Source-sink regulation by sugar and stress. Current Opinion in Plant Biology **2**:198-206.

Rolland,F., Moore,B., and Sheen,J. (2002) Sugar sensing and signaling in plants. Plant Cell **14**:185-205.

Rook,F. and Bevan,M.W. (2003) Genetic approaches to understanding sugar-response pathways. Journal of Experimental Botany **54**:495-501.

Rook,F., Gerrits,N., Kortstee,A., van Kampen,M., Borrias,M., Weisbeek,P., and Smeekens,S. (1998) Sucrose-specific signaling represses translation of the *Arabidopsis* ATB2 bZIP transcription factor gene. Plant Journal **15**:253-263.

Rossel,J.B., Wilson,I.W., and Pogson,B.J. (2002) Global changes in gene expression in response to high light in *Arabidopsis*. Plant Physiology **130**:1109-1120.

Roven,C. and Bussemaker,H.J. (2003) REDUCE: an online tool for inferring *cis*-regulatory elements and transcriptional module activities from microarray data. Nucleic Acids Research **31**:3487-3490.

Rüdiger,W., Thummler,F., Cmiel,E., and Schneider,S. (1983) Chromophore structure of the physiologyogically active form (Pfr) of Phytochrome. Proceedings of the National Academy of Sciences of the United States of America **80**:6244-6248.

Russin,W.A., Evert,R.F., Vanderveer,P.J., Sharkey,T.D., and Briggs,S.P. (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export defective1 maize mutant. Plant Cell **8**:645-658.

Saier,M.H., Jr., Beatty,J.T., Goffeau,A., Harley,K.T., Heijne,W.H., Huang,S.C., Jack,D.L., Jahn,P.S., Lew,K., Liu,J., Pao,S.S., Paulsen,I.T., Tseng,T.T., and Virk,P.S. (1999) The major facilitator superfamily. Journal of Molecular Microbiology and Biotechnology **1**:257-279.

Sakr,S., Noubhani,M., Bourbouloux,A., Riesmeier,J., Frommer,W., Sauer,N., and Delrot,S. (1997) Cutting, ageing and expression of plant membrane transporters. Biochimica et Biophysica Acta **1330**:207-16.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sanger,F., Nicklen,S., and Coulson,A. (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America **54**:5467.

Sauer,N., Baier,K., Gahrtz,M., Stadler,R., Stolz,J., and Truenit,E. (1994) Sugar-transport across the plasma-membranes of higher-plants. Plant Molecular Biology **26**:1671-1679.

Sauer,N., Caspari,T., Klebl,F., and Tanner,W. (1990b) Functional Expression of the Chlorella Hexose Transporter in *Schizosaccharomyces pombe*. Proceedings of the National Academy of Sciences of the United States of America **87**:7949-7952.

Sauer,N., Friedlander,K., and Graml-Wicke,U. (1990a) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. EMBO Journal **9**:3045-3050.

Sauer,N. and Tanner,W. (1993) Molecular-Biology of Sugar Transporters in Plants. Biochimica et Biophysica Acta-Biomembranes **106**:277-286.

Sauer,N. and Stadler,R. (1993) A sink-specific H^+/monosaccharide co-transporter from *Nicotiana tabacum*: cloning and heterologous expression in baker's yeast. Plant Journal **4**:601-610.

Sauer,N. and Stolz,J. (1994) SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. Plant Journal **6**:67-77.

Schena, M., D. Shelon,R.W. Davis, and Borwn,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270**:467-470.

Schmitt,J., Dudley,S.A., and Pigliucci,M. (1999) Manipulative approaches to testing adaptive plasticity: Phytochrome-mediated shade-avoidance responses in plants. American Naturalist **154**:S43-S54.

Schneidereit,A., Scholz-Starke,J., and Buttner,M. (2003) Functional characterization and expression analyses of the glucose-specific AtSTP9 monosaccharide transporter in pollen of *Arabidopsis*. Plant Physiology **133**:182-190.

Scholz-Starke,J., Büttner,M., and Sauer,N. (2003) AtSTP6, a New Pollen-Specific H^+-Monosaccharide Symporter from *Arabidopsis*. Plant Physiology **131**:70-77.

Schoof,H., Zaccaria,P., Gundlach,H., Lemcke,K., Rudd,S., Kolesov,G., Arnold,R., Mewes,H.W., and Mayer,K.F.X. (2002) MIPS *Arabidopsis thaliana* Database (M*At*DB): an integrated biological knowledge resource based on the first complete plant genome. Nucleic Acids Research **30**:91-93.

Schroeder,D.F., Gahrtz,M., Maxwell,B.B., Cook,R.K., Kan,J.M., Alonso,J.M., Ecker,J.R., and Chory,J. (2002) De-etiolated 1 and damaged DNA binding protein 1 interact to regulate *Arabidopsis* photomorphogenesis. Current Biology **12**:1462–1472,

Schuchhardt,J., Beule,D., Malik,A., Wolski,E., Eickhoff,H., Lehrach,H., and Herzel,H. (2000) Normalization strategies for cDNA microarrays. Nucleic Acids Research **28**:e47.

Schulz,A., Kühn,C., Riesmeier,J., and Frommer,W. (1998) Ultrastructural effects in potato leaves due to antisense inhibition of the sucrose transporter indicate an apoplasmic mode of phloem loading. Planta **206**:533-543.

Schulze,W., Weise,A., Frommer,W.B., and Ward,J.M. (2000) Function of the cytosolic N-terminus of sucrose transporter AtSUT2 in substrate affinity FEBS Letters **485**:189-194.

Sessions,A., Burke,E., Presting,G., Aux,G., McElver,J., Patton,D., Dietrich,B., Ho,P., Bacwaden,J., Ko,C., Clarke,J.D., Cotton,D., Bullis,D., Snell,J., Miguel,T., Hutchison,D., Kimmerly,B., Mitzel,T., Katagiri,F., Glazebrook,J., Law,M., and Goff,S.A. (2002) A high-throughput *Arabidopsis* reverse genetics system. Plant Cell **14**:2985-2994.

Shah.N.H., King,D.C. Shah,P.N. and Fedoroff,N.V. (2003) A tool-kit for cDNA microarray and promoter analysis. Bioinformatics **19**:1846-1848.

Shakya,R. and Sturm,A. (1998) Characterization of source- and sink-specific sucrose/H^+ symporters from carrot. Plant Physiology **118**:1473-1480.

Sharrock,R.A. and Quail,P.H. (1989) Novel phytochrome sequences in *Arabidopsis thaliana* - structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes & Development **3**:1745-1757.

Sheen,J. (2003) Metabolic repression of transcription in higher plants. Plant Cell **2**:1027-1038.

Sheen,J., Zhou,L., and Jang,J.C. (1999) Sugars as signaling molecules. Current Opinion in Plant Biology **2**:410-418.

Shellhammer,J. and Meinke,D. (1990) Arrested embryos from the *bio1* auxotroph of *Arabidopsis thaliana* contain reduced levels of biotin. Plant Physiology **93**:1162-1167.

Sherson,S.M., Hemmann,G., Wallace,G., Forbes,S., Germain,V., Stadler,R., Bechtold,N., Sauer,N., and Smith,S.M. (2000) Monosaccharide/proton symporter AtSTP1 plays a major role in uptake and response of *Arabidopsis* seeds and seedlings to sugars. Plant Journal **24**:849-857.

Short,T.W. (1999) Overexpression of *Arabidopsis* phytochrome B inhibits phytochrome A function in the presence of sucrose. Plant Physiology **119**:1497-1505.

Simpson,F., Whitehead,J.P., and James,D.E. (2001) GLUT4 - At the cross roads between membrane trafficking and signal transduction. Traffic **2**:2-11.

Smalle,J., Haegman,M., Kurepa,J., VanMontagu,M., and VanderStraeten,D. (1997) Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proceedings of the National Academy of Sciences of the United States of America **94**:2756-2761.

Smeekens,S. (1998) Sugar regulation of gene expression in plants. Current Opinion in Plant Biology **1**:230-234.

Smeekens,S. (2000) Sugar-induced signal transduction in plants. Annual Review Plant Physiology and Plant Molecular Biology **51**:49–81.

Smeekens,S. and Rook,F. (1997) Sugar sensing and sugar-mediated signal transduction in plants. Plant Physiology **115**:7–13.

Smith,H. (1995) Physiological and ecological function within the phytochrome family. Annual Review of Plant Physiology and Plant Molecular Biology **46**:289-315.

Speulman,E., Metz,P.L.J., van Arkel,G., Hekkert,P.T.L., Stiekema,W.J., and Pereira,A. (1999) A two-component Enhancer-Inhibitor transposon mutagenesis system for functional analysis of the *Arabidopsis* genome. Plant Cell **11**:1853-1866.

Spray,D.C. and Bennett,M.V.L. (1985) Physiology and pharmacology of gap-junctions. Annual Review of Physiology **47**:281-303.

Stadler,R., Brandner,J., Schulz,A., Gahrtz,M., and Sauer,N. (1995) Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. Plant Cell **7**:1545-1554.

Stadler,R., Buttner,M., Ache,P., Hedrich,R., Ivashikina,N., Melzer,M., Shearson,S.M., Smith,S.M. and Sauer,N. (2003) Diurnal and Light-Regulated Expression of AtSTP1 in Guard Cells of Arabidopsis. Plant Physiology **133**:528-537.

Stadler,R. and Sauer,N. (1996) The *Arabidopsis thaliana AtSUC2* gene is specifically expressed in companion cells. Botanica Acta **109**:299-308.

Stadler,R., Truernit,E., Gahrtz,M., and Sauer,N. (1999) The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in *Arabidopsis*. Plant Journal **19**:269-278.

Stolz,J., Ludwig,A., Stadler,R., Biesgen,C., Hagemann,K., and Sauer,N. (1999) Structural analysis of a plant sucrose carrier using monoclonal antibodies and bacteriophage lambda surface display. FEBS Letters **453**:375-379.

Sugden,C., Crawford,R.M., Halford,N.G. and Hardie,D.G. (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5'-AMP. Plant Journal **19**:433-439.

Sullivan,J.A. and Deng,X.W. (2003) From seed to seed: the role of photoreceptors in *Arabidopsis* development. Developmental Biology **260**:289-297.

Swarup,R., Parry,G., Graham,N., Allen,T., and Bennett,M. (2002) Auxin cross-talk: integration of signalling pathways to control plant development. Plant Molecular Biology **49**:411-426.

Sze,H., Li,X.H., and Palmgren,M.G. (1999) Energization of plant cell membranes by H^+-pumping ATPases: Regulation and biosynthesis. Plant Cell **11**:677-689.

Tabata,S. (2002) Impact of genomics approaches on plant genetics and Physiology. Journal of Plant Research **115**:271-275.

Tague,B.W., Gallant,P., and Goodman,H.M. (1996) Expression analysis of an *Arabidopsis* C2H2 zinc finger protein gene. Plant Molecular Biology **32**:785-796.

Taiz,L. and Zeiger,E. (1998) Plant Physiology. Sinauer Associates, Inc.

Tepperman,J.M., Zhu,T., Chang,H.S., Wang,X., and Quail,P.H. (2001) Multiple transcription-factor genes are early targets of phytochrome A signaling. Proceedings of the National Academy of Sciences of the United States of America **98**:9437-9442.

The *Arabidopsis* Genome Project (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**:796-815.

Theodoulou,F.L. (2000) Plant ABC transporters. Biochimica et Biophysica Acta-Biomembranes **1465**:79-103.

Thoiron,S. and Briat,J.F. (1999) Differential expression of maize sugar responsive genes in response to iron deficiency. Plant Physiology and Biochemistry **37**:759-766.

Thomine,S., Wang,R., Ward,J.M., Crawford,N.M. and Schroeder,J.I. (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. Proceedings of the National Academy of Sciences of the United States of America **97**:4991-4996.

Thompson,J.D., Higgins,D.G., and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22**:4673-80.

Thum,K.E., Shasha,D.E., Lejay,L.V., and Coruzzi,G.M. (2003) Light and carbon signaling pathways. Modeling circuits of interactions. Plant Physiology **132**:440-452.

Tissier,A.F., Marillonnet,S., Klimyuk,V., Patel,K., Torres,M.A., Murphy,G., and Jones,J.D. (1999) Multiple independent defective suppressor-mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. Plant Cell **11**:1841-1852.

Tobin,E.M. and Silverthorne,J. (1985) Light regulation of gene-expression in higher-plants. Annual Review of Plant Physiology **36**:569-593.

Truernit,E. (2001) Plant Physiology: The importance of sucrose transporters. Current Biology **11**: 169-R171.

Truernit,E. and Sauer,N. (1995) The promoter of the *Arabidopsis thaliana SUC2* sucrose-H^+ symporter gene directs expression of β-glucuronidase to the phloem - evidence for phloem loading and unloading by SUC2. Planta **196**:564-570.

Truernit,E., Schmid,J., Epple,P., Illig,J., and Sauer,N. (1996) The sink-specific and stress-regulated *Arabidopsis STP4* gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. Plant Cell **8**:2169-2182.

Truernit,E., Stadler,R., Baier,K., and Sauer,N. (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. Plant Journal **17**:191-201.

Trumbly,R.J. (1992) Glucose repression in the yeast *Saccharomyces cerevisiae*. Molecular Microbiology **6**:15-21.

Turgeon,R. and Beebe,D.U. (1991) The evidence for symplastic phloem loading. Plant Physiology **96**:349-354.

Turgeon,R. and Gowan,E. (1990) Phloem loading in *Coleus blumei* in absence of carrier-mediated uptake of export sugar from the apoplast. Plant Physiology **94**:1244-1249.

Uemura,M. and Steponkus,P.L. (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. Plant Cell and Environment **26**:1083-1096.

van Bel,A.J.E., Ammerlaan,A., and van Dijk,A.A. (1994) A three-step screening procedure to identify the mode of phloem loading in intact leaves. Planta **192**:31-39.

Vaughn,M.W., Harrington,G.N., and Bush,D.R. (2002) Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. Proceedings of the National Academy of Sciences of the United States of America **99**:10876-10880.

Vera,J.C., Rivas,C.I., Fischbarg,J., and Golde,D.W. (1993) Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature **364**:79-82.

Verwoerd,T.C., Dekker,B.M.M., and Hoekema,A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Research **17**:2362.

Vierstra,R.D. and Quail,P.H. (1986) Phytochrome; the protein. In Photomorphogenesis in Plants. R.E.Kendrick and G.H.M.Kronenberg, eds Martinus Nijhoff, Dordrecht), pp. 35-50.

Vierstra,R.D. (1993) Illuminating phytochrome functions. Plant Physiology **103**:679-684.

von Schaewen,A., Stitt,M., Schmidt,R., Sonnewald,U., and Willmitzer,L. (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO Journal **9**:3033-3044.

Wang,H.Y., Ma,L.G., Habashi,J., Li,J.M., Zhao,H.Y., and Deng,X.W. (2002) Analysis of far-red light-regulated genome expression profiles of phytochrome A pathway mutants in *Arabidopsis*. Plant Journal **32**:723-733.

Ward,J.M. (2001) Identification of novel families of membrane proteins from the model plant *Arabidopsis thaliana*. Bioinformatics. **17**:560-563.

Ward,J.M., Kühn,C., Tegeder,M., and Frommer,W.B. (1998) Sucrose transport in higher plants. International Review of Cytology **178**:41-71.

Weber,A., Servaites,J.C., Geiger,D.R., Kofler,H., Hille,D., Groner,F., Hebbeker,U., and Flugge,U.I. (2000) Identification, purification, and molecular cloning of a putative plastidic glucose translocator. Plant Cell **12**:787-801.

Weber,H., Borisjuk,L., and Wobus,U. (1997) Sugar import and metabolism during seed development. Trends in Plant Science **2**:169-174.

Weig,A., Franz,J., Sauer,N., and Komor,E. (1994) Isolation of a family of cDNA clones from *Ricinus-Communis l* with close homology to the hexose carriers. Journal of Plant Physiology **143**:178-183.

Weise,A., Barker,L., Kühn,C., Lalonde,S., Buschmann,H., Frommer,W.B., and Ward,J.M. (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. Plant Cell **12**:1345-1355.

Whitelam,G.C. and Devlin,P.F. (1997) Roles of different phytochromes in *Arabidopsis* photomorphogenesis. Plant, Cell and Environment **20**:752-758.

Whitelam,G.C., Johnson,E., Peng,J.R., Carol,P., Anderson,M.L., Cowl,J.S., and Harberd,N.P. (1993) Phytochrome-A null mutants of *Arabidopsis* display a wild-type phenotype in white-light. Plant Cell **5**:757-768.

Whitelam,G.C., McCormac,A.C., Boylan,M.T., and Quail,P.H. (1992) Photoresponses of *Arabidopsis* seedlings expressing an introduced oat *PHYA* cDNA - persistence of etiolated plant type responses in light-grown plants. Photochemistry and Photobiology **56**:617-621.

Whitelam,G.C., Patel,S., and Devlin,P.F. (1998) Phytochromes and photomorphogenesis in *Arabidopsis*. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **353**:1445-1453.

Williams,L.E., Lemoine,R., and Sauer,N. (2000a) Sugar transporters in higher plants - A diversity of roles and complex regulation. Trends in Plant Science **5**:283-290.

Williams,L.E., Nelson,S.J., and Hall,J.L. (1990) Characterization of solute transport in plasma-membrane vesicles isolated from cotyledons of *Ricinus communis*. II. Evidence for a proton-coupled mechanism for sucrose and amino acid uptake. Planta **182**:540-545.

Williams,L.E., Pittman,J.K. and Hall,J.L. (2000b) Emerging mechanisms for heavy metal transport in plants. Biochimica et Biophysica Acta – Biomembranes **1465**:104–126.

Wilson,C.A., Dawson,J., Russell,J., and Mulligan,B.J. (1991) Exploited plant: *Arabidopsis thaliana*. Biologist **38**:163-169.

Wimmers,L.E. and Turgeon,R. (1991) Transfer cells and solute uptake in minor veins of *Pisum sativum* leaves. Planta **186**:2-12.

Winzer,T., Lohaus,G., and Heldt,H.W. (1996) Influence of phloem transport, N-fertilization and ion accumulation on sucrose storage in the taproots of fodder beet and sugar beet. Journal of Experimental Botany **47**:863-870.

Wisman,E. and Ohlrogge,J. (2000) *Arabidopsis* microarray service facilities. Plant Physiology **124**:1468-1471.

Wright,K.M., Roberts,A.G., Martens,H.J., Sauer,N., and Oparka,K.J. (2003) Structural and functional vein maturation in developing tobacco leaves in relation to *AtSUC2* promoter activity. Plant Physiology **131**:1555-1565.

Wu,S.H., Ramonell,K., Gollub,J., and Somerville,S. (2001) Plant gene expression profiling with DNA microarrays. Plant Physiology and Biochemistry **39**:917-926.

Wullschleger,S.D. and Difazio,S.P. (2003) Emerging use of gene expression microarrays in plant physiology. Comparative and Functional Genomics **4**:216-224.

Yang,Y.H. and Speed,T. (2002) Design issues for cDNA microarray experiments. Nature Reviews Genetics **3**:579-588.

Young,J.C., Liscum,E., and Hangarter,R.P. (1992) Spectral-dependence of light-inhibited hypocotyl elongation in photomorphogenic mutants of *Arabidopsis* - Evidence for a UV-A photosensor. Planta **188**:106-114.

Yuen,T., Wurmbach,E., Pfeffer,R.L., Ebersole,B.J., and Sealfon,S.C. (2002) Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. Nucleic Acids Research **30**:48.

Zamski,E. and Schnaffer,A.A. (1996) Photoassimilates, Distribution in Plants and Crops. New York: Decker.

Zhou,L., Jang,J.-C., Jones,T.L., and Sheen,J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proceedings of the National Academy of Sciences USA **95**:10294–10299.