Phloem vascular tissues transport sugars synthesized by photosynthesis in mature leaves by a process called phloem loading in source tissues and unloading in sink tissues. Phloem loading in source leaves is catalyzed by Suc/H⁺ symporters (SUTs) which are energized by proton motive force. In Arabidopsis the principal and perhaps exclusive SUT catalyzing phloem loading is AtSUC2. In mutant plants harboring a T-DNA insertion in each of the functional SUT-family members, only Atsuc2 mutants demonstrate overtly debilitating phloem transport. Analysis of a mutant allele (Atsuc2-4) of AtSUC2 with a T-DNA insertion in the second intron showed severely stunted phenotype similar to previously analyzed Atsuc2 null alleles. However unlike previous alleles Atsuc2-4 produced viable seeds.

Analysis of phloem specific promoters showed that promoter expression was regulated by Suc concentration. Unlike AtSUC2p, heterologous promoter CoYMVp was not repressed under high Suc conc. Further analysis was conducted using CoYMVp to test the capacity of diverse clades in SUT-gene family for transferring Suc in planta in Atsuc2 -/ - mutant background. AtSUC1 and ZmSUT1 from maize complemented Atsuc2 mutant plants to the highest level compared to all other transporters. Over-expression of the above SUTs in phloem showed enhanced Suc loading and transport, but against expectations, plants were stunted. The implications of SUT over-expression to enhance phloem transport and loading are discussed and how it induces a perception of phosphate imbalance is presented.
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Kasturi Dasgupta
I gratefully thank my major professor, Dr. Brian Ayre for his constant support, mentorship and guidance to accomplish this work. I appreciate his interest in accepting me as a graduate student even though my experience in the field of plant physiology and biochemistry was minimal. I also extend my deepest thanks to my committee members, Dr.Kent Chapman, Dr. Jyoti Shah, Dr. Ron Mittler and Dr. Douglas Root for their patience and cooperation in serving on my dissertation committee and helping me for anything that contributed to my degree experience. I would like to thank the Department of Biological Sciences, University of North Texas for providing me with financial support during the course of my study and for the facilities to carry out my research. I would like to thank all the past and present members of the Ayre lab specially Dr. Avinash Srivastava, Dr. Roisin McGarry, Aswad Khadilkar, Ipsita Lahiri, Sarah Prewitt, Dr. Umesh Yadav, Justin Laughlin, Heather Thames, Bernice Yau, Angela Chia-Chi Fu, Crystal Jain, Sindhu Manivasagam, Idicula Mathew and last but not the least Te Cao for being a wonderful companion and colleague during my initial years at UNT. I would also like to thank Dr. Stevens Brumley for providing all the help I needed to complete my research. The members of the Dickstein lab for making third floor of the life science building a happy place to work in and my friend Gauri Khandekar for being a wonderful companion during the initial challenging times as a graduate student.

I am grateful to my parents and sister, Kaushani Dasgupta for being the pillar of support in my life and the many personal sacrifices they made to help me achieve this goal. Finally, I am greatly indebted to my husband and love of my life Dr. Suman Mandal for being the best friend, philosopher and guide.
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COMPREHENSIVE LIST OF ABBREVIATIONS

BR – Brassinosteroid

AtSUC2 – Arabidopsis thaliana sucrose transporter 2

C - Carbon

CoYMV - Commelina yellow mottle virus

EDTA - Ethylene diamine tetraacetic acid

EF - Elongation factor

Fru - Fructose

FW - Fresh weight

Glc - Glucose

GUS - β-Glucoronidase

HPLC - High performance liquid chromatography

NaCl - Sodium chloride

NaHCO₃ - Sodium bicarbonate

Pᵢ - Phosphate

PCR - Polymerase chain reaction

qRT-PCR - Quantitative reverse transcription polymerase chain reaction

Suc - Sucrose

SE-CCC - Sieve element-companion cell complex

SE - Standard error

UBQ - Ubiquitin

WT - Wild type
CHAPTER 1

INTRODUCTION

The vascular tissue comprising of xylem and phloem transports fluid and nutrients internally in the plants. Xylem is mainly responsible for transporting water and minerals from the soil to leaves whereas phloem is involved in the transport of water, minerals and organic molecules from mature photosynthetic source leaves to non-photosynthetic sink tissues. Indeed, as much as 50-80% of the CO₂ photoassimilated in a mature leaf is transported out of the leaf in the phloem to satisfy the needs of the heterotrophic organs of the plant (Ainsworth and Bush, 2011). Understanding the transport of photoassimilate through the phloem is important for understanding carbon partitioning and plant productivity (Taiz and Zeiger, 2006).

Phloem tissue consists of mainly two types of cell: sieve elements and companion cells. Sieve elements are elongated cells which are connected to each other via sieve plates to form a continuous tube system that spreads out through the entire plant. The sieve elements contain very little cytoplasm and lack nuclei, vacuoles, golgi bodies and ribosome. They are lined up end-to-end to form sieve tubes through which the sap flows. This tube contains cross-walls at intervals, perforated by many pores to allow the sap to flow. Hence the cross-walls are called sieve plates and the tubes as sieve tubes. Adjacent to the sieve tubes are small cells, each with a nucleus, and dense cytoplasm. These are companion cells. Because of their many active processes, they have large numbers of mitochondria to produce the required amount of ATP. These cells carry out the metabolic processes using ATP as the energy source, such as loading of the sugars in the tubes and appear with the sieve tube element forming the sieve element-companion cell complex (SE-CCC) (Haritatos et al., 2000). One or more companion cells may be
associated with a single sieve element and together the SE-CCC function in several roles, including phloem loading and unloading, long-distance transport, and as a quality control center for monitoring the nature of substances passing into and out of the phloem (Oparka and Turgeon, 1999). The functioning of the phloem essentially hinges on the co-operation between SE and CC. An indication of the nature of their co-operation lies in the relative size of SE and CC along the phloem pathway (Van bel AJE, 1996)

Mature leaves are referred to as source tissues and regions of growth and storage are termed sink tissues. At the source end of the phloem (area where sugar is synthesized), sugars are moved into the phloem sieve elements. This increase in solute decreases the water potential of the cell and causes water to flow in from surrounding areas by osmosis (Turgeon and Wolf, 2009). The increase in the volume of water in the cell causes an increase in pressure which forces the sap solution to move toward the sink tissue. At the sink tissue, the sugars are off-loaded which increases the water potential and causes water to flow out of the phloem by osmosis. The sieve elements must remain alive and keep a functioning plasma membrane in order to help control the flow of sugars into and out of the sieve element. The hydrostatic pressure gradient results in bulk flow of water and dissolved sugars from source to sink tissues (Giaquinta, 1983).

1.1. Phloem Loading

Phloem loading is the starting point for export of carbohydrates and other nutrients from leaves. Three distinct methods can be employed by plants to move sugars into the phloem: symplastic phloem loading, apoplastic phloem loading and passive phloem loading. The first mechanism, known as symplastic phloem loading or polymer trapping (Turgeon and
Wolf, 2009), requires energy (active) although the initial step is not. In the initial step, sucrose (Suc) diffuses from the mesophyll into the minor vein companion cells through specialized plasmodesmata. The Suc in the companion cells is then used to synthesize raffinose and stachyose, a process that increases the concentrations of these sugars in the SE-CCC (Turgeon and Wolf, 2009; Turgeon and Ayre, 2005; Schulz, 2005). The second mechanism, known as apoplastic loading, uses proton motive force to carry Suc, and in some cases sugar alcohols, into the phloem via transporters against a concentration gradient (Lalonde et al., 2004; Sauer, 2007; Braun and Slewinski, 2009). In contrast to these active mechanisms, passive loading is energetically downhill. In the leaves of these plants, sugar levels are higher in the mesophyll than in the phloem. Sugars diffuse through plasmodesmata at each interface, without a concentrating step (Turgeon and Medville, 1998; Reidel et al., 2009; Rennie and Turgeon, 2009; Turgeon, 2010).

1.1.1. Symplastic Phloem Loading

Suc derivatives such as raffinose, stachyose and verbascose are prominent transport sugars in plants such as *Cucumis melon* and *Alonsoa meridionalis* which use the symplastic phloem-loading mechanism (Turgeon, 1991; Turgeon, 1996; Oparka and Turgeon, 1999), and the polymer trap model was proposed to explain the mechanism of symplastic phloem loading (Turgeon, 1991). The conversion from Suc to RFOs reduces the concentration of Suc to maintain the Suc diffusion gradient from mesophyll cells to intermediary cells and allows continued diffusion. RFOs are predicted to be unable to diffuse back to the mesophyll cells because of the diameter of the raffinose molecules exceeding the molecular exclusion limit of the plasmodesmata between mesophyll and intermediary cell. This prevents back-flow to the
mesophyll cells and consequently RFO accumulation to increase the overall solute concentration and generate hydrostatic pressure. RFOs then enter the sieve elements of the phloem through the wider plasmodesmata-pore units that connect these two cells (Turgeon and Ayre, 2005).

1.1.2. Apoplastic Phloem Loading

In apoplastic phloem loading Suc moves from the mesophyll cells into the cell wall space or apoplast first and is then loaded into the SE-CCC from the apoplast. The loading of Suc from apoplast to companion cell is an active process and requires symporters which use the proton motive force to move sugars into SE-CCC (Sauer, 2007). Fig. 1.1 illustrates the mechanism of apoplastic phloem loading. The movement of Suc across the plasma membrane from the apoplast into the SE-CCC against a concentration gradient is accomplished by Suc transporters (SUTs) using the proton motive force for energized symport. SWEETS are a recently described family of transporter participating in passive movement of Suc from mesophyll cells to the apoplast (Chen et al., 2012).

1.2. Suc/H⁺ Symporters (SUTs)

Genes encoding SUTs form a small family in all species studied and contain 12 transmembrane domains with N- and C-termini on the cytoplasmic side of the membrane (Shiratake, 2007). These domains form a pore which permits Suc transport through the membrane. SUTs are important for plant growth and development and are involved in processes like phloem loading, pollen tube growth, Suc signaling and others (Sauer, 2007; Ayre, 2011). On the other hand, SUTs differ in their substrate affinity, specificity and sub cellular
localization and different members show differences in expression patterns and affinities (Kuhn and Grof, 2010).

With increasing numbers of SUT sequences available, phylogenetic analysis divided the symporters between three to five groups (Braun and Slewinski, 2009; Aoki et al., 2003; Sauer, 2007; Kuhn and Grof, 2010). Based on sequence homology and biochemical activity of SUTs, Aoki and colleagues identified Type I, II, and III as distinct branches in one of the earliest trees (Aoki et al., 2003). Similarly Lalonde and colleagues divided the SUTs into three Clades (Clade I, II, and III) (Lalonde et al., 2004). As more sequences were identified, the number of major branches has increased from three to four (Sauer, 2007), to the current five by further dividing the Type II (Clade III) branch (Braun and Slewinski, 2009; Kuhn and Grof, 2010). The convention followed in this document is based on the convention used by Braun and Slewinski which has divided the symporters into 5 groups (Fig. 1.2) (Braun and Slewinski, 2009).

Group 1 consist of moderate affinity monocot transporters involved in phloem loading (Carpaneto et al., 2005; Aoki et al., 2006; Scofield et al., 2007) and grain filling (Aoki et al., 2002). Group 2 consist of high affinity dicot specific transporters expressed in both source and sink tissues (Lalonde et al., 2003; Sauer, 2007). Group 2 SUTs are thought to have the highest specificity for Suc among the SUT groups (Sun et al., 2010). Group 3 members are from monocots and dicots and have more amino acids and a different exon / intron structure than the other groups. The function of this group is still not very clear and these are low affinity transporters or potential Suc sensors without symporter activity (Barker et al., 2000). Most of the information about Group 4 comes from tonoplast localized low affinity transporters such as monocot barley HvSUT2, Lotus japonicus LjSUT4, Arabidopsis AtSUC4, and Solanum tuberosum.
StSUT4 (Kuhn and Grof, 2010; Liesche et al., 2011; Reinders et al., 2008). Group 4 SUTs are roughly 47% similar in sequence to Group 2 SUTs and have approximately 10-fold lower affinity for Suc (Weise et al., 2000; Reinders et al., 2008). Group 5 is monocot-specific and was split from Group 1 (Braun and Slewinski, 2009) because of their high affinity for SUTs. OsSUT5 of rice, the only member of this group has been biochemically analyzed while the role of most other group 5 transporters is still unknown (Sun et al., 2010).

Functional demonstration of Suc/H⁺ symporter activity and kinetics are from studies in yeast (Aoki et al., 2006; Meyer et al., 2000; Riesmeier et al., 1992; Saur et al., 1994; Weise et al., 2000; Zhou et al., 2000) and *Xenopus oocytes* (Boorer et al., 1996; Carpaneto et al., 2005; Chandran et al., 2003; Ramsperger et al., 2004; Reinders et al., 2008; Sivitz et al., 2008). Expression patterns have been used to infer physiological roles in source and sink tissues, and have been determined by promoter fusions to reporter genes, whole-gene fusions, immunolocalization, or in situ hybridization (Barker et al., 2000; Kuhn et al., 1997; Meyer et al., 2000; Reismeier et al., 1993; Saur et al., 2004; Schmitt et al., 2008; Sivitz et al., 2007; Stadler et al., 1999; Truernit et al., 1995). Efforts to establish function *in planta* have centered on T-DNA insertions in Arabidopsis (Gottwald et al., 2000; Meyer et al., 2000; Sauer et al., 2004; Sivitz et al., 2007; Sivitz et al., 2008) and RNA-mediated repression in Solanaceae species (Burkle et al., 1998; Chincinska et al., 2008; Hackel et al., 2006; Riesmeier et al., 1994). However the function of these transporters in plants other than some clue about their involvement in phloem loading is not very clear. Therefore there exists a need to gain insight into the capacity of specific symporters by testing their ability to overcome a defined deficiency.
Suc transport and compartmentalization is fundamental to our understanding of plant physiology and biology. Manipulating the SUTs for efficient Suc transport can lead to strategies for manipulating sinks with biomass to increase yield and also promote photosynthesis for greater primary productivity (Ainsworth and Bush, 2011). Further recent release of plant and fungal genomes as well as transcriptomic databases allowed the generation of an inventory of genes encoding sugar transporter that are involved in plant–fungal interactions (Doidy et al., 2012). These reflect the importance of understanding the mechanisms underlying sugar exchanges from host plants to fungi.

1.2.1. Function of SUTs

Despite the fact that SUTs predate vascular plants, it is their current function in phloem loading that contributes to Suc distribution and plant productivity. It made them mainstays of plant physiology well before their molecular characterization (Giaquinta, 1983; Bush, 1999) and focuses on the importance of SUTs from an evolutionary stand point. Understanding the function and regulation at present is therefore the key to manipulate them for biotech purposes.

The Arabidopsis genome contains the largest SUT gene family characterized to date. It has nine SUT-like genes, although two are categorized as pseudo genes (Sauer et al., 2004). Of the remaining seven, five are group 2 members, one is a group 3 member, and the last is a group 4 member. The rice genome contains five SUT genes: two group 1 members and a single gene each from groups 3, 4, and 5 (Aoki et al., 2003). Analyses of the draft genomes of maize and sorghum indicate that they contain the same five SUT genes as rice. All data currently
available on monocot SUTs are derived solely from grasses. In comparison with dicots, few monocots have been characterized in terms of leaf anatomy and phloem-loading mechanism.

AtSUC2 from group 2 in Arabidopsis has been characterized extensively with respect to its function in phloem loading. The functional homologs in Solanaceae and Maize namely LeSUT1 from group 2 and ZmSUT1 from group 1 have been shown as the main SUT, with similar function as AtSUC2 (Carpaneto et al., 2005; Scofield et al., 2007; Matsukura et al., 2000; Rae et al., 2005). Mutation in the symporters of AtSUC2, ZmSUT1 and LeSUT1 results in severe stunting and hyper accumulation of sugar and starch, caused by impaired phloem transport (van Bel and Hafke, 2005). Using tissue specific promoters to complement an Atsuc2 mutant, it was shown that the only essential function in photoassimilate distribution for AtSUC2 was to load Suc into the phloem in the leaf minor veins (Srivastava et al., 2008). Similar to AtSUC2, LeSUT1 protein localization is reported to the plasma membrane of companion cells suggesting that both Solanaceae and Arabidopsis plants are involved in loading Suc into the phloem companion cells (Sivitz et al., 2008).

Other members from group 2, like AtSUC1, are expressed in pollen, trichomes and root. AtSUC1 functions in Suc uptake during germination. It is important for pollen germination and Suc induced anthocyanin accumulation (Sivitz et al., 2008; Feuerstein et al., 2010). AtSUC9, another important member from group 2 is distinctive as having a very low Km value making it by far the symporter with the greatest Suc affinity (Sivitz et al., 2007). AtSUC3 from group 3 localizes in the sieve elements of the Arabidopsis phloem and is not colocalized with the companion cell specific AtSUC2. AtSUC3 and SUT2 orthologs from group 3 have sequence, size, and number of exons and introns different from the other groups. For example LeSUT2 from
group 3 of tomato was originally described as a potential Suc sensor without symporter activity. More recently, LeSUT2-repressed plants showed debilitated fruit development and LeSUT2 was implicated in pollen-tube growth and phloem unloading in sinks tissues (Hackel et al., 2006).

Several group 4 SUTs localize to the tonoplast and may be involved in mediating Suc transport between the cytoplasm and vacuole (Endler et al., 2006). Most of the work from group 4 has been done on HvSUT2 from barley which was shown to be localized on tonoplast (Endler et al., 2006; Schulz et al., 2011). Similar to HvSUT2, AtSUT4 and LjSUT4 are tonoplast localized (Reinders et al., 2008). However some SUT4 transporters like StSUT4 and LeSUT4 from Solanaceace are claimed to be localized to the plasma membrane as well as the tonoplast (Chincinska et al., 2008). Table 1.1 summarizes the characteristics of the five SUT groups and their characterized representative members.
Figure 1-1 Apoplastic phloem loading mediated by the SUTs.

SWEET proteins ( ) localized on the plasma membrane of phloem parenchyma cells efflux Suc into the phloem apoplast. Sucrose proton symporters (SUTs) on the plasma membrane of the sieve element (SE)-companion cell (CC) complex uptake Suc from the apoplast, thereby concentrating Suc into sieve elements of the phloem.
Figure 1-2 Phylogenetic tree of all grass and selected dicot and monocot SUTs.
The five different groups of SUTs are indicated with brackets and are based on sequence homology and biochemical activity. The tree was rooted with the SUT-like sequence from *Aspergillus fumigatus* (AfSUT; accession no. EAL92728) as an outgroup. At=*Arabidopsis thaliana*; Le=*Lycopersicon esculentum*; St=*Solanum tuberosum*; Nt=*Nicotiana tabacum*; 
Pm=*Plantago Major*; Ps=*Pisum sativum*; Lj=*Lotus japonicas*; Hv=*Hordeum vulgare*; Os=*Oryza sativa*; Sb=*Sorghum bicolor*; Zm=*Zea Mays*; Bo=*Bambusa oldhamii*; Bd=*Brachypodium distachyon*; Sh=*Saccharum officinarum L*; Ta=*Triticum aestivum L* (Braun et al., 2009).
<table>
<thead>
<tr>
<th>Group</th>
<th>Representative member</th>
<th>Distribution/function</th>
<th>Location/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1/SUT3</td>
<td>OsSUT1</td>
<td>Monocot, moderate affinity</td>
<td>Broadly, seed filling, not loading</td>
</tr>
<tr>
<td></td>
<td>ZmSUT1</td>
<td></td>
<td>Phloem &amp; sinks, loading</td>
</tr>
<tr>
<td>Group 2/SUT1</td>
<td>AtSUC1</td>
<td>Dicot, high-affinity uptake</td>
<td>Numerous sinks, uptake</td>
</tr>
<tr>
<td></td>
<td>AtSUC2</td>
<td></td>
<td>Phloem, loading/retrieval</td>
</tr>
<tr>
<td></td>
<td>AtSUC9</td>
<td></td>
<td>Broadly, high-affinity retrieval</td>
</tr>
<tr>
<td></td>
<td>LeSUT1</td>
<td></td>
<td>Phloem and source, loading</td>
</tr>
<tr>
<td></td>
<td>PsSUF1</td>
<td></td>
<td>Phloem and sinks, loading/retrieval</td>
</tr>
<tr>
<td>Group 3/SUT2</td>
<td>LeSUT2</td>
<td>Monocot and dicot, low affinity</td>
<td>Sink organs, pollen tube growth</td>
</tr>
<tr>
<td></td>
<td>AtSUC3</td>
<td></td>
<td>Sinks and wounded tissue</td>
</tr>
<tr>
<td>Group 4/SUT4</td>
<td>HvSUT2</td>
<td>Dicot and monocot, low affinity</td>
<td>Mesophyll, tonoplast transport</td>
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<tr>
<td></td>
<td>LjSUT4</td>
<td></td>
<td>Nodules, tonoplast transport</td>
</tr>
<tr>
<td></td>
<td>StSUT4</td>
<td></td>
<td>Broadly, plasma membrane</td>
</tr>
<tr>
<td></td>
<td>PsSUF4</td>
<td></td>
<td>Bidirectional Suc facilitator</td>
</tr>
<tr>
<td>Group 5/SUT5</td>
<td>OsSUT5</td>
<td>Monocot, high-affinity</td>
<td>Broadly</td>
</tr>
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1.3. Chapter References


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CHAPTER 2

CHARACTERIZATION OF Atsuc2 MUTANTS AND TESTING EXOTIC PROMOTERS AS A TOOL FOR MANIPULATING PHLOEM TRANSPORT

2.1. Abstract

In Arabidopsis AtSUC2 encodes a phloem localized Suc/H⁺ symporter involved in Suc transport from source to sink tissues. Previous studies have shown that plants with T-DNA insertions in the gene encoding the AtSUC2 transporter accumulates sugar, starch in the mature leaves and shows delayed development and stunted growth. These plants fail to produce viable seeds at the end of their life cycle. In this study a new AtSUC2 allele with a T-DNA in the second intron (SALK_038124, designated Atsuc2-4) was analyzed. This allele failed to produce full length transcript and a truncated protein translated from sequences upstream of the insertion site did not catalyze Suc uptake in yeast cells. Detailed growth characterization of Atsuc2-4 plants showed phenotypes similar to previously analyzed Atsuc2 null alleles. However unlike previous alleles Atsuc2-4 produced viable seeds.

2.2. Introduction

Apoplastic phloem loading is the main mode of Suc transport in Arabidopsis and AtSUC2 is the predominant member involved in this process (Gottwald et al., 2000; Lalonde et al., 2004; Sauer, 2007). In mutant Arabidopsis plants harboring a T-DNA insertion in each of the functional SUT-family members, only Atsuc2 mutants demonstrate overtly debilitated phloem transport

1Part of this work has been published in Annals of Botany 104: 1121–1128, 2009 and is reproduced here with permission from Oxford University Press.
In this study a mutant allele of AtSUC2 (Atsuc2-4) with a T-DNA insertion in the second intron was analyzed. The SALK_038124 T-DNA insertion is in the second intron, 1473 nucleotides downstream of the start codon in the genomic sequence. Transcripts of this allele were previously demonstrated to be missing the third and fourth exons (Srivastava et al., 2008) but transcripts corresponding to exons one and two were present at greatly reduced levels relative to wild type. This leaves the possibility that a truncated protein with residual SUT activity might be produced. Mutants harboring this allele did not accumulate $[^{14}\text{C}]\text{Suc}$ in the minor veins of mature leaves (Srivastava et al., 2009). In this study, the truncated cDNA was expressed in yeast cultures and Suc uptake measured relative to controls to establish further that this allele is a null allele along with detailed characterization of the mutant $\text{Atsuc2-}^{-/-}$ plants (Srivastava et al., 2009).

The phenotype of this allele is identical to the previously described mutants, with the important exception that viable seeds are produced. These results argue that AtSUC2-catalysed transport and activity is not essential for the plant to complete its life cycle, and alternative mechanisms for transporting photoassimilated carbon into maturing seeds are discussed.

2.3. Results

2.3.1. $\text{Atsuc2::T-DNA Mutation Analysis}$

Prior analysis of the T-DNA insert location, transcript analysis and phenotype of $\text{Atsuc2-4}$ plants implied that the T-DNA insertion in SALK_038124 creates a null allele (Srivastava et al., 2008). Full length $\text{AtSUC2}$ transcript was not detected in $\text{Atsuc2-4}$ mutant however low quantities of transcript corresponding to 5’ end of gene (upstream of the T-DNA insert were
identified) such that there was a possibility that a truncated protein could be produced and have residual SUT activity. If translated, the AtSUC2 protein would have 440 of 512 amino acids and would be missing 72 carboxyl-terminal amino acids, corresponding to two transmembrane domains, an extracellular domain, and the C-terminal cytoplasmic domain (Bush, 1999; Gottwald et al., 2000).

To test if such a protein may have residual symporter activity, a cDNA representing AtSUC2 truncated after the second exon (corresponding to 440 amino acid) to mimic the potential truncated SUT2 in the Atsuc2-4 mutant was constructed, expressed in yeast from a strong ADH1 promoter, and [14C] Suc uptake measured (Fig. 2.1). Yeast cultures harboring the truncated AtSUC2 cDNA had Suc uptake rates identical to empty-vector controls, whereas uptake by cells harboring full-length cDNA was significantly greater (Fig. 2.2). This analysis supports the hypothesis that the T-DNA insertions in SALK_038124 and the previously identified lines (Gottwald et al., 2000) created true null alleles.

2.3.2. Phenotype Analysis of Mutants

Plants homozygous for the T-DNA (Atsuc2::T-DNA/Atsuc2::T-DNA) or Atsuc2 -/- were severely stunted, and accumulated anthocyanin in mature leaves (Fig. 2.3). Anthocyanin accumulation in rosette leaves was prominent, as described previously for other Atsuc2 mutants (Srivastava et al., 2008; Gottwald et al., 2000), but was less evident in cauline leaves and inflorescence stems (contributed by Srivastava A). Siliques from fertilized flowers in Atsuc2-4 plants developed seeds but were shorter than wild type siliques and the number of siliques with seeds were significantly lower than wild-type (Fig. 2.4). Seed yields were also dramatically reduced and germination rates varied substantially among seed batches from individual Atsuc2-
4 plants, whereas they were consistently high among wild type plants (Table 2.1) indicating that the overall growth of these plants were severely affected. However despite compromised growth and development, Atsuc2-4 plants produced viable seeds. Progeny of Atsuc2-4 plants grew with the same morphology such as stunted, delayed development and growth and anthocyanin accumulation and had similar fecundity as Atsuc2-4 plants segregating from heterozygous AtSUC2 +/ - parents (data not shown).

2.3.3. Transient Carbohydrate Distribution in the Complemented Lines

Suc is the predominant transport sugar in Arabidopsis. When Suc is not efficiently transported out of photosynthesizing leaves, soluble sugars and starch accumulates. To assess the effect of the Atsuc2-4 mutation on carbon partitioning, the distribution of the major forms of transport and storage carbohydrate was analyzed among WT and Atsuc2-4, 30 d after germination. Atsuc2-4 plants had 20-fold or greater levels of soluble sugar and starch, relative to WT control plants (Table 2.2).

2.4. Discussion

AtSUC2 is important for phloem loading and transport but based on the results above Atsuc2-4 KO plants can complete their life cycle and produce viable seed in its absence. Atsuc2-4 was found to be a true null allele based on yeast uptake studies (Fig. 2.1, Fig. 2.2). The phenotype of this allele was identical to the previously described Atsuc2 mutants, with the important exception that viable seeds were produced (Fig. 2.4). These results argue that AtSUC2-catalysed phloem loading is not essential for the plant to complete its life cycle but the growth deficiences were extreme, and the phloem loading role of AtSUC2 is crucial for plant vigor.
Arabidopsis may have alternative mechanisms for transporting photoassimilate to sink tissues such as maturing seeds. For example other members of the SUT gene family may have a role in controlling carbon partitioning during the life cycle of Arabidopsis. However these genes are well characterized and none have expression patterns compatible with a phloem loading function (Srivastava et al., 2009). Sugars such as raffinose family of oligosaccharides and sugar alcohols may also function as transport sugars and in Arabidopsis, raffinose is found in phloem sap (Haritatos et al., 2000). The contribution of these sugars to phloem transport may be elevated in Atsuc2-4 plants. In addition, phloem transport in Atsuc2-4 plants may rely on other solutes for hydrostatic pressure such as $\text{K}^+$ which is active in the phloem (Lacombe et al., 2000). Earlier work has shown increased phloem $\text{K}^+$ levels associated with decreased Suc availability (Lacombe et al., 2000). In addition amino acids are known to contribute to phloem hydrostatic pressure (Lalonde et al., 2004).

Atsuc2-4 leaves accumulate approx. 20-fold higher levels of Suc in mature leaves than wild type (Table 2.2). The high level of Suc accumulation can facilitate “passive loading” and long-distance transport (Turgeon and Medville, 1998). In passive loading the highest solute conc. is in the mesophyll cells and plasmodesmatal connections are proposed to allow diffusion into the phloem and translocation stream. In sieve tube members, bulk flow initiates because the hydrostatic pressure exceeds the resistance imposed by sieve pores in the sieve plates. Turgeon and colleagues showed through a survey of 45 diverse dicotyledonous species that ‘passive’ phloem loading may be more widespread than previously appreciated (Rennie and Turgeon, 2009). Arabidopsis leaves have sufficient plasmodesmatal connections from mesophyll cells to SE-CCC such that they are compatible with, but not optimized for passive
loading. Considering the above, it will be interesting to test the capacity of other SUTs to transport Suc and their ability to phloem load in Atsuc2-4 mutant background (See chapter 3).

2.5. Materials and Methods

2.5.1. Plasmid Construction and Transport Assays in Yeast

Plasmids pRS424::ADH-MCS and pRS424::ADH-cSUC2 were previously described (Srivastava et al., 2008) and are the empty vector control and full-length AtSUC2 cDNA, respectively, used for [14C] Suc uptake studies in Saccharomyces cerevisiae (yeast).

pRS424::ADH-Ex1-2 is an equivalent plasmid harboring AtSUC2 cDNA truncated to include only the first two exons. The truncated cDNA was created by PCR using pRS424::ADH-cSUC2 as a template and forward oligonucleotide AtSUC25 (5’-TTCAAGGTACCAAATATGGTCAGCCATCCAATG -3’) and reverse oligonucleotide AtSUC2-Ex2Rstop (5’-GAATTCGAGCTCATTGGCCGGCAC CGGAATTGGTTG-3’), which incorporates a stop codon downstream of the amino acid encoding sequences in the second exon of AtSUC2. Phusion Hot Start polymerase (NEB, Beverly, MA) was used according to the manufacturer’s instructions. The PCR product was digested with restriction endonucleases KpnI and SacI (NEB); the recognition sequences are underlined in the oligonucleotide sequences), and ligated into the same sites of pRS424::ADH-MCS to create pRS424::ADH-Ex1-2. Transformation of yeast strain SuSy7-URA, growth of cultures, and [14C] Suc uptake assays were as previously described (Srivastava et al., 2008).

2.5.2. Plant Growth Analysis

Atsuc2-4 plants along with WT were germinated and grown under low light conditions in a controlled-environment chamber (Percival AR 95 L; Percival Scientific, Iowa) at 110 to 150
µmol photons m\(^{-2}\) s\(^{-1}\), 14 h of light at 22\(^{\circ}\) C, and 10 h of dark at 19\(^{\circ}\) C. Germination rate was recorded and number of siliques with seeds was recorded at maturity.

2.5.3. Transient Carbohydrate Analysis

Atsuc2-4 plants along with WT were grown for 30 d under the conditions described above. The first three adult leaves were excised at the stem, and fresh weights were established. All tissues were collected between 4 and 6 h into the light period, with plants removed from the chamber immediately before sampling. Tissues were immersed in 300 mL of ice-cold MCW extraction solution (methanol:chloroform:water, 12:5:3) containing 10 µM lactose as a standard and kept on ice until all samples were collected. The samples were extracted at 50\(^{\circ}\) C for 15 min, and the extraction was repeated two more times in MCW without lactose. Extracts were combined and phases separated by the addition of 0.6 volumes of water. The methanol:water phase was reduced to approximately 200 mL in a vacuum centrifuge, and the neutral fraction was eluted from a column consisting of AG 50W-X4 cation-exchange resin (Bio-Rad), polyvinyl polypyrrolidone (Sigma-Aldrich), and AG 1-X8 anion-exchange resin (formate form; Bio-Rad), 250-, 100-, and 250 mL bed volumes, respectively (top to bottom), and washed with 1.0 mL of water. The collected flow through was quantified against standards by high-performance anion-exchange chromatography with pulsed-amperometric detection using a CarboPac PA20 column at 40\(^{\circ}\) C, 50 mM NaOH eluent, and quadruple waveform, as recommended by the instrument manufacturer (Dionex). Values were normalized against lactose. The insoluble fraction of each sample was tested for starch content with the Total Starch Assay Procedure Kit from Megazyme (amyloglucosidase/a-amylase...
method) scaled down 10 times. Calculations and statistical analyses were done using Microsoft Excel.
Figure 2-1 Site of T-DNA insertion in *Atsuc2-4* (SALK_038124) and expression cassettes used for uptake in yeast.

(A) T-DNA insertion site in the second intron of *AtSUC2* (At1g22710) in *Atsuc2-4* with flanking nucleotides indicated; exons are indicated as white boxes and intergenic regions are indicated by the solid black line; numbering is relative to the start of the 5’ UTR, based on the gene model AT1G22710.1 at http://www.arabidopsis.org; T-DNA is indicated as labeled above the gene model. (B) The three expression cassettes used for [14C]Suc uptake into yeast cells; full-length and truncated *AtSUC2* cDNA as labeled with fused exons indicated as white boxes; ATG start and TGA stop codons as labeled; MCS, multiple-cloning site in the empty-vector control, *ADHp*, yeast *ALCOHOL DEHYDROGENASE* promoter; *ADHt* yeast *ADH* terminator.
Figure 2-2 Uptake of $[^{14}\text{C}]$ Suc uptake into yeast cultures harboring the indicated plasmids. Yeast cultures harboring this truncated $\text{AtSUC2 cDNA}$ had Suc uptake rates identical to empty-vector controls, whereas uptake by cells harboring full-length $\text{cDNA}$ was significantly greater.

Figure 2-3 Phenotype characterization of WT and $\text{Atsuc2-4}$ mutant. Rosettes of $\text{Atsuc2-4}$ plants, relative to a single plant segregating as WT, 21 d after germination. Scale bar = 0.8 cm.
Figure 2-4 Rosette, inflorescence and siliques from Atsuc2-4 plants.
(A) Several Atsuc2-4 plants in a single pot, 90 d after germination. Three plants have inflorescences with maturing siliques (white arrows) and one plant has a rosette without an inflorescence (white arrowhead); rosette leaves have visibly evident anthocyanin (white circle) and cauline leaves have less (black circle). Scale bar = 10 mm. (B) Atsuc2-4 plants have shorter siliques (top three) than wild type (bottom three), and do not accumulate anthocyanin. Scale bars ¼ 2 mm. Siliques with seeds from Atsuc2-4 plants and WT were counted individually; n = 6, variation is expressed as standard error.
Table 2.1 *Atsuc2-4* plants produce viable seeds and seedlings to complete their life cycle. Seeds from *Atsuc2-4* plants were counted individually; seeds from wild-type plants were calculated by mass assuming 50 seeds per milligram (Weigel and Glazebrook., 2002). Variation is expressed as SE; n = 6. *a*Student’s t test, P<0.05, relative to WT.

<table>
<thead>
<tr>
<th>Seed line</th>
<th>Siliques with seeds</th>
<th>Seeds per plant</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>224±83</td>
<td>8950±1300</td>
<td>98±1</td>
</tr>
<tr>
<td><em>Atsuc2-4</em></td>
<td>9.8±3.7*a</td>
<td>143±62*a</td>
<td>69±34*a</td>
</tr>
</tbody>
</table>

Table 2.2 Accumulation of transient carbohydrates in 21 day old leaves of the indicated lines. Sugars were measured to assess whether photo assimilate was efficiently transported out of leaves. The first three adult leaves from a single plant were pooled for extraction, and carbohydrate levels were measured. All values are nmoles per milligram fresh weight, and starch is expressed as Glc equivalents. Variation is expressed as SE; n=6 sibling plants. *a*Student’s t test, P<0.05, relative to WT.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>WT</th>
<th><em>Atsuc2-4</em></th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.94 ± 0.5</td>
<td>23.2 ± 7.2*a</td>
<td>24.7</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.35 ± 0.2</td>
<td>8.23 ± 2.1*a</td>
<td>23.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.56 ± 0.7</td>
<td>28.0 ± 6.1*a</td>
<td>17.9</td>
</tr>
<tr>
<td>Starch</td>
<td>29.2 ± 7.1</td>
<td>719 ± 114*a</td>
<td>24.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>32.5 ± 7.8</td>
<td>779 ± 127*a</td>
<td>24.0</td>
</tr>
</tbody>
</table>
2.6. Chapter References


symporter necessary for efficient phloem transport, are able to complete their life cycle and produce viable seed. Annals of Botany 104: 1121-1128


CHAPTER 3

ESTABLISH SUT CAPACITY FOR SUCROSE UPTAKE IN PLANTA USING RESTORATION OF
PHLOEM LOADING AS A READILY SCORED BIOASSAY

3.1. Abstract

In Arabidopsis, SUTs mediate processes central to growth and development but their role in Suc distribution is poorly characterized. Analysis of complemented Atsuc2-4 mutants with AtSUC2 cDNA expressed from phloem specific promoters showed that promoter expression was regulated by Suc concentration. Higher levels of exogenous Suc reduced expression from AtSUC2p; but expression from a promoter element of Commelina Yellow Mottle Virus, CoYMVp increased. The differential regulation of Suc on the expression of the promoters and their impact on Suc transport is discussed. Further the capacity of SUTs to move Suc across the plasma membrane was tested by assessing their ability to complement Atsuc2-4 using CoYMVp as the promoter of choice. The study shows that despite sequence conservation and similar functions in dicots, only one of the tested group 2 SUTs (AtSUC1) complemented the Atsuc2-4 phenotype, whereas, a divergent, monocot-specific group 1 SUT (ZmSUT1) also effectively restored growth. This establishes the symporters that are strong candidates for loading carbohydrate into desired organs for biotechnology. No or very poor complementation was achieved with representatives from group 3 and group 4 SUTs indicating that there exists a need to reassess their presumed function in the tissues where they are naturally expressed.

3.2. Introduction

The capacity of each SUT family member for transferring Suc from the apoplast into the cytoplasm is fundamental in understanding carbohydrate partitioning. Testing SUTs
representing the subgroups for their ability to complement an Atsuc2-4 mutation can provide meaningful information about transport capacity in planta. For example, it has been shown that potato-tuber yield can be increased by expressing rice SUTs OsSUT5 and OsSUT2 in the tuber (Sun et al., 2011). This indicates the importance of understanding the transport capacity of SUTs to exploit them for different biotechnology applications.

SUTs from Arabidopsis and other plant species were used for this and are represented in Table 3.1. They were chosen because they represent four groups (Grp 1, 2, 3, and 4) previously studied to various extents. All the proteins of the chosen SUTs are functional in yeast and/or Xenopus oocytes, indicating that tissue-specific, species-specific, or even plant-specific modification or co-factors are not required for activity (Aoki et al., 2006; Boorer et al., 1996; Carpaneto et al., 2005; Chandran et al., 2003; Meyer et al., 2000; Ramsperger et al., 2004; Reinders et al., 2008; Riesmeier et al., 1992; Sauer and Stolz, 1994; Sivitz et al., 2007; Weise et al., 2000; Zhou et al., 2007). This allows cross referencing the findings with existing models.

CoYMVp (Commelina Yellow Mottle Virus promoter) was used for expressing the SUTs because previous studies have indicated that the exotic promoter CoYMVp, when used for expressing cSUC2 in an Atsuc2-/- mutant background can complement the mutation to wild type levels in terms of rosette area and carbohydrate accumulation (Srivastava et al., 2009). CoYMV infects the monocot Commelina diffusa but contains a DNA element that confers strong, companion cell-specific expression to diverse species (Matsuda et al., 2002; Medberry et al., 1992). Activity of CoYMVp was tested under different environmental conditions in this study and compared with AtSUC2p. One of the key physiological conditions studied was the effect of Suc concentration on promoter activity. Previous studies have indicated that Suc
concentration down regulates promoter activity (Chiou and Bush, 1998). A high level of Suc in the phloem reduces symporter expression and ultimately photosynthesis through feedback mechanisms (Vaughn et al., 2002; Ainsworth and Bush, 2011). The hypothesis was that CoYMVp can bypass Suc specific regulation since it is an exotic promoter. By uncoupling promoter expression from natural regulation, phloem transport rates may remain high under conditions they may normally be reduced.

Results indicated that CoYMVp expression was stronger than AtSUC2p, and it was not repressed in some of the environments that regulate AtSUC2p activity. AtSUC2 was used as a positive control for complementation studies, and transgenic plants were characterized for general growth, sugar distribution and transport efficiency. Only one of the tested group 2 SUTs (AtSUC1) and one monocot-specific group 1 SUT (ZmSUT1) complemented Atsuc2-4 plants and restored growth to near wild type levels. The characterization of the complemented plants is further discussed in this study in relation to SUTs that have higher capacity for phloem loading in planta.

3.3. Results

3.3.1. CoYMVp Phloem-Specific Promoter is activated by Suc While the AtSUC2 Promoter is repressed

CoYMVp has been shown to compliment Atsuc2-/- knockout mutation when fused to AtSUC2 cDNA (Srivastava et al., 2009). It confers strong companion cell-specific expression, and compliments the Atsuc2-/- knockout mutant up to similar levels as AtSUC2p when fused to AtSUC2 cDNA (Fig. 2.1). To establish if CoYMVp and AtSUC2p sequences are differentially regulated, quantitative GUS assays were performed with plants grown on increasing levels of
Suc harboring \textit{AtSUC2} cDNA (\textit{cSUC2}) fused to the \textit{uidA} reporter gene downstream of \textit{AtSUC2p} and \textit{CoYMVp} promoter. Consistent with previous work, GUS activity in the \textit{AtSUC2p::cSUC2::uidA} line decreased with increasing levels of Suc, however it was enhanced in the \textit{CoYMVp::cSUC2::uidA} line (Fig. 2.2A). This demonstrates that \textit{CoYMVp} does show different fine-tuning in response to carbohydrate (Suc, specifically) and may maintain high expression and transport levels when the natural gene is repressed.

To test the relative response of \textit{AtSUC2p} and \textit{CoYMVp} to additional environmental conditions quantitative GUS assays were conducted after exposing the plants to osmotic stress induced by mannitol and salinity stress induced by NaCl. Under osmotic stress conditions both lines showed GUS activity increase with increasing osmoticum (Fig. 2.2B). Similarly GUS activity increases in both lines with increasing salt stress except that \textit{AtSUC2p} dropped somewhat with the highest concentration treatment (Fig. 2.2C). These results imply that under drought, osmotic or salt stress, \textit{CoYMVp} may not enhance phloem transport beyond the capacity of the natural \textit{AtSUC2} promoter as it does in response to Suc levels.

3.3.2. \textit{SUT}s Used for the Study and Vectors Constructed

Having established that \textit{CoYMVp} can drive \textit{cSUC2} expression in a manner sufficient to compliment \textit{Atsuc2-4} (Srivastava et al., 2009), and that activity increases when the endogenous promoter is repressed, it was used to test the capacity of other \textit{SUT}s to restore or improve phloem loading and transport in \textit{Atsuc2-4}. Representative \textit{SUT}s from each of the four major branches of the gene family were sub-cloned as cDNA downstream of \textit{CoYMVp} by Gateway recombination (generically referred to as \textit{CoYMVp::SUTX}) (Fig. 3.3).
3.3.3. Characterization of SUTs

Homozygous Atsuc2 -/- plants are unsuitable for transformation by floral dip because of their severe stunting and low fecundity. AtSUC2 + / - (SALK_038124) is suitable for probing the function of AtSUC2 in whole-plant carbon partitioning by complementation with genes that have altered activity or expression pattern (Srivastava et al., 2008). The binary vectors harboring the CoYMVp::cSUCX cassettes, therefore, were transformed into heterozygous AtSUC2 + / - plants, and transgenic progeny (T1) were genotyped as AtSUC2 + / +, + / - , or - / - by PCR. For each construct, 25-30 independent lines were identified that, based on a 3:1 segregation ratio for glufosinate ammonium resistance in the subsequent generation, contained T-DNA at a single locus. T3 or T4 plants harboring homozygous CoYMVp::cSUCX cassettes, and homozygous Atsuc2 - / -, were analyzed for vegetative growth at 21 d after germination.

The independent lines showed varying ranges of growth characteristics presumably reflecting differing levels of cDNA expression. The average rosette areas of the two most-robust, independent transformants of each transporter were chosen for phenotype analysis (Fig. 3.4). Table 3.2 summarizes the level of complementation of different SUTs in an Atsuc2 - / - background. WT, Atsuc2-4 and CoYMVp::AtSUC2 plants were used as controls in this study. The average rosette area of the two independent lines of CoYMVp::ZmSUT1 and CoYMVp::AtSUC1 transformants were not significantly different from CoYMVp::AtSUC2 control plants (Fig. 3.4). Compared to WT, AtSUC2, ZmSUT1 and AtSUC1 showed the best complementation in terms of growth and development.
3.3.4. Sequence Similarity is not a Strong Indicator of SUT Capacity to Complement Atsuc2-4 Mutants

Transgene expression in the lines of each cassette was determined by RT-qPCR and in all cases; transcript abundance was 50% to 80% that of AtSUC2 in WT plants (Fig. 3.5). The degree of phenotype complementation (i.e., restoration of vegetative growth) did not match well with transcript abundance in some cases. Despite the relative equivalency among the transcripts, only some constructs showed complementation and others did not, and the SUT cassettes could be readily placed into one of two groups: those that promoted complementation (Group 2 AtSUC2 and AtSUC1, and Group 1 ZmSUT1) and those that did not (Group 2 AtSUC9, LeSUT1, NtSUT3; Group 3 AtSUC3 [also called AtSUT2], LeSUT2; Group 4 LeSUT4). AtSUC2, AtSUC1, and ZmSUT1 was chosen as candidates for further study and detailed analysis.

3.3.5. Transient Carbohydrate Distribution in the Complemented Lines

To assess the effect of the CoYMVp::cSUCX expression on carbon partitioning, the distribution of the major forms of transport and storage carbohydrates was analyzed among WT and complemented lines in Atsuc2 -/- at 21 d after germination. CoYMVp::AtSUC2 showed similar levels as WT; CoYMVp::AtSUC1 had 2 to 3 fold more soluble sugar than WT whereas CoYMVp::ZmSUT1 complemented lines had 1 to 2 fold higher than WT plants in shoot (Table 3.3).

3.3.6. Photosynthesis Measurement

The fully expanded leaves of transgenic lines showed photosynthetic rates similar to that of the WT control (Fig. 3.6). High levels of photoassimilate are known to negatively regulate photosynthesis (Stitt et al., 2010; Ainsworth and Bush 2011). This was observed in the
**Atsuc2** - / - plants used for this experiment as controls. However, *CoYMVp:cSUCX* complemented lines (*AtSUC2*, *AtSUC1*, and *ZmSUT1*) in Fig. 3.6 photosynthetic rates were similar to WT plants suggesting sufficient transport of Suc with no feedback inhibition on photosynthesis.

### 3.4. Discussion

To bypass potential transcriptional regulation, *AtSUC2p* was compared with *CoYMVp* and results show that it is not repressed in some of the environments that regulate *AtSUC2p* (Fig. 3.2). Higher levels of exogenous Suc reduced expression from *SUC2p* in sugar beet (Vaughn et al., 2002) similar to what we observed here. Similarly it was shown by Dan Bush and colleagues that SUT activity declined in plasma membrane vesicles isolated from leaves fed exogenous Suc via the xylem transpiration stream (Chiou and Bush, 1998). Symporter activity dropped to 35–50% of water controls when the leaves were fed with 100 mM Suc and to 20–25% of controls with 250 mM Suc. In contrast, alanine symporters and glucose transporter activities did not change in response to Suc treatments. Glucose and Suc regulation of *AtSUC2* was also previously investigated by using *AtSUC2p::GUS* gene construct to visualize the expression pattern of the symporters in excised leaves and young seedlings (Truernit and Sauer, 1995). In that system, no sugar dependent changes in gene expression were observed after 24 h incubations of excised tissues on sugar-containing medium. One possible explanation for such results could be that the sensitive cells were not exposed long enough to the elevated levels of sugar. In the experiments conducted in this chapter exposing the seedlings to 72 h of high Suc concentration resulted in repression of *AtSUC2p* activity.
Different phloem specific promoters show different fine tuning specifically with regard to regulation by Suc. Using promoters to maintain high levels of expression when the endogenous gene may otherwise be repressed can be a mechanism for maintaining high levels of phloem transport under diverse conditions. This in turn may reduce Suc levels in mesophyll and perhaps prevent the well-established feedback inhibition on photosynthetic activity (Stitt et al., 2010; Ainsworth and Bush 2011).

The study with CoYMvp and different symporters helped in establishing a comparative analysis of SUTs in terms of phloem loading and also provided candidates for manipulating carbohydrate loading into desired organs for biotechnology. Only one of the tested groups 2 SUTs (AtSUC1) complemented the Atsuc2 -/- phenotype, and a divergent, monocot-specific group 1 SUT (ZmSUT1) also restored growth to AtSUC2 levels. No or very poor complementation was achieved with representative group 3 and group 4 SUTs. Based on the restoration of growth bioassay, the SUTs tested immediately fell into one of two groups: those that did restore phloem transport and those that did not.

The inability of SUTs from group 3 to restore growth is consistent with this group being characterized as a low affinity / high capacity, or Suc sensors without Suc uptake capacity: the kinetic parameters of this group are sufficiently different from group 2 transporters that it is not surprising that they did not complement (Barker et al., 2000). Group 4 SUTs used here also did not complement. Group 4 SUTs from Arabidopsis, barley and rice localize to the tonoplast, whereas Solanaceae LeSUT4 is shown to localize to the plasma membrane (Kuhn and Grof, 2010; Liesche et al., 2011; Reinders et al., 2008). Because of this discordance, full-length LeSUT4 sequence was tested in Arabidopsis with the rationale that restoration of phloem transport
would support plasma membrane localization. However, transport was not restored and this construct was not pursued, such that this negative result does not illuminate the question of LeSUT4 localization.

Among the Grp 2 SUTs, of which AtSUC2 is one, only AtSUC1 restored phloem transport, as previously reported (Sauer and Stolz, 1994; Sivitz et al., 2008). This was previously shown when AtSUC1 cDNA was fused to the AtSUC2 promoter (Wippel and Sauer, 2012), whereas here it restored phloem transport with a heterologous gene driven from a heterologous promoter. Perhaps the more surprising outcome here relates to the transporters that did not restore phloem transport. AtSUC9 has the highest affinity for Suc among the plant SUTs and is expressed broadly in sink organs, whereas NtSUT3 and LeSUT1 are the major transporters for phloem loading in the Solanaceae species tobacco and tomato (Weise et al., 2008; Sivitz et al., 2007; Lemoine et al., 1999; Hackel et al., 2006). These negative results are worthy of mention because each of these SUTs transport Suc in heterologous organisms (Yeast and/or Xenopus oocytes) and thus do not have an obligate need of plant-specific co-factor for activity, and in the case of NtSUT3 and LeSUT1, these function in phloem loading in the endogenous organism. Notably, these transgenic lines grew moderately better than Atsuc2-/- lines, implying a low level of improved transport, and also had less transgene transcript than those that did complement. However, this lower level of transcript does not seem sufficiently low to account completely for the poor growth. Further experiments are required to test the protein levels in these lines to confirm the presence the protein and yet failure of some of the transgenic lines transformed with CoYMVp::SUTs in an Atsuc2-/- background.
ZmSUT1 did efficiently restore phloem transport, even though Zea mays is more diverged from Arabidopsis than the Solanaceae, and ZmSUT1 is in the distinctly monocot Grp1 family. It is also documented that HvSUT1 from Barley can restore phloem transport to Atsuc2-/- knockouts (Sivitz et al., 2005; Reinders et al., 2012). These results illuminated the transport potential of each in the tissues where they are naturally expressed. Furthermore, the symporters that best restored phloem loading in this study are strong candidates for being the best for loading carbohydrate into desired organs for biotechnology.

3.5. Materials and Methods

3.5.1. Quantitative GUS Analysis

AtSUC2p::AtSUC2::uidA and CoYMVp::AtSUC2::uidA lines used for quantitative GUS analysis is described in Srivastava et al., 2009. Transgenic lines were tested for analyzing promoter strength under different environmental conditions. Seeds were initially germinated on MS+1% Suc and grown for 7 days and then transferred to plates under different Suc concentrations (0%, 1%, 3%, 5% or 0 mM, 30 mM, 80 mM, 146 mM) or NaCl concentrations (0 mM, 50 mM, 100 mM, 200 mM) or Mannitol concentrations (0 mM, 100 mM, 200 mM, 300 mM). After 3 days seedlings were pooled and used for quantitative GUS analysis. Quantitative MUG assays for GUS activity with 4-methylumbelliferyl-β-D-glucuronide hydrate as substrate was measured using a Biorad Versa Xuor Fluorometer and were performed as described (Weigel D, 2002).

3.5.2. Plasmid Construction

Full-length open reading frames of SUTs were amplified by PCR from plasmid backbone in which they were received from Dr. John Ward’s lab (University of Minnesota). The pENTR
directional TOPO cloning kits were used to directionally clone a blunt end PCR product into a vector for entry into the Gateway system available from Invitrogen (Carlsbad, CA, USA). The Gateway-compatible, “Destination” binary vector pGPTV::CoYMVp::ccdB::CmR was constructed by standard procedures (Sambrook et al., 2001). The CoYMVp-ccdB-CmR cassette of pGEM::CoYMVp::ccdB::CmR was received from Dr. Brian Ayre (University of North Texas), was subcloned into pGPTV-Bar (Becker et al., 1992).

_AtSUC1_ was received in pCR8::AtSUC1 backbone and was transferred into pGPTV::CoYMVp::ccdB::CmR by LR recombination reaction using Gateway LR Clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) to create pGPTV::CoYMVp::AtSUC1. _AtSUT2_ and _AtSUC9_ were also received as pCR8::AtSUT2 and pCR8::AtSUC9, and were similarly transferred to the destination vector by LR recombination reaction to create pGPTV::CoYMVp::AtSUT2, pGPTV::CoYMVp::AtSUC9. _ZmSUT1_ was received as pENTR::ZmSUT1 from Dr. David Braun (University of Missouri) and was transferred into pGPTV::CoYMVp::ccdB::CmR by LR recombination reaction using Gateway LR Clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) to create pGPTV::CoYMVp::ZmSUT1.

_AtSUC2_ was amplified from pGEM::SUC2p::cSUC2 (Srivastava et al., 2008) using oligonucleotides AtSUC2F (5’ CACCATGGTCAGCCATCCAATGGAGAAAGCTGC 3’) and AtSUC2R (5’ ATGAAATCCCATAGCTTTGAACGCAGGAGC 3’) to produce a blunt end PCR product. Oligonucleotides were obtained from Invitrogen. PCR reactions were run with high fidelity Phusion DNA polymerase (Finnzymes OY, Espoo, Finland). Using LR Clonase II, the PCR product was transferred to pENTR by LR recombination reaction to create pENTR::cSUC2. This was further recombined with pMDC7 vector (Invitrogen) (Curtis and Grossniklaus., 2003) using LR
recombination reaction to create pMDC7::cSUC2. pMDC7::cSUC2 was recombined with pDONOR-Zeo (Invitrogen) to create pDONOR::cSUC2 using gateway BP Clonase II enzyme (Invitrogen). pDONOR::cSUC2 was finally recombined with destination binary vector pGPTV::CoYMVp::ccdB::CmR to create pGPTV::CoYMVp::AtSUC2 using LR Clonase II enzyme. Dr. John Ward also provided cDNA for NtSUT3 (p195xE::NtSUT3), LeSUT2 (pDR195::LeSUT2), LeSUT4 (pDR195::LeSUT4) and LeSUT1 (p112A1::LeSUT1). Each was PCR amplified using oligonucleotides NtSUT3F (5’ CACCATGGAGAGTGGTAGTATGGGAATG 3’) and NtSUT3R (5’ AAACCTTGGTTTGATGTCCAGTATTTTGTC 3’), LeSUT2F (5’ CACCATGGATGCGGTATCGATCAGAGTACCGT 3’) and LeSUT2R (5’ ACCAAATGGAAGCCAGGTTGATT TG 3’), LeSUT4F (5’ CACCATGGAGAATGGTACAAAAGGGAAACT 3’) and LeSUT4R (5’ ACCAAATGGAAGCCAGGTTGATT TG 3’), LeSUT1F (5’ CACCATGGAGAATGGTACAAAAGGGAAACT 3’) and LeSUT1R (5’ AATGGAAACCGCCATGGCGACTGCTGG 3’) respectively and TOPO cloned into pENTR vector in a similar way as described above, and further recombined with pGPTV::CoYMVp::ccdB::CmR using LR clonase II enzyme to create pGPTV::CoYMVp::NtSUT3, pGPTV::CoYMVp::LeSUT2, pGPTV::CoYMVp::LeSUT4, pGPTV::CoYMVp::LeSUT1. All clones incorporating a PCR product were sequenced to ensure accuracy (Eurofins MWG Operon). These promoter::cSUCX cassettes were then electroporated into Agrobacterium tumefaciens strain GV3101mp90 as described (Ayre and Turgeon, 2004).

3.5.3. Plant Material

Heterozygous plants (AtSUC2 +/-) were transformed with the CoYMVp::cSUCX constructs by the floral dip procedure (Clough and Bent, 1998). T1 generation seeds were sown
on SunGro Metro-Mix in square pots (9X9 cm²) at approximately 1,000 seeds per pot and stratified for 72 h. Plants were then transferred to a controlled-environment chamber (Percival AR 95 L; Percival Scientific, Perry, IA, USA), 14 h of light at 22°C, and 10 h of dark at 21°C. Transgenic seedlings were selected by spray application of glufosinate ammonium (20 mg L⁻¹; "Finale", Farnam Companies, Phoenix, AZ) for 5 alternate days. Resistant plants were genotyped as \( \text{AtSUC2/AtSUC2} \) (designated \( \text{AtSUC2}^+/- \)), \( \text{AtSUC2/Atsuc2} \) (designated \( \text{AtSUC2}^+/- \)) , or \( \text{Atsuc2/Atsuc2} \) (designated \( \text{Atsuc2}^-/- \)) by PCR using the RED Extract-N-Amp plant PCR kit (Sigma-Aldrich) according to the manufacturer’s instructions. The AtSUC2-specific oligonucleotides were \( \text{AtSUC2F1054} \) (5’GGATTGGTGGAAATTGGGAGGAG-3’) and \( \text{AtSUC2IVS1210} \) (5’CGCGTATATATGGTCACTCAAACG 3’), and the T-DNA-specific oligonucleotide was \( \text{LB280} \) (5’GATTTCGGAACCACCATCAAACAGG3’). The cycling parameters were 15 s of denaturation at 94°C, 15 s of annealing at 65°C, and 1 min of elongation at 72°C, for 30 cycles. Twenty five or more glufosinate ammonium-resistant independent lines segregating \( \text{AtSUC2}^+/- \) at the genomic locus were obtained from the T1 generation. T1 seedlings segregating \( \text{AtSUC2}^+/- \) were grown to seed and glufosinate ammonium-resistant homozygous \( \text{Atsuc2}^-/- \) and \( \text{AtSUC2}^+/- \) were PCR selected from the T2 generation. Ultimately 3 or more lines independently transformed with the \( \text{cDNA} \) constructs showing highest level of complementation in terms of vegetative growth in \( \text{Atsuc2}^-/- \) background was selected and grown for T3 or T4 generation. The independent lines from T3 or T4 generation were also tested to be homozygous for the \( \text{cDNA} \) by growing them on glufosinate ammonium. \( \text{AtSUC2}^+/- \) sibling from the same T1 independent line at the genomic locus and homozygous for the \( \text{cDNA} \) were obtained in a similar way as described above from either the T3 or T4 generation.
For growth analysis, seeds from 2 or 3 independent lines for each construct (i.e. T3 or T4 generation) homozygous for the cDNA were germinated in individual cells of a 36-cell flat (T.O. Plastics), and rosettes were digitally photographed at 25 d after germination. Rosette surface area was measured with ImageJ version 1.38 x (Rasband WS, 2007).

3.5.4. Transcript Analysis

Total RNA was isolated from rosette leaves of 21 d old plants using Trizol (Invitrogen Carlsbad, CA) according to the manufacturer’s instructions and treated with RNase-free DNase I (Ambion TURBO DNase, Grand Island, NY, USA). 500 ng RNA from each plant was reverse transcribed with 50 µM oligo(dT) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Primers used for quantitative real-time PCR are listed in Table 3.4. Real-time PCR was performed with Sybr Green PCR Master Mix (Applied Biosystems) on an Vii7 (Applied Biosystems) using the following amplification protocol: 10 min polymerase activation and denaturation at 95°C, 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. This was followed by a product melt to confirm a single PCR product. Three biological and three technical reps were used for each SUT line and WT tested. The level of SUTs expression was normalized to that of EF1α by subtracting the cycle threshold value of EF1α from the cycle threshold value of SUTs (Livak and Schmittgen, 2001).

3.5.5. Transient Carbohydrate Analysis

Transgenic lines along with controls were grown for 21 days on soil under the conditions described above. The rosette was excised at the petioles and sugar extraction was performed. All tissues were collected between 4 and 6 h into the light period, with plants removed from the chamber immediately before sampling. Tissues were immersed in 500 µL of ice-cold MCW
extraction solution (methanol: chloroform: water, 12:5:3) containing 10 µM lactose as a standard. The samples were extracted at 50° C for 15 min, and the extraction was repeated one more time in MCW with 10 µM lactose. Extracts were combined and phases separated by the addition of 0.6 volumes of water. The methanol:water phase was reduced to approximately 200 µL in a vacuum centrifuge, and neutral sugars were eluted from a mix bed ion exchange column consisting of AG50-X8 cation-exchange resin (Bio-Rad, Hercules, CA; hydrogen form), polyvinyl polypyrrolidone (Sigma-Aldrich, St.Louis, MO), and AG1-X8 anion-exchange resin (Bio-Rad, Hercules, CA; formate form), 250-, 100-, and 250 mL bed volumes, respectively (top to bottom), and washed with 1.0 mL of water. The collected flow through was resolved and quantified against commercial standards (galactinol, trehalose, glucose, galactose, fructose, sucrose, lactose, raffinose, stachyose) by high-performance anion-exchange chromatography with pulsed-amperometric detection using a CarboPac PA20 column (Dionex). Values were normalized against lactose (Srivastava et al., 2008).

3.5.6. Photosynthesis Measurement

A LI-COR 6400 XT with a whole plant Arabidopsis chamber (6400-17 whole plant Arabidopsis chamber and 6400-18 RGB light source) (Li-Cor, Inc, Lincoln, NE, USA) was used for measuring photosynthesis per unit surface area. The conditions were constant light source (PAR=225 mol photons m⁻² s⁻¹) at air temperature 23° C. Seedlings were germinated and grown on 2.5 in (65 mm) pots for 3 weeks before taking the measurements.
Table 3.1 SUTs used in this study with sub family grouping and their natural expression and function as indicated.

Groups based on (Braun and Slewinski, 2009) classification.

<table>
<thead>
<tr>
<th>Representative members</th>
<th>Expression and function of selected SUTs (Adapted from Ayre, 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmSUT1 Maize (Grp 1)</td>
<td>Mainly source leaves. Monocot specific, capable of mediating both the sucrose uptake into the phloem in mature leaves and probably desorption of sugar from the phloem vessels into heterotrophic tissues.</td>
</tr>
<tr>
<td>AtSUC2 Arabidopsis (Grp 2)</td>
<td>Companion cells. Encodes a phloem-localized SUT necessary for efficient Suc transport from source tissues to sink tissues.</td>
</tr>
<tr>
<td>AtSUC1 Arabidopsis (Grp 2)</td>
<td>Pollen, root elongation zone. Required for pollen germination and sucrose-induced anthocyanin accumulation.</td>
</tr>
<tr>
<td>AtSUC9 Arabidopsis (Grp 2)</td>
<td>Expressed mainly in sink in shoot and flower, mutants have an early flowering phenotype, high affinity, low substrate specificity.</td>
</tr>
<tr>
<td>LeSUT1 Tomato (Grp 2)</td>
<td>Companion cells, xylem parenchyma, guard cells trichomes. Phloem-specific inhibition of LeSUT1 antisense plants showed a phenotype consistent with an essential role in phloem loading.</td>
</tr>
<tr>
<td>NtSUT3 Tobacco (Grp 2)</td>
<td>Detected only in pollen and is restricted to late pollen development, pollen germination and pollen tube growth.</td>
</tr>
<tr>
<td>AtSUC3 Arabidopsis (Grp 3)</td>
<td>Sieve elements, wound tissue, pollen, guard cells, seed coats, root tips. Low-affinity sucrose transporter compatible with a role as a second low-affinity sucrose transporter or as a sucrose sensor.</td>
</tr>
<tr>
<td>LeSUT2 Tomato (Grp 3)</td>
<td>Developing sink organs. LeSUT2 inhibition impairs pollen tube growth. Possibly important role in pollen loading.</td>
</tr>
<tr>
<td>LeSUT4 Tomato (Grp 4)</td>
<td>Sieve elements, pollen, guard cells, seed coats, root tips. Plays important role in sucrose signaling.</td>
</tr>
</tbody>
</table>
Table 3.2 Complementation levels of SUTs expressed from CoYMVp::cSUCX in an Atsuc2 -/- background.

Groups (Grp) based on (Braun and Slewinski, 2009) and complementation levels calculated based on the two chosen representative line for each SUT (Data used from Fig 3.4A)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Complementation level relative to WT based on rosette area from Fig 3.4A</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSUC2 (Grp 2)</td>
<td>80-85%</td>
</tr>
<tr>
<td>ZmSUT1 (Grp 1)</td>
<td>75-80%</td>
</tr>
<tr>
<td>AtSUC1 (Grp 2)</td>
<td>70-75%</td>
</tr>
<tr>
<td>LeSUT4 (Grp 4)</td>
<td>10-15%</td>
</tr>
<tr>
<td>NtSUT3 (Grp 2)</td>
<td>8-12%</td>
</tr>
<tr>
<td>AtSUC9 (Grp 2)</td>
<td>5-7%</td>
</tr>
<tr>
<td>AtSUC3 (Grp 3)</td>
<td>4-5%</td>
</tr>
<tr>
<td>LeSUT1 (Grp 2)</td>
<td>4-5%</td>
</tr>
<tr>
<td>LeSUT2 (Grp 3)</td>
<td>3-5%</td>
</tr>
</tbody>
</table>
Table 3.3 Sugar and starch in whole rosette of the complemented SUT lines in Atsuc2 -/- background.

Accumulation of transient carbohydrates in whole rosette of the indicated lines was measured to assess whether photoassimilate was efficiently transported out of leaves. All values are in nanomoles per milligram fresh weight. Variation is expressed as SE; n = 6. *Student’s t test P<0.05 relative to WT. AtSUC2 complemented lines represented as At2-1-3 and At2-5-7, ZmSUT1 complemented lines as Zm1-6-3, Zm1-8-5, and AtSUC1 complemented lines as At1-4-1, At1-1-1.

<table>
<thead>
<tr>
<th>Line #</th>
<th>Glc</th>
<th>Fructose</th>
<th>Suc</th>
<th>Starch</th>
<th>Total soluble sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT±</td>
<td>1.56±0.1</td>
<td>0.64±0.1</td>
<td>2.65±0.7</td>
<td>15.8±0.4</td>
<td>4.85±1.1</td>
</tr>
<tr>
<td>At2-1-3</td>
<td>1.28±0.5</td>
<td>0.43±0.3</td>
<td>3.44±0.8</td>
<td>17.2±0.4</td>
<td>5.14±0.3</td>
</tr>
<tr>
<td>At2-5-7</td>
<td>2.02±0.9</td>
<td>0.68±0.3</td>
<td>4.74±0.3</td>
<td>24.2±2.9</td>
<td>7.44±0.6</td>
</tr>
<tr>
<td>Zm1-6-3</td>
<td>2.56±0.2</td>
<td>0.64±0.06</td>
<td>5.28±0.3</td>
<td>22.5±1.4</td>
<td>8.47±0.5</td>
</tr>
<tr>
<td>Zm1-8-5</td>
<td>3.25±0.3</td>
<td>2.04±0.2</td>
<td>5.54±0.9</td>
<td>25.9±1.3</td>
<td>10.8±1.7</td>
</tr>
<tr>
<td>At1-4-1</td>
<td>4.34±0.1</td>
<td>2.25±0.1</td>
<td>6.84±0.2</td>
<td>39.4±6.7</td>
<td>13.4±0.1</td>
</tr>
<tr>
<td>At1-1-1</td>
<td>5.62±0.6</td>
<td>3.31±0.5</td>
<td>7.39±1.0</td>
<td>50.8±6.5</td>
<td>16.3±2.6</td>
</tr>
<tr>
<td>Primer</td>
<td>Forward (5’→3’)</td>
<td>Reverse (5’→3’)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1α</td>
<td>GAGCCCAAGTTTTTGAAGA</td>
<td>CTAACAGCGAAACGTCCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSUC2</td>
<td>TAGCCATTGTCGTCCTCAGATG</td>
<td>ATGAAATCCCATAGAGCTTTGAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSUC1</td>
<td>GTCGTCTTTTCATCGCCACC</td>
<td>TTGTTGGCTACGTCGAGGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmSUT1</td>
<td>AGACGCAGGCCATTATCC</td>
<td>GGAGAAGTCAAGGAGCCAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeSUT4</td>
<td>CAGCCTCTAGATCCCAGTCG</td>
<td>ACAAGCAGGATCACCACAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NtSUT3</td>
<td>CAGAAGCCTGTGGTGTTCAA</td>
<td>TGATCTTCTGTGGCAGCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSUC9</td>
<td>CCCTCCTACCAATGCCATCAGA</td>
<td>GCCACCGGAACCTGGCTGGAAATAATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeSUT1</td>
<td>CCATAGCTGCTGGTGTTCAA</td>
<td>ACCAGAAATGGGGTCACAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeSUT2</td>
<td>CCGCTATCATTAGCGTGTTT</td>
<td>GCAAGAGGAATGCCAGAGAAG</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AtSUC3</td>
<td>TTCGGCTGATGGTGAAATCTGTGT</td>
<td>AAGCATGCGATATTCCAAAGGTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-1 Growth characteristics of controls and mutant plants complemented with promoter::cSUC2 cassettes.

Representative 21-day old wild type (*AtSUC2*+/+), homozygous mutant *Atsuc2*-4 (*Atsuc2*-/−), and homozygous mutant plants independently transformed with the indicated constructs. Scale bar A through D = 0.8 cm.
Figure 3-2 CoYMVp and AtSUC2p phloem-specific promoter activity in presence of different solutes.

(A) Quantitative GUS analysis of AtSUC2p::AtSUC2::uidA and CoYMVp::AtSUC2::uidA plants grown in presence of 0%, 1%, 3%, 5% Suc. Data expressed relative to 0% Suc as control. (B) Quantitative GUS analysis of CoYMVp::uidA and AtSUC2p::uidA plants grown in presence of 0 mM, 100 mM, 200 mM, 300 mM Mannitol. Data expressed relative to 0 mM Mannitol as control. (C) Quantitative GUS analysis of CoYMVp::uidA and AtSUC2p::uidA plants grown in presence of 0 mM, 50 mM, 100 mM, 200 mM NaCl. Data expressed relative to 0mM NaCl as control. n = 6, variation is expressed as standard error.
Figure 3-3 T-DNA cassettes used in the study

Schematic representation of T-DNA cassettes in (A) Representative *SUTs* from each of the four major branches of the *SUT* gene family were sub-cloned as cDNA downstream of *CoYMVp* by Gateway recombination (generically referred to as CoYMVp::SUTX). (B) pGPTV-CoYMVp::ccdB::CmR used as empty vector controls. LB: T-DNA left border; RB: T-DNA right border; *Pnos*-BAR-*pAnos*: nopaline synthase promoter – glufosinate ammonium cDNA – nopaline synthase poly-adenylation signal; *CoYMVp*: Commelina yellow mottle virus. attR1, attR2, attB1 and attB2-Gateway recombination
Figure 3-4 Growth characteristics of WT, Atsuc2-/- and Atsuc2-/- lines harboring cSUC2 CoYMVp::cSUCX cassettes.

(A) Rosette area (cm²) of 21 d Atsuc2-/- plants transformed with CoYMVp::SUTX construct along with wild type Arabidopsis and Atsuc2-/- (KO). as controls. Variation is expressed as SE; n=6 sibling plants. The SUTs are in the following order AtSUC2 (At2-1-3, At2-5-7), ZmSUT1 (Zm1-6-3, Zm1-8-5), AtSUC1 (At1-4-1, At1-1-1), LeSUT4 (Le4-1-8, Le4-28-2), NtSUT3 (Nt3-10-8, Nt3-19-6), AtSUC9 (At9-32-2, At9-13-2) AtSUC3 (At3-9-1, At3-6-1), LeSUT1 (Le1-14-7, Le1-23-2), LeSUT2 (Le2-20-1, Le2-22-2). (B) Representative 25 d WT, Atsuc2-/-, and homozygous Atscu2-/- plants independently transformed with CoYMVp::cSUCX which showed highest level of complementation. Scale bar = 2.7 cm
Figure 3-5 RT-qPCR, transcript levels of SUTs.

Transcript levels relative to EF1α transcript, as internal control, n = 3 for three biological reps and three technical reps, variation is expressed as standard error. The SUTs are in the following order AtSUC2 (At2-1-3), ZmSUT1 (Zm1-6-3), AtSUC1 (At1-4-1), LeSUT4 (Le4-1-8), NtSUT3 (Nt3-10-8), AtSUC9 (At9-32-2) AtSUC3 (At3-9-1), LeSUT1 (Le1-14-7), LeSUT2 (Le2-20-1) based on their level of complementation. See materials and methods for details.
Figure 3-6 Measurements of photosynthesis rates of WT and CoYMVp::cSUCX complemented lines.

Photosynthesis measurements were taken on 21 d plants. Net Photosynthesis expressed as $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$. Variation is expressed as SE; n= 6 siblings. The SUTs are in the following order AtSUC$_2$ (At2-1-3, At2-5-7), ZmSUT1 (Zm1-6-3, Zm1-8-5), AtSUC$_1$ (At1-4-1, At1-1-1).
3.6. Chapter Reference


CHAPTER 4
OVEREXPRESSION OF SUTs AND UNDERSTANDING ITS REGULATION ON HYPERLOADING THE PHLOEM

4.1. Abstract

Plants respond to changes in carbon partitioning by exhibiting a suite of developmental and physiological alterations to maintain carbon homeostasis. Studies have indicated that reduction in the expression of SUTs have deleterious effect on plant growth and development, however little evidence exists about the possibility of improving plant performance by enhancing the expression of SUTs in Arabidopsis. In this study heterologous SUTs were overexpressed in a WT background with the hypothesis that it might be possible to improve plant performance. AtSUC2 +/+ lines harboring CoYMV::cSUCX genes had two different symporters in the companion cells of Arabidopsis. Detailed characterization showed unexpected growth aberration which included stunted phenotype, darker green leaves and reduced rosette area compared to WT plants. Our studies provide compelling evidence that overexpression of SUTs enhances phloem transport and loading but impacts plant responses to carbohydrate partitioning and phosphate requirements.

4.2. Introduction

In the Arabidopsis genome there are nine annotated SUT genes and most of the work published involved characterizing mutants for those genes (Sivitz et al., 2008; Feuerstein et al., 2010; Sivitz et al., 2007; Gottwald et al., 2000; Srivastava et al., 2008, Sauer et al., 2004). There are relatively few reports of over-expression studies in planta with either constitutive or tissue-specific promoters aiming to characterize SUTs in their native organism, or for applied
productivity gains. With respect to ectopic expression of *SUTs* to study the impact on carbon partitioning, constitutive expression of *SoSUT1* of spinach from the *CaMV 35S* promoter in potato resulted in shifts in metabolite levels with little effect on tuber morphology (Leggewie et al., 2003) while over-expression of *OsSUT5* and *OsSUT2* of rice from a tuber-specific promoter was reported to increase tuber yield (Sun et al., 2011). In other species, ectopic expression of *StSUT1* in the storage parenchyma of pea cotyledons during seed development enhanced Suc influx and enhanced cotyledon growth rates (Rosche et al., 2002) and *SUT* over-expression in wheat grains increased levels of storage protein indicating that enhanced Suc transport has benefits beyond carbohydrate accumulation (Weichert et al., 2010).

In addition, altered phloem transport of Suc by manipulating the *SUTs* involved in phloem loading has been put forward as a means to improve plant productivity. As one example, Suc loading increases and decreases in response to the physiological and environmental needs of the plant, and it was proposed that heterologous promoters that are uncoupled from this natural regulation may be useful to keep loading rates constantly high (Srivastava et al., 2009). Increased Suc transport from sources leaves to sink organs is proposed to be an effective method to enhance crop productivity (Ainsworth et al., 2011). This hypothesis stems from well-established research showing that diminished transport promotes Suc accumulation in source leaves, which in turn causes product inhibition on photosynthesis (Stitt et al., 2010).

In addition, the regulation of SUT activity is poorly understood. Work in Solanaceae species has shown that SUT activity is in part regulated by forming homo and/or heterodimers (Krugel et al., 2008; Reinders et al., 2002). However such dimerization of symporters is still
questionable. In Arabidopsis, lines with a foreign symporter gene expressed in the same cells as
the AtSUC2 might shed light in understanding such putative interactions of symporters, and
how that contributes to regulating carbon partitioning.

The objective of this research was to test the ability of phloem-specific SUT over-
expression to increase phloem transport and productivity. Results showed that over-expression
of SUTs from phloem-specific promoters enhanced Suc loading and transport, but against
expectations, plants were stunted and accumulated Suc in rosette leaves. This effect was traced
to a perceived phosphate deficiency, and could be relieved by adding phosphate to the growth
medium. The implications of SUT over-expression to enhance phloem transport and loading are
discussed and a model of how enhanced Suc transport induces perception of a phosphate
limitation is presented.

4.3. Results

4.3.1. Phloem-Specific SUT Overexpression Does Not Improve Productivity

WT siblings (AtSUC2 +/+ ) of the KO lines (Atsuc2 -/- ) complemented lines with
CoYMVp::AtSUC2, CoYMVp::AtSUC1 and CoYMVp::ZmSUT1 from chapter 3 were generated to
test if overexpression of SUTs can ‘hyperload’ the phloem and enhance growth and
development. These AtSUC2 +/+ lines with CoYMVp::SUTX cassettes thus have SUT expression
from two phloem-specific promoters (AtSUC2p and CoYMVp) with different regulation, and
other than CoYMVp::AtSUC2 plants, also have two different symporters participating in the
phloem loading process.

AtSUC2, AtSUC1 and ZmSUT1 cDNA fused to CoYMVp restores phloem loading to
appreciable levels in the Atsuc2 -/- background, and an additive capacity to phloem load was
predicted in WT (AtSUC2 + / +) background. However the growth of SUT over-expressing lines (SUT OE) included stunted phenotype with darker green leaves, and reduced rosette area compared to both WT plants and the respective complemented KO siblings (Fig. 4.1). Similar phenotype was not observed in plants harboring empty vector in AtSCU2 + / + background (data not shown). AtSUC1 and ZmSUT1 showed phenotype similar to AtSUC2 OE plants. At2-1-6 (representative lines expressing CoYMVp::cAtSUC2 in AtSUC2 + / + background), At1-4-4 (representative lines expressing CoYMVp::cAtSUC1 in AtSUC2 + / + background) and Zm1-6-8 (representative lines expressing CoYMVp::cZmSUT1 in AtSUC2 + / + background) were used for all further analysis.

4.3.2. Transcript Analysis of Phloem-Specific SUT Overexpression

SUT expression in the SUT OE lines was tested to confirm additive expression by RT-qPCR (Fig. 4.2). Based on the expression pattern, there is clearly higher combined expression of AtSUC2 and cSUC2 in line At2-1-6 than of AtSUC2 alone in WT plants. AtSUC1 naturally expresses in sink organs and throughout leaves, and the additional AtSUC1 expression observed in line At1-4-4 is attributed to phloem-specific cAtSUC1 expression. cZmSUT1 expression in Zm1-6-8 in this experiment is equivalent to AtSUC2 expression, and Fig. 4.2 shows the different level of phloem-specific SUT expression. These results argue that expression levels are indeed additive in the SUT OE lines.

4.3.3. Phloem-Specific SUT Overexpression Affects Carbon Partitioning

To assess if the observed phenotype is linked to carbon partitioning, the major soluble carbohydrates were quantified at the end of day (EOD) and end of night (EON) time periods. At 21 days post germination, SUT OE lines had more soluble sugars and starch in rosettes than WT
at both time points, but this was less pronounced for Fru (Fig. 4.3). Generally, carbohydrate levels in the rosettes of SUT OE lines were comparable to those in the complemented lines suggesting that reduced transport and carbohydrate accumulation is not the cause of the more extreme phenotype.

Starch content of SUT OE lines was also higher at the end of day than WT. However the level of starch content at the end of night was of comparable levels (Fig. 4.3). This could be due to increased photosynthesis and enhanced phloem transport. In relative terms, roughly one half of the starch that accumulates by the end of the day is digested during the dark period for both WT and the SUT OE lines. However, in absolute amounts, the SUT OE lines accumulate more than twice the starch during the day and degrade more at night. In terms of soluble sugar, it is notable that the SUT OE lines had more Glc, Fru and Suc at the end of the night when these are usually lower at the end of the dark period (Blasing et al., 2005). Earlier works by others show that starch accumulation during the day and degradation during the dark periods is regulated by the circadian clock, and not by sugar levels (Graf et al., 2010; Stitt and Zeeman, 2012). Taken together, an explanation for additional soluble sugar at the end of the night is circadian control of starch degradation but altered patterns of phloem transport to sink organs, such that starch degradation and export are not in sync.

Further analysis of sugars levels in shoot and roots of WT and SUT OE lines grown on ½ MS was conducted to assess whether the sink tissues are carbon-starved or carbon-excess. Roots of SUT OE lines showed higher amount of soluble sugars in the roots compared to WT suggesting higher rate of transport from shoot to root (Fig. 4.4). Both shoots and roots of SUT
OE lines showed higher soluble sugars levels compared to WT, arguing that sugar transport was improved rather than compromised.

4.3.4. Overexpression of SUTs Enhances Phloem Transport

To investigate the transport efficiency among SUT OE lines from shoot to root plants were photosynthetically labeled with $^{14}$CO$_2$ and phloem exudates were analyzed for soluble sugars. SUT-OE lines were tested for enhanced phloem transport by measuring the rate of $^{14}$C exuded from cut petioles into EDTA solution. In these experiments the first 20 minutes of exudation, which could contain the contents of cut cells, was discarded, and $^{14}$C exudation over the next 2x one-hour intervals was measured. Exudation results expressed as CPM per mg fwt per hour showed that phloem exudation rate was 4-5 fold higher than WT (Fig. 4.5) indicating more efficient transport of carbon through the phloem. The fairly constant rates of exudation over the first and second one-hour intervals argue that the values obtained were reliable.

To further confirm if SUT OE increases the phloem transport from source to sink organs, rosettes were photosynthetically labeled with $^{14}$CO$_2$, and transport of label into roots was measured by scintillation counting. As a percentage of total label in the plant, all three SUT OE lines transported more label to the root, indicating that more photoassimilate produced during the 20 minute labeling period was loaded into the phloem and transported (Fig. 4.5). When these values were standardized against WT, the SUT OE lines transport 25% to 30% more label to the root. Relative to WT, these differences are statistically different, but relative to each other, there was no statistical difference between the SUT OE lines. Both results imply enhanced accumulation of photoassimilate in the phloem, relative to WT (Fig. 4.5) suggesting ‘hyperloading’ of carbon in the phloem.
4.3.5. Overexpression of SUTs Enhances Phloem Loading

The results above show enhanced phloem transport but do not address enhanced phloem loading in the SUT OE lines directly. To compare phloem loading among WT and SUT OE lines, leaf disks were infiltrated with a $^{14}$C-Suc solution for 20 minutes, washed thoroughly, freeze-dried, and uptake of $^{14}$C into the veins was measured by scintillation counting (See materials and methods for experimental set up). The results demonstrate that there is higher loading in the SUT OE lines compared to WT ($P<0.5$) expressed either as CPM/mg fwt or CPM/leaf surface area (Fig. 4.6). Taken together these results suggest higher availability of carbon in the source as well as sink tissues which can be used by the plants for various metabolic reactions.

4.3.6. Availability of Phosphate in the Media Rescues the SUT OE Growth

Sugars such as Suc or Glc or Frc need to be phosphorylated for further metabolism. For example, the initial phosphorylation of glucose is required to activate the molecule for cleavage into two pyruvate molecules (Paul and Pellny, 2002; Koch, 2004; Voet and Voet, 2004). Therefore, availability of phosphate is an important factor for utilization of available carbon for plants. Further there are evidences that there is a direct or indirect link between sugar signaling or concentration and phosphate starvation response (Veneklaas et al., 2012; Rolland et al., 2006; Liu et al., 2005; Karthikeyan et al., 2007; Hammond et al., 2008; Lei et al., 2011). More recently, Lei and colleagues identified AtSUC2 in an activation tagging screen as a gene that causes a Pi-limitation when constitutively overexpressed from CaMV 35S enhancer and promoter element, and that additional P$_i$ on sterile plates relieved the phenotype similar to the recovery of phenotype in SUT OE lines here (Lei et al., 2011).
Indeed the phenotype of SUT OE lines when grown on Pi medium was fully recovered and was similar to WT plants (Fig. 4.7). In the absence of added Pi, roots of 8-day old SUT OE plants were approximately 2/3 the length of WT roots and had fewer lateral roots. On ½ strength MS medium supplemented with an additional 1.2 mM Pi, however, root length of the SUT OE lines equaled or exceeded WT root length (Fig. 4.8).

On sterile media supplemented with additional Pi, the rosettes, like the roots, recovered from the stunted phenotype. In order to further confirm the rescue of phenotype, SUT OE lines were further grown on MS media with increasing concentration of phosphate (Fig. 4.9). Results showed increase root growth with increase in phosphate concentration at 1.24 mM, 1.83 mM and 3 mM phosphate suggesting that phosphate is required for phenotype rescue of SUT OE lines. The availability of phosphate restores normal growth and phenotype in the SUT OE lines and Suc availability in the shoot and root plays a role in mediating such responses.

Since addition of phosphate relieves the Pi-limited phenotype, the impact of additional phosphate on carbohydrate transport from leaves to roots was studied. As shown in Fig. 4.10, SUT OE line At2-1-6 had additional soluble sugar in both shoots and roots relative to wild-type plants, when grown on ½ media with all Pi supplemented conditions. When plants in sterile medium with Pi supplements were photosynthetically labeled with $^{14}$CO$_2$, roots consistently accumulated a larger percentage of total assimilated carbon, implying enhanced transport to sinks tissues (Fig. 4.11).
4.3.7. Overexpression of SUTs Enhances Pi Starvation-Induced Gene Expression and Correlates with Shoot Pi Levels

It has been observed that the level of induction of Pi starvation-induced (PSI) genes in Arabidopsis seedlings are positively correlated with the concentration of Suc present in the culture medium (Rolland et al., 2006). To test if overexpression of SUTs is also enhancing PSI gene expression, expression of AtPT2 was tested by RT-qPCR. AtPT2 is a major high-affinity Pi transporter in Arabidopsis whose expression is also highly induced by Pi starvation (Karthikeyan et al., 2007). The enhanced induction of the endogenous AtPT2 gene was confirmed in the SUT OE compared to the WT plants (Fig. 4.12).

In addition to AtPT2, the expression of another PSI gene that encodes Pi transporter PHT2;1 (AtPhT2) was also analyzed (Karthikeyan et al., 2007). Similar to AtPT2 the induction of PHT2;1 gene was also enhanced in SUT OE plants compared with wild-type plants (Fig. 4.12). This indicated that the over expression of SUTs in the phloem is sufficient to induce PSI gene expression.

To sustain normal growth and development, it is important for plants to have a mechanism to maintain ion homeostasis, including Pi, as they respond to external stress. To examine the effects of hyperloading on phosphate homeostasis in plant cells, free Pi levels were analyzed in the shoots of wild-type and SUT OE on soil. The contents of cellular free Pi in SUT OE plants decreased significantly in shoot tissues suggesting that more phosphate is being used for metabolism (Fig. 4.13 A). However the total Pi levels in the shoot between WT and SUT OE lines did not vary significantly (Fig. 4.13 B).
4.3.8. Overexpression of SUTs from the Natural AtSUC2 Promoter Shows Growth Aberration

Similar to CoYMVp::SUT Lines in AtSUC2 +/- Background

CoYMVp promoter has the same spatial expression as the AtSUC2 promoter but it responds differently to various stimuli, particularly exogenous Suc (Fig. 3.1). It was thus tested if over-expression of SUC2 cDNA from an AtSUC2 promoter similarly promotes a Pi limited results. Line kd1039 (Atsuc2 -/- background) was previously described as complemented line with AtSUC2 cDNA expressed from 2 kb of AtSUC2 promoter (Srivastava et al., 2009). kd476 (AtSUC2 +/-) is the sibling plant of kd1039. As shown in Fig. 4.14, plants over-expressing SUC2 cDNA from the AtSUC2 promoter have the same phenotype as plants with SUC2 expressed from CoYMVp. This result further argues that it is not a disruption in the pattern of Suc distribution in these transgenic plants that results in the stunted phenotype, but that the phenotype is directly linked to the quantity of Suc being distributed to sink organs. Thus, we believe that it is the level of Suc being transported through phloem rather than the activity of a particular Suc transporter or phloem specific promoter that regulates the extent of plant responses to Pi starvation.

4.4. Discussion

Plants expressing CoYMVp::SUTs in the Atsuc2 -/- background were modestly stunted and accumulated carbohydrate in rosette leaves, implying that Suc transport was not restored to WT levels, but was sufficient for near-normal growth (Chapter 3 results). RT-qPCR revealed that SUT expression was less than that in WT, so compromised transport is not surprising. However, sibling lines segregating AtSUC2 +/- were more stunted and accumulated more carbohydrate in rosettes than their Atsuc2 -/- counterparts. AtSUC1 and ZmSUT1 may have
post-transcriptional regulation distinct from AtSUC2 to further uncouple phloem loading from endogenous, self-correcting regulation but showed phenotype similar to AtSUC2 OE plants. Photosynthesis yield of SUT-OE lines were reduced relative to WT (data not shown), probably because accumulated carbohydrates resulted in feedback repression on the photosynthetic machinery.

Although the SUT-OE rosettes had more carbohydrate in general, the pattern of soluble sugars and starch accumulation are noteworthy: starch demonstrated the expected pattern of accumulation at the end of the day and reductions at the end of the night, but soluble sugars had the unexpected pattern of end of night levels being higher than end of day levels. Stunting and carbohydrate accumulation in the SUT-OE lines was most unexpected since our premise was that up-regulating SUT gene expression would enhance phloem transport and growth. Further analysis revealed that these plants are indeed loading and transporting more Suc than WT: In intact plants, more $^{14}$C was transferred to roots after photosynthetic labeling; in EDTA exudation experiments, in which transport is not limited by sink capacity to receive the contents of the translocation stream, soluble carbohydrates levels were more than two-fold greater; and $^{14}$C-Suc uptake into the veins of leaf disks, which is direct measure of phloem loading also showed higher loading of $^{14}$C-Suc/mg fwt or $^{14}$C-Suc/leaf area.

There is growing evidence that Suc plays an important role in Phosphate Starvation Induced (PSI) responses. Several reports have shown that Pi starvation led to increased accumulation of Suc in plant leaves (Liu et al., 2005; Karthikeyan et al., 2007). In Arabidopsis, Pho3 mutation was identified in a screen identifying mutants that failed to produce acid phosphatase, and was found to be an allele of Atsuc2. Further previous studies have shown that
change in photosynthate level had a strong effect on PSI gene expression in white lupin and Arabidopsis (Liu et al., 2005; Karthikeyan et al., 2007). This was demonstrated through stem girdling and photoperiod manipulation. Therefore transfer of Suc from shoot to root seems to be a crucial part of signaling during Pi starvation, and SUTs have an important role in regulating sugar movement. Lei and colleagues showed hypersensitive to phosphate starvation1 (hps1), results in activation of AtSUC2 from the tetramer of CaMV 35S enhancers at the left border of the mutagenizing T-DNA such that AtSUC2 becomes ectopically expressed and alters the pattern or Suc distribution in the plant (e.g., accumulation of Suc in areas where it would not normally accumulate, and preventing distribution to other areas) (Lei et al., 2011). In the work presented here, the spatial expression of the SUTs was not altered but merely enhanced, and still resulted in a Pi-limited phenotype. Furthermore the different regulation of CoYMVP and AtSUC2p is not responsible for the phenotype since SUT expression from 2 kb of AtSUC2p gave the same result (Fig. 4.14). The interaction of Suc and Pi signals in regulating hyperloading of phloem suggests that Suc acts as a major regulator of the expression of genes that are involved in plant responses to nutrient availability.

The production of sugars during photosynthesis and the conversion of these sugars into energy during respiration enable the plant to perform all other life-functions. When respiration is restricted due to a P shortage, sugars are not converted into energy and they accumulate within the plant tissue. The accumulation of unused sugars leads to the purple coloration often seen with P deficiency (Lei et al., 2011). The low energy level within the plants is the underlying cause of the stunted growth typically seen with P deficiency. When energy is low, all plant
processes suffer. Flowering and reproduction place a high demand for energy on plants (not to mention the need for DNA in seed production after fertilization).

In summary, the work demonstrates that plants have strong regulation on amount of sugar being transported through phloem and available to the roots (Fig. 4.15). Through our analyses, we showed that elevated levels of SUT expression affects the expression of PSI genes that are involved in Pi signaling, transport, mobilization, and allocation between shoots and roots. A challenging task ahead is to identify signaling components that are the direct targets of Suc and understand how this carbohydrate signal is perceived and transmitted at the molecular level. This study also provides a framework for rational design of future experiments aimed at using membrane transporters to target nutrient partitioning to specific tissues and organs.

Although the focus here is on SUTs, these experiments provided a paradigm for other carbohydrate transport systems (e.g., cell-wall invertases and monosaccharide transporters), amino acid transport systems, or ion transport systems for tailoring the overall nutritional value of harvested organs and understand phloem loading.

4.5. Materials and Methods

4.5.1. Plant Material and Growth Conditions

Heterozygous plants (AtSUC2 + / -) were transformed with the CoYMVp::cSUCX constructs by the floral dip procedure (Clough and Bent, 1998). T1 generation seeds were sown on SunGro Metro-Mix in square pots (9 X 9 cm²) at approximately 1,000 seeds per pot and stratified for 72 h. Plants were then transferred to a controlled-environment chamber (Percival AR 95 L; Percival Scientific), 14 h of light at 22°C, and 10 h of dark at 21°C. Transgenic seedlings were selected by spray application of glufosinate ammonium (20 mg L⁻¹; "Finale", Farnam
Companies, Phoenix, AZ) for 5 days. Resistant plants were genotyped as AtSUC2/AtSUC2 (designated AtSUC2 +/+), AtSUC2/Atsuc2 (designated AtSUC2 +/-), or Atsuc2/Atsuc2 (designated Atsuc2 -/-) by PCR using the RED Extract-N-Amp plant PCR kit (Sigma-Aldrich) according to the manufacturer’s instructions. AtSUC2-specific oligonucleotides were AtSUC2F1054 (5’GGATTGGTGGAAATTGGGAGGAG3’) and AtSUC2IVS1210 (5’CGCGTATATATGGTCACTCAAACG3’), and the T-DNA-specific oligonucleotide was LB280 (5’GATTTCGGAACCACCATCAAACAGG3’). The cycling parameters were 15 s of denaturation at 94°C, 15 s of annealing at 65°C, and 1 min of elongation at 72°C, for 30 cycles. Twenty five or more glufosinate ammonium-resistant independent lines segregating AtSUC2 +/ - at the genomic locus were obtained from the T1 generation. T1 seedlings segregating AtSUC2 +/ - were grown to seed and glufosinate ammonium-resistant homozygous Atsuc2 -/- and AtSUC2 +/- were PCR selected from the T2 generation. Ultimately 3 or more lines independently transformed with the cDNA constructs and segregating homozygous Atsuc2 -/- or AtSUC2 +/- sibling from the same T1 independent line at the genomic locus were obtained from either the T3 or T4 generation. For growth analysis, seeds from the 2 or 3 independent lines for each construct (i.e. T3 or T4 generation) homozygous for the cDNA were germinated in individual cells of a 36-cell flat and rosettes were digitally photographed at 21 d after germination. Rosette surface area was measured with ImageJ version 1.38x (Rasband WS, 2007).

4.5.2. Transcript Analysis

Total RNA was isolated from rosette leaves of 21 d old plants using Trizol (Invitrogen Carlsbad, CA) according to the manufacturer’s instructions and treated with RNase-free DNaseI (Ambion TURBO DNase, Grand Island, NY, USA). 500 ng RNA from each plant was reverse
transcribed with 50 µM oligo(dT) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed with Sybr Green PCR Master Mix (Applied Biosystems) on a Vii7 (Applied Biosystems) using the protocol followed in chapter 3. The list of primers used for the RT-qPCR analysis is in Table 4.1.

4.5.3. Transient Carbohydrate Analysis

Transgenic lines along with controls were grown for 21 days on soil or for 5 days in MS+0% Suc after germination in 1% Suc media. The rosette was excised at the petioles and sugar extraction was performed separately on shoots and roots. All tissues were collected between 4 and 6 h into the light period, with plants removed from the chamber immediately before sampling. Tissues were immersed in 500 µl of ice-cold MCW extraction solution (methanol: chloroform: water, 12:5:3) containing 10 µM lactose as a standard. The samples were extracted based on the protocol as mentioned in chapter 3. Values were normalized against lactose. The insoluble fraction of each sample was tested for starch content with the Total Starch Assay Procedure Kit from Megazyme (amyloglucosidase/a-amylase method) scaled down 10 times. Calculations and statistical analyses were done using Microsoft Excel.

For sugars extracted from plants grown on different phosphate concentration, wild type plants and SUT OE lines were germinated in 1% Suc media. The plants were then transferred to MS+0% Suc media in different concentrations of phosphate 0.62 mM, 1.24 mM, 1.83 mM and 3 mM after 5 days. The phosphate in the media was altered by adding KH₂PO₄, and the addition of potassium was balanced with K₂SO₄.
4.5.4. Radiolabeling and EDTA Exudates Analysis

In order to investigate transport efficiency among the transgenic lines an EDTA-exudation method (Srivastava et al., 2009) was used to collect phloem sap from cut petioles on the representative SUT OE lines along with appropriate controls lines. Plants were grown for 21 days on salad boxes (15X5X13.5 cm) and then labeled using $^{14}$CO$_2$. The advantage of growing plants in salad boxes containing soil was that they could be used directly as chambers for injecting $^{14}$CO$_2$. The salad box formed a tight seal which prevented any escape of $^{14}$CO$_2$. $^{14}$CO$_2$ was generated by injecting 5 ul of radiolabeled NaHCO$_3$ and lactic acid followed by a pulse time for 20 mins. Individual plants were excised at the stem after 40 mins total (chase + pulse) time and their fresh weights measured. Leaves were cut again under 5 mM EDTA and arranged into a small chamber(24 well micro titre plate) containing 1 ml of 5 mM EDTA (pH 6.0), such that the cut petioles were submerged. The chambers were capped to maintain near 100% humidity and minimize the amount of solution drawn into the leaves by transpiration and xylem transport. The fraction of exudates obtained was analyzed by scintillation counter.

4.5.5. Radiolabeling

To analyze Suc uptake and Suc transport in SUT OE and WT plants, plants were grown under long day conditions for 5 days in MS+0% Suc after germination in MS+1% Suc media. Plants were labeled in a similar way as described above using $^{14}$NaHCO$_3$ and lactic acid. After 40 mins total (chase + pulse) time, for each individual plant shoots and roots were cut and amount of radioactivity was detected by scintillation counter.

For $[^{14}\text{C}]$ Suc uptake studies, individual leaves of 21 d plants were harvested by cutting the hypocotyls, fresh weight was established, and plants were submersed in MES buffer (20
mM, pH 5.5 with KOH) plus 2mM CaCl₂, supplemented with [¹⁴C] Suc (1 mM; 30 KBq ml⁻¹), and weighted down with 4mm glass beads. Each replicate contained two or four pooled leaves from the same plant. The leaves were vacuum-infiltrated for 5 min and incubated at room temperature for 20 mins, followed by three, washes in fresh buffer without labeled sugar. The leaves were gently blotted dry after washing, placed between sheets of filter paper, and frozen in powdered dry ice. Frozen rosettes were lyophilized in a -30°C chamber for 48 h, pressed flat between steel plates in a large vice and exposed to X-ray film (Kodak BioMax MR Film, Rochester NY) for 24 h. The leaves were then cleared with 1 mL of 95% ethanol for 30 mins, and then bleached with 1 mL commercial bleach for 15 mins. Five mL of scintillation fluid was added and [¹⁴C Suc] uptake expressed as cpm/mg fwt or cpm/leaf surface area. The leaf surface area was measured with ImageJ version 1.38x (Rasband WS, 2007).

4.5.6. **Pᵢ Assay**

For free Pᵢ assay tissues plants were grown for 14 days on soil, whole rosette cut, rinsed in distilled water, blotted dry, weighed, frozen, and ground to a fine powder in liquid nitrogen. The ground tissues were suspended in 1% glacial acetic acid and mixed thoroughly. The samples were incubated for 30 mins at 42°C. After a brief centrifugation to pellet cellular debris, aliquots of the solution were assayed for Pᵢ using a phosphomolybdate colorimetric assay mix. The assay mix contains 10% ascorbic acid and 0.42% ammonium molybdate in H₂SO₄ made fresh. Samples were incubated for 30 mins at 42°C after addition of assay mix. The absorbance readings were taken at OD₈₂₀. For calculations, standard curve was generated using different concentration of KH₂PO₄ made from 1 mM stock as described previously (Ames, 1966). For total Pᵢ assay tissues were rinsed in distilled water, blotted dry, weighed and transferred to glass
tubes. Mg(NO₃)₂ solution was added to each sample followed by dry and ash by shaking over a strong flame until the brown fumes disappear. The blank and standards were also include in the dry and ash process after addition of Mg(NO₃)₂. 0.5 N HCl was added after the tubes cooled followed by heating at 65⁰C for 30 mins. Aliquots were transferred to new tubes, assay mix added, vortexed and incubated for 42⁰C for 30 mins. The absorbance readings were taken at OD₈₂₀. The color is stable for several hours. The absorbance readings were taken using Synergy 2 multi-mode microplate reader (Biotek, Winooski, VT, USA).
Table 4.1 Primers used in this study for real time PCR

<table>
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Figure 4-1 Growth characteristics of WT, Atsuc2 -/- complemented lines, AtSUC2 +/+ lines harboring the cSUC2 cassettes CoYMv::cSUC2 and Atsuc2 -/- plants.

(A) Representative 21-day old WT, Atsuc2 -/- plants independently transformed with CoYMv::cSUCX (cAtSUC2, cAtSUC1, cZmSUT1) which showed highest level of complementation, WT sibling of the above complemented lines in the same order. The overexpressing SUT lines are in the following order AtSUC2 (At2-1-6), ZmSUT1 (Zm1-6-8), AtSUC1 (At1-4-4). (B) Rosette area (cm²) of 21-day old Atsuc2 -/- plants transformed with CoYMv::SUTX construct along with wild type and Atsuc2 -/- as controls. Variation is expressed as SE; n=6 sibling plants. Scale bar = 1cm. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-2 SUT transcript abundance in representative SUT OE lines.

RT-qPCR, transcript levels relative to EF1α transcript, as internal control, n = 3 for 3 biological reps and 3 technical reps, variation is expressed as standard error. (A) The expression levels of AtSUC2 in WT and representative SUT OE lines (B) The expression levels of AtSUC1 in WT and AtSUC1 SUT OE line (C) The expression levels of ZmSUT1 in WT and ZmSUT1 SUT OE line. See materials and methods for details.
Figure 4-3 Sugar and starch in whole rosette of the indicated lines.
Accumulation of transient carbohydrates in whole rosette of the indicated lines was measured to assess whether photoassimilate was efficiently transported out of leaves. (A) Major soluble carbohydrates quantified at the end of day (EOD) and end of night (EON) time periods along with (B) starch expressed as Glc equivalents in nmoles per milligram fresh weight. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-4 Sugar in whole rosette and 1 cm root tip of the indicated lines grown on plates.

(A) Accumulation of transient carbohydrates in whole rosette of the indicated lines was measured to assess whether photoassimilate was efficiently transported out of leaves in nmoles per fwt (mg). (B) Amount of soluble sugars was measured in 1 cm root tip to test whether sink tissues are carbon starved or carbon excess. Amount of soluble sugars in root tip expressed in nmoles per cm. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-5 Exudation of $[^{14}C]$ from entire rosette and retention of $[^{14}C]$ in the shoot and roots after photosynthetic labeling with $^{14}$CO$_2$.

(A) Plants were photosynthetically labeled with $^{14}$CO$_2$ and phloem exudation into EDTA-containing solution was collected from plants grown on soil and analyzed by scintillation counting. (B) Plants were photosynthetically labeled with $^{14}$CO$_2$ and shoots and roots were cut and analyzed by scintillation counting separately. CPM in roots represented as a percent of total incorporated. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT ($P < 0.05$, t test).
Figure 4-6 Uptake of $^{14}$C Suc into leaves of WT and SUT OE lines.
(A) $^{14}$C Suc uptake in WT and SUT OE lines as shown in autoradiograph. $^{14}$C Suc uptake in WT and SUT OE lines expressed as (B) Total CPM/leaf area in cm$^2$ (C) Total CPM/fwt in gm. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-7 Comparisons of growth morphologies between WT and SUT OE seedlings. Seeds of wild-type (WT) and SUT OE plants were on ½ MS medium with (P+) or without (P−) supplemented inorganic Pi and grown for 8 d on vertically oriented petri plates. Scale bar=0.5cm.

Figure 4-8 Root length of representative 8 d old plants on sterile ½ MS medium. Variation is expressed as SE; n= 8 siblings. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-9 Comparisons of root morphologies between WT and SUT OE seedlings.
Seeds of wild-type (WT) and SUT OE plants were on ½ MS medium with supplemented inorganic Pᵢ and grown for 11 d on vertically oriented petri plates. Scale bar= 0.5 cm.
Figure 4-10 Sugar and starch in whole rosette and root tip of the indicated lines with Pi supplements on media.

Accumulation of transient carbohydrates in whole rosettes of the indicated lines was measured to assess whether photoassimilate was efficiently transported out of leaves. (A) Major soluble carbohydrates quantified in rosette and expressed in nmoles per milligram fresh weight. (B) Amount of soluble sugars was measured in 1 cm root tip to test whether sink tissues are carbon starved or carbon excess. Amount of soluble sugars in root tip expressed in nmoles per cm. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-11 Accumulation of [$^{14}$C] in the sink tissues of SUT OE lines.

Plants were photosynthetically labeled with $^{14}$CO$_2$ for 20 minutes. Shoots and roots were cut and analyzed by scintillation counting separately. Total counts per minute in roots expressed as a percentage of the total counts in shoots and roots in the representative lines. Variation is expressed as SE; n = 6. Asterisks indicate significant differences compared with the WT (P < 0.05, $t$ test).
Figure 4-12 Quantitative analysis of phosphate starvation induced gene expression

(A) Expression of *AtPhT2* and (B) *AtPT2* encoding P$_i$ transporters. RT-qPCR, transcript levels relative to *EF1α* transcript, as internal control, n = 3 for three biological rep and three technical rep; variation is expressed as standard error. See materials and methods for details. Asterisks indicate significant differences compared with the WT (P < 0.05, t test).
Figure 4-13 Comparison of Pi contents between WT and SUT OE lines.
(A) The free cellular Pi, expressed in nmoles/mg fwt. (B) Total Pi content in nmoles/mg fwt.
Asterisks indicate significant differences compared with the WT ($P < 0.05$, $t$ test). Variation is expressed as SE; $n = 6$. 
Figure 4-14 Overexpression of *SUTs* with the *AtSCU2* promoter shows a phosphate starvation phenotype.

Representative 21-day old transgenic lines with WT and *Atsuc2 -/-* as controls. Scale bar = 1 cm.
Figure 4-15 Overexpression of SUTs causes additional Suc mobilization to the sink organs and induces phosphate starvation response.
4.6. Chapter References


CHAPTER 5

SUMMARY

SUTs are fundamental for whole plant growth and development and play key roles in phloem loading and unloading processes. They are also key regulators of phloem transport processes and form integral components of signal transduction between sink and source metabolism. Understanding and manipulating the transport process mediated by the SUTs may provide rational strategies for biomass partitioning.

Characterization of an Atsuc2 allele from Chapter 2 clearly indicated the importance of the symporters in Arabidopsis. The initial characterization of suc2 mutant laid the foundation to understand the capacity of symporters in transporting Suc and its subsequent effect on overall phenotype of the plant. The results from promoter analysis under high Suc concentration indicated that CoYMVP was upregulated by Suc signaling as opposed to AtSUC2p repression. The regulatory cascades that govern solute accumulation in the phloem evolved to ensure plant survival in natural environments, and these may not be optimal in domesticated crops where yield of harvested organs is the primary concern. Different regulations control the amount of carbon being distributed by balancing sink source relations. They can have some other specific role which will prevent the carbon available to reach the tissues/organs being harvested.

Therefore exotic or engineered promoters along with symporters that do not reduce expression under these conditions can maintain high levels of phloem transport and consequently contribute to increased productivity. All further studies were conducted in Atsuc2 mutant background with CoYMVP to understand the capacity of other symporters in phloem loading (Chapter 3).
One of the key aspects for targeted biomass partitioning is to increase plant productivity with less impact on agricultural land and its nutrient resource. This is a major challenge because growth at elevated [CO₂] did not stimulate photosynthesis, biomass, or yield in maize grown on field (Leakey et al., 2006). Higher carbon availability appears to also increase the need of other nutrients. Based on the findings of this research there is a need for more phosphorus with increase in carbon availability (Chapter 4). Higher carbohydrate levels or carbon availability induces a need for more phosphorous (P), which is both a non-renewable yield determinant and a polluting component of agricultural runoff. Enhancing photosynthesis to enhance plant productivity for food, fiber, and fuel is a prominent goal of the plant biology community (Ainsworth and Bush, 2011; Evans et al., 2011; Stitt 2010). Increasing P-use efficiency is also prominent to minimize the need for this non-renewable resource in agricultural production and to reduce the deleterious impacts of P used in fertilizers (Vance et al., 2011; Gaxiola et al., 2012; Veneklaas et al., 2012). However, the evidence is now clear for a link between increased carbohydrate production and delivery and the need for other nutrients such as phosphate, such that achieving both of these goals simultaneously will be difficult unless they can be uncoupled.

This work sheds light on new aspects of nutrient partitioning and the need to understand these links with carbohydrate partitioning. One hypothesis for such interactions could be that the link between Suc transport and P-deficiency is predominantly due to metabolic constraints. Although Suc is the major transported carbohydrate, it is not a prominent storage molecule in most species under physiological conditions. Rather, it is metabolized through a network of pathways and many of the intermediates are phosphorylated (Sulpice et al., 2010). Enhanced Suc transport to sink organs may sequester too
much phosphate into phosphorylated intermediates. Another possibility could be that a
signaling pathway between carbohydrate and phosphate exists which is ill equipped to
coordinate the higher transport rates, and activates a P-limitation response when none exists.
In this scenario, the higher Suc levels in the transport stream may be perceived as a disruption
in the C:P balance, provoking the plant to prepare for a limitation. Combining, genetics,
genomics, metabolomics and physiology, the influence of metabolic constraints and signaling
can be dissected and rational solutions to this could be obtained which can have a major impact
on the agriculture sector and plant productivity.

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APPENDIX A

To IDENTIFY THE CAUSE OF GROWTH ANOMOLIES IN TRANSGENIC PLANTS CARRYING A VECTOR SYSTEM TO GENERATE “ON-DEMAND” KNOCKOUT OF AtSUC2 GENE


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A.1. Introduction

This study initiated as an effort to create a vector system in which a cDNA sequence of interest could be excised upon delivery or activation of a site-specific recombinase. It was designed with dual selection for recombination. After FLP-mediated recombination at the FRT sites, the positive selection marker bar (also pat; phosphinonothricin aminotransferase) was to be activated by being placed adjacent to a CaMV 35S promoter (Davies et al, 1999) and the negative selection marker P450SU1 was to be inactivated by being excised from the genome along with the cDNA of interest (cDNA encoding the AtSUC2 in this specific case). The P450SU1 gene from the soil bacteria Streptomyces griseolus encodes an inducible cytochrome P450, CYP105A1, capable of metabolizing sulfonylurea herbicides via dealkylation (O'Keefe Odell, 1994). However, the activity of CYP105A1 also results in the metabolism of the sulfonylurea pro-herbicide 2-methylethyl-2, 3-dihydro-N-[(4, 6-dimethoxypyrimidin-2-yl) aminocarbonyl]-1, 2-benzoisothiazole-7-sulfonamide-1, 1-dioxide (R7402) to a highly phytotoxic metabolite, such that plants expressing P450SU1 are killed by R7402 treatment at levels that are benign to plants without P450SU1 expression. This has allowed P450SU1 to be used in conjunction with R7402 as a negative-selection marker to select for plants that lack P450SU1 as a transgene (O'Keefe et al., 1994). Negative selection markers like P450SU1 are useful in experiments where selecting for the loss of genes linked to the marker is desired. Independent transgenic lines generated to test on demand knock of AtSUC2 displayed a range of aberrant phenotypes. Therefore the objective of this study was to identify the cause of growth anomalies observed in the transgenic plants. Experiments were designed to correlate the severity of the phenotypes with P450SU1 expression
levels and AtSUC2 expression levels, and additional experiments were conducted to test if CYP105A1 from S. griseolus disrupts brassinosteroid homeostasis in these transgenic plants.

A.2. Results

A.2.1. Arabidopsis Lines Overexpressing P450SUL1 Show Abnormal Growth

The plasmid pART-P450-cSUC2-BAR was used to create transgenic plants with an excisable AtSUC2 cDNA (cSUC2) adjacent to the negative selection marker P450SUL1. AtSUC2 encodes the predominant Suc/H+ symporter required for efficient phloem loading and transport, and plants harboring a homozygous mutation are severely debilitated (Srivastava et al., 2008; Gottwald et al., 2000). Transgenic plants with an excisable cSUC2 cassette would be a valuable research tool and alleviate some of the difficulties associated with null mutants. The negative-selection gene P450SUL1 was incorporated into the excisable cassette as a marker for effective excision. P450SUL1 encodes CYP105A1, a CYP from Streptomyces griseolus which converts the relatively benign pro-herbicide R7402 into a highly phytotoxic product. In the presence of R7402, whole plants or tissues expressing P450SUL1 die while those having lost the sequences retain viability (O’Keefe et al., 1994). Similarly, plasmids pART-cSUC2-BAR and pART-uidA-BAR were used to create transgenic plants used as controls in the experiments (Fig. A.1). Growth aberrations on sterile media during selection on kanamycin and in potting mix were noted among a large proportion of independent T1 seedlings harboring pART-P450-cSUC2-BAR (referred to as OCP lines; Overexpressing Cytochrome P450SUL1). In plants displaying the most severe phenotype, these aberrations included severe stunting, darker green and purplish leaves characteristic of anthocyanin accumulation, thicker leaves in the abaxial/adaxial orientation, delayed flowering, shortened inflorescence internodes, reduced apical dominance and
numerous unexpanded siliques with no or very few seeds. In addition, plants with the most severe phenotype demonstrated counter-clockwise leaf curling that gave rosettes a distinctive ‘twirled’ appearance (Fig. A.1). Similar phenotypes were not observed in T1 plants (n > 20) harboring pART-cSUC2-BAR or pART-uidA-BAR, or in any WT plants.

The two antibiotic genes, nptII and bar, are common markers that are present in all three T-DNA sequences: they are unlikely to be responsible for the growth abnormalities observed in plants transformed with pART-P450-cSUC2-BAR. Reduced or ectopic expression of genes encoding Suc/H+ symporters can disrupt patterns of carbon partitioning and cause growth anomalies, such as stunting, anthocyanin accumulation, and low seed yield (Leggewie et al., 2003; Srivastava et al., 2009; Schulz et al., 1998). However, growth aberrations were not observed among pART-cSUC2-BAR plants (referred as cSUC2 lines), and altered carbon partitioning does not account for the full spectrum of phenotypes observed among pART-P450-cSUC2-BAR plants. P450SU1 has been used as a negative-selection marker in tobacco, Arabidopsis and barley (O'Keefe et al., 1994; Koprek et al., 1999; Tissier et al., 1999). In barley, “striking morphological differences” were observed in transgenic plants compared to non-transgenic plants (Koprek et al., 1999). However, elaboration of those differences was not provided, and no morphological changes are described for Arabidopsis or tobacco.

A.2.2. Transcript Levels of P450SU1 Correlate with the Aberrant Phenotype

The extent of the phenotype varied among OCP lines independently transformed with pART-P450-cSUC2-BAR and suggested a correlation with expression of one of the transgene: most likely P450SU1 but possibly AtSUC2. P450SU1 and AtSUC2 transcript levels were analyzed relative to UBQ10 transcripts (encoding ubiquitin) by semi-quantitative RT-PCR in 17 OCP lines.
as well as in WT and cSUC2 lines, and those transformed with pART-uidA-BAR (uidA lines). In Fig. A.2, the OCP lines were ranked by height for severity of phenotype in 50-day old plants and there is a strong correlation between $P450_{SU1}$ transcript level and phenotype: Lines with the most severe phenotype had the highest levels of $P450_{SU1}$ transcript while those with intermediate and no phenotype had lesser and no transcript, respectively. Conversely, $AtSUC2$ and $cSUC2$ transcript levels (the oligonucleotides used for qRT-PCR detect transcript from both) showed variation among lines with no obvious correlation to phenotype. These findings strongly suggest that expression levels of $P450_{SU1}$, and thus levels of CYP105A1 protein, interfere with plant growth and development.

A.2.3. Overexpression of $P450_{SU1}$ Affects Vegetative and Reproductive Growth

Having established a correlation between $P450_{SU1}$ expression and phenotype, a more detailed analysis of OCP growth and development was conducted. Representative lines demonstrating severe, intermediate, and mild phenotypes were analyzed relative to WT, cSUC2 and uidA lines as controls. As shown in Table I.1, the reproductive phase of the OCP lines was significantly delayed: Under long-day conditions, WT, cSUC2 and uidA lines had visible floral organs within 24-26 days while $P450_{SU1}$ expression associated with delayed transition to flowering (Table I.1). Plants overexpressing $P450_{SU1}$ also had fewer siliques and individual siliques had fewer seeds, resulting in an overall lower seed yield (Fig. A.3).

To gain insight into why fecundity in OCP lines was compromised, scanning electron microscopy was used to analyze flower development. Most conspicuous was the near absence of pollen in severe OCP lines, which may account partially or entirely for the reduced seed yield.
Additionally, OCP lines had delayed senescence: 60-day old OCP plants had green leaves and siliques while WT and cSUC2 lines had completely senesced (Fig. A.5).

A.2.4. Overexpression of \textit{P450}_{SU1} Impacts Brassinosteroid Homeostasis

The morphological and developmental anomalies observed among OCP lines are characteristic of plants defective in brassinosteroid (BR) synthesis and signaling. Plants defective in BR synthesis and signaling display characteristic phenotypes that include severe stunting, darker color from anthocyanin accumulation, epinastic round leaves, delayed flowering, late senescence, reduced male fertility, and compromised germination (Szekeres et al., 1996; Li et al., 1996; Li et al., 2001; Clouse et al., 1996; Clouse et al., 1996; Ye et al., 2010). Seedlings deficient in BR signaling also undergo abnormal skotomorphogenesis (Li et al., 1996). Unlike the elongated hypocotyls, closed cotyledons and prominent apical hooks of WT Arabidopsis seedlings germinated and grown in the dark, BR-deficient seedlings exhibit short and thickened hypocotyls, open and expanded cotyledons, and the emergence of true leaves characteristic of the de-etiolation that occurs during photomorphogenesis (Song et al., 2009; Turk et al., 2003). Exogenous BR can stimulate cell division and expansion and rescue biosynthetic mutants. In WT plants, exogenous BR can cause supraoptimal effects and result in abnormal development from chaotic growth (Szekeres et al., 1996).

To test if \textit{P450}_{SU1} expression in the OCP lines affects BR signaling, the impact of exogenous 24-epibrassinolide (24-epiBL) on skotomorphogenesis was analyzed in dark grown seedlings. In the absence of 24-epiBL, severe OCP lines showed moderate reductions in hypocotyl elongation relative to less severe lines and controls. In the presence of supraoptimal 1 \mu M 24-epiBL, importantly, severe OCP lines showed no significant alteration in growth while
WT and other control seedlings displayed substantial morphological disruptions including chaotic growth in hypocotyls and cotyledons and generally shorter hypocotyls (Fig. A.6).

BR levels are also known to impact root development. Mutants deficient in BR or BR signaling have shorter roots than WT and in the presence of supraoptimal exogenous BR, root development can be severely impaired (Mussig et al., 2003; Clouse et al., 1993; Sathiyamoorthy P, 1990).

Root growth was measured in OCP and WT lines on vertically-oriented sterile media. In the absence of exogenous 24-epiBL, OCP lines had shorter roots than WT but this did not correlate strongly with the severity of the above-ground phenotype. In the presence of 1 µM 24-epiBL, the length of WT roots was reduced to 22% of roots grown in the absence of 24-epiBL, whereas roots of the most severe OCP lines were reduced to only 65% to 75% relative to those grown without exogenous 24-epiBL (Fig. A.7). These findings that exogenous 24-epiBL severely affects WT root and aerial growth, but has little impact on the most severe OCP lines, combined with a growth pattern that phenocopies BR deficient mutants (described above), strongly suggests that the CYP105A1 enzyme encoded by the \textit{P450SU1} gene is affecting BR homeostasis directly or indirectly.

A.2.5. \textit{Overexpression of P450SU1} does not Impact Gibberellin or Auxin Mediated Growth Characteristics

Gibberellin and auxin metabolism are also impacted by CYP activity, and hypocotyl- and root-growth experiments were conducted to test if CYP105A1 visually affects growth responses to these hormones. Exogenous application of GA$_3$ or IAA is known to modestly increase hypocotyls length of etiolated seedlings (Golovatskaya, 2007; Collett et al., 2000; Cowling,
This was observed in wild type and control plants, but the effect was identical among even the most severe OCP lines (Fig. A.8; the slight decrease in observed in OCP9 is not statistically significant). Conversely, exogenous GA$_3$ or IAA treatment is known to result in decreased root elongation in etiolated seedlings (Szekeres et al., 1996; Golovatskaya, 2007; Hobbie and Estelle, 1995). In our experiments with 1 μM of either hormone, OCP and control lines showed identical extents of reduced root elongation. These results show that $P450_{SU1}$ expression does not mitigate the influence of exogenous GA$_3$ or IAA (Fig. A.8) as it did for exogenous 24-epiBL (Fig. A.7), and argues that the CYP105A1 enzyme impacts BR homeostasis, but not that of IAA or GA$_3$.

A.3. Discussion

The $P450_{SU1}$ gene from *Streptomyces griseolus* has been used as a negative-selection marker in conjunction with the pro-herbicide R7402 since plants expressing the gene are killed by R7402 while those not expressing it retain viability. However, in the absence of R7402, plants with high $P450_{SU1}$ expression show aberrant growth characteristic of defects in brassinosteroid synthesis and perception. When exposed to supraoptimal exogenous brassinosteroids, the growth habit of these plants is relatively normal compared to wild type. As controls, plants transformed with T-DNA that retained the AtSUC2 cDNA but had $P450_{SU1}$ deleted were phenotypically normal, as were plants lacking both AtSUC2 cDNA and $P450_{SU1}$ and instead expressing *uidA* encoding β-glucuronidase. The combined results of (1) the close correlation between $P450_{SU1}$ expression and a phenotype resembling a deficiency in BR synthesis or perception, (2) $P450_{SU1}$ expression mitigating the effects of exogenous 24-epiBL, and (3) the process of eliminating other candidate genes indicate that the CYP105A1 enzyme is
acting on exogenous BR and affects endogenous BR by altering BR homoeostasis. Together, these results indicate that both endogenous and exogenous brassinosteroids are a target of the \textit{P450}_{SUI} encoded CYP105A1 monooxygenase.

Work by others has shown that CYP105A1 can hydroxylate vitamin D2 and D3 at multiple positions (Sawada et al., 2004) and can catalyze the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin by O-dealkylation (Hussain and Ward, 2003). Detoxification of sulfonylurea herbicides and N-dealkylation of the pro-herbicide R7402 to produce a toxic metabolite are additional activities (O’Keefe et al., 1994), and collectively, these reactions suggest that CYP105A1 substrate selection and mode of action may be quite broad, but does not extend to IAA or GA3. Weeds with enhanced CYP-mediated detoxification can be difficult to control because resistance can develop against multiple, unrelated classes of herbicide (Siminszky B, 2006; Powles and Yu, 2010). However, in the limited species that have been subjected to analysis, there is a fitness cost associated with elevated CYP levels: In the absence of the selective pressure imparted by the herbicide, herbicide-resistant varieties of \textit{Lolium rigidum} showed up to 30% reduced vitality relative to their herbicide-sensitive counterparts (Vila-Aiub et al., 2005). Therefore understanding and manipulating the association between herbicides and herbicide-resistance genes is therefore a prominent goal for agricultural biotechnology.

A.4. Materials and Methods

A.4.1. Plasmid Construction

Unless stated otherwise, plasmids were created by standard protocols (Sambrook et al., 2001), enzymes were obtained from New England Biolabs (Beverly, MA) and correct constructs were verified by sequencing (SeqWright, Houston TX) (Davies et al., 1999). The plasmid pGEM-
P450-cSUC2-BAR was constructed by a former researcher in Brian Ayre’s laboratory to study FLP mediated conditional loss of an essential gene. Plasmid pSSU-SU11 with a \( P450_{SU1} \) gene cassette consisting of a promoter from the small subunit of Rubisco, a chloroplast targeting sequence fused to the \( P450_{SU1} \) open reading frame and a polyadenylation signal from Rubisco was obtained from Daniel O’Keefe (O’Keefe et al., 1994). pGEM-P450-cSUC2-BAR was digested with \( NtI \) and the P450-cSUC2-BAR fragment ligated into \( NtI \) site of the binary vector pART27 (Gleave, 1992) generating pART-P450-cSUC2-BAR. Similarly, pGEM-cSUC2-BAR and pGEM-uidA-BAR were digested with \( NtI \) to introduce the cSUC2-BAR and uidA-BAR cassettes, respectively, into pART27 to generate pART-cSUC2-BAR and pART-uidA-BAR. In all binary vectors, the orientations of the genes in the cassettes were the same as the pART27 \( nptII \) gene.

A.4.2. Plant Material and Growth Conditions

Seeds were stratified at 4°C for 48 hours prior to germination, and plants were grown in a Percival AR95L chamber (Percival Scientific, Perry, IA) with 14 h light / 10 h dark at 21°C. Plants with the \( Atsuc2 \) allele (SALK_038124) have a T-DNA insertion in \( AtSUC2 \) (At1g22710) (Srivastava et al., 2008). Heterozygous plants (\( AtSUC2/Atsuc2 -/- \)) were transformed (Clough and Bent, 1998) with pART-P450-cSUC2-BAR, pART-cSUC2-BAR, and pART-uidA-BAR, and T1 seedlings selected on Murashige and Skoog basal medium with Gamborg vitamins (Phytotechnology Laboratories, Shawnee Mission, KS) containing 100 mg L-1 of kanamycin for seven days before transferring to MetroMix 360 potting media (Sun Gro Horticulture, Vancouver, Canada). Rosettes were digitally photographed 21 days post-germination, just before WT plants transitioned to flowering, such that all aerial growth was represented in the rosette area. For root and hypocotyl growth analysis, seeds were germinated on vertically-
oriented MS plates, supplemented with 100 mg L\(^{-1}\) kanamycin and 1 µM 24-epibrassinolide (24-epiBL), gibberellic acid (GA3) (both from PhytoTechnology Laboratories), or indole acetic acid (IAA) (Sigma) as indicated and seedlings were analyzed after 7 days. For experiments with dark-grown seedlings, stratified seeds on sterile medium were exposed to light for three hours to induce germination and then covered with aluminum foil for five days. Digitally-photographed plants were analyzed using Image J (Rasband WS, 2007). To assess pollen abundance, flowers of 40-day old plants were imaged with a Hitashi TM-1000 scanning electron microscope after removing some of the sepals and petals.

A.4.3. Transcript Analysis

Total RNA was isolated from rosette leaves of 21-day old plants using Trizol (Invitrogen Carlsbad, CA) according to the manufacturer’s instructions and treated with RNase-free DNaseI (Invitrogen). 500 ng RNA from each plant was reverse transcribed with 50 µM oligo(dT) and Super Script III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. For semi quantitative PCR, 1 µL of cDNA was amplified in the presence of 250 µM dNTP and 500 nM each forward and reverse primer in 25 µL reactions with Red Taq Genomic DNA Polymerase (Sigma-Aldrich, St. Louis, MO). Cycling parameters were 94°C for 10 s, 60°C for 15 s, and 72°C for 50 s. 25, 30, and 35 cycles (in separate tubes) were tested for increasing band intensities, and three replicates of 30 cycles and 35 cycles were used to quantify band intensity with ImageJ (Rasband WS, 2007) by resolving 5 to 10 µL on 1.5% agarose gels. Oligonucleotides amplifying AtSUC2 sequences downstream of the T-DNA insert were AtSUC2Ex3Ex4F (5’-TAGCCATTGTGCCTCCTAGTG-3’; spans the junction between exons 3 and 4) and SUC2-3-ORF (5’-ATGAAATCCATAGTGGTGAAG-3’). Oligonucleotides specific to P450SUI were RT5P450

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(5’-GTGCAGTCCACGGACGC GCAGAG-3’) and P4501RT3 (5’-CGATGGCGAGGTAGCGGAGCAGTTCC-3’). Transcript abundance was standardized to UBQ10 (encoding ubiquitin), using oligonucleotides UBQ1 (5’-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3’) and UBQ2 (5’-CGACTTGTCA TTGAAAGAAAGAGATAACAGG-3’) (Weigel D, 2002).

Table A.1 Effect of P450SU1 on flowering time in OCP lines

<table>
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<th>Plant line</th>
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<tr>
<td>OCP-1</td>
<td>51.2 ± 1.1a</td>
<td>42.5 ± 1.8a</td>
</tr>
<tr>
<td>OCP-10</td>
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<td>OCP-3</td>
<td>42.6 ± 2.3a</td>
<td>38.1 ± 1.9a</td>
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<td>OCP-9</td>
<td>32.7 ± 3.5a</td>
<td>25.7 ± 2.6a</td>
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<td>OCP-13</td>
<td>34.7 ± 3.2a</td>
<td>27.0 ± 2.2a</td>
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<td>19.7 ± 3.4</td>
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<td>12.3 ± 0.4</td>
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<tr>
<td>cSUC2-1</td>
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<td>14.8 ± 2.1</td>
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<tr>
<td>uidA-1</td>
<td>24.8 ± 2.5</td>
<td>13.5 ± 2.5</td>
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</tbody>
</table>

Data represents mean values ± standard deviation of 12 plants from different OCP and control lines.

a Student’s T-test, p < 0.05, relative to wild type (WT).
Figure A-1 T-DNA cassettes used and representative Arabidopsis plants displaying a range of growth patterns.


Representative 21-day old rosettes of (D) transgenic line OCP-1 (Overexpressing Cytochrome P450SU1) harboring pART-P450-cSUC2-BAR and displaying a severe phenotype, (E) transgenic
line cSUC2-1 harboring pART-cSUC2-BAR, and (F) wild type Arabidopsis. (G) Representative 35-day old OCP-17, OCP-9 (both displaying severe phenotypes), OCP-2 (displaying a moderate phenotype), wild type, and cSUC2-1, as indicated. (H, inset) Representative 50-day old OCP-1 plant showing anthocyanin accumulation and ‘twirled’ rosette. Scale bar in D - H is 1 cm.
Figure A-2 Relationships between aberrant growths, represented as plant height, and AtSUC2 and P450Su1 transcript abundance.

(A) OCP, WT, cSUC2, and uidA lines arranged by phenotype severity, with plant height of the indicated lines at full maturity (i.e., senescent and ready for seed harvesting), n = 6, variation is expressed as standard deviation. (B) Semi-quantitative RT-PCR of P450Su1 (black bars) and AtSUC2 (white bars) transcript levels relative to UBQ10 transcript, encoding ubiquitin, n = 3, variation is expressed as standard deviation. (C) Representative gel used to calculate transcript abundance.
Figure A-3 Fecundity analyses of representative OCP lines relative to WT, cSUC2 and uidA control lines.

(A) Number of siliques per plant on the indicated lines at maturity. (B) Seed yield per plant harvested from indicated lines. OCP lines are arranged by phenotype severity and variation is expressed as standard deviation, n = 10. Fecundity analyses of representative OCP lines relative to WT, cSUC2 and uidA control lines.
Figure A-4 Scanning electron micrographs of a WT and OCP flower. (A) WT flower showing copious pollen on anthers and carpels (arrows) and (B) OCP-1 flower with a dearth of pollen (arrowheads). Flowers in (A) and (B) are the same age with respect to opening (anthesis), some petals and sepals were removed to view the internal organs, scale bar is 100 μm.
Figure A-5 Delayed senescence in OCP lines relative to WT and cSUC2 lines. 60-day old representative plants of the indicated lines. Note the shortened internodes and lack of senescence among the OCP plants; OCP-1 still has active blooms. Scale bar = 5 cm.
Figure A-6 Expression of \(P450_{SU1}\) affects hypocotyl and root growth in the dark in the presence and absence of exogenous 24-epibrassinolide.

Images of dark-grown 5-day old seedlings from OCP-1 and wild type in the (A) absence and (B) presence of exogenous 1μM 24-epiBL. Scale bar = 1 mm.

Figure A-7 Hypocotyl and root length in the absence and presence of 24-epiBL.

(C) Hypocotyl length and (D) root length in the absence (black bars) and presence (white bars) of 1 μM 24-epiBL. (E) Hypocotyl length and (F) root length in the presence of 1 μM 24-epiBL relative to sibling plants grown in the absence of exogenous hormone. OCP lines are arranged by phenotype severity, and variation is expressed as SD; \(n = 12\) sibling plants.
Figure A-8 Expression of P450SUI does not influence the impact of GA₃ or IAA on hypocotyl and root growth.

Images of dark-grown 5-day old seedlings from OCP-1 and wild type in (A) the presence of 1 μM GA₃, and (B) the presence of 1 μM IAA. Scale bar is 1 mm. (C, D) Hypocotyl length and (E, F) root length in the presence of 1 μM GA₃ (C, E) and 1 μM IAA (D, F) relative to sibling plants grown in the absence of exogenous hormone. OCP lines are arranged by phenotype severity, and variation is expressed as SD; n = 12 sibling plants.
A.5. Chapter References


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