METABOLIC ENGINEERING IN PLANTS TO CONTROL SOURCE/SINK RELATIONSHIP AND BIOMASS DISTRIBUTION

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Traditional methods like pruning and breeding have historically been used in crop production to divert photoassimilates to harvested organs, but molecular biotechnology is now poised to significantly increase yield by manipulating resource partitioning. It was hypothesized that metabolic engineering in targeted sink tissues can favor resource partitioning to increase harvest. Raffinose Family Oligosaccharides (RFOs) are naturally occurring oligosaccharides that are widespread in plants and are responsible for carbon transport, storage and protection against cold and drought stress. Transgenic plants (GRS47, GRS63) were engineered to generate and transport more RFOs through the phloem than the wild type plants. The transgenic lines produced more RFOs and the RFOs were also detected in their phloem exudates. But the $^{14}\text{CO}_2$ labeling and subsequent thin layer chromatography analysis showed that the RFOs were most likely sequestered in an inactive pool and accumulate over time. Crossing GRS47 and GRS63 lines with MIPS1 plants (that produces more myo-inositol, a substrate in the RFO biosynthetic pathway) did not significantly increase the RFOs in the crossed lines. For future manipulation of RFO degradation in sink organs, the roles of the endogenous $\alpha$-galactosidases were analyzed. The alkaline $\alpha$-galactosidases ($\text{AtSIP1}$ and $\text{AtSIP2}$ in Arabidopsis) are most likely responsible for digesting RFOs in the cytoplasm and may influence the ability to manipulate RFO levels in engineered plants. $\text{Atsip1/2}$ ($\text{AtSIP1}/\text{AtSIP2}$ double-knockout plants) were generated and phenotypically characterized based on seed germination patterns, flowering time, and sugar content to
observe the impact on RFO sugar levels. The observations and analysis from these lines provide a basis for further insight in the manipulation of resource allocation between source and sink tissues in plants for future research.
ACKNOWLEDGEMENTS

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CHAPTER 1
THE POTENTIAL ROLE OF RAFFINOSE FAMILY OLIGOSACCHARIDES IN METABOLICALLY ENGINEERED ARABIDOPSIS PLANTS FOR MANIPULATING RESOURCE ALLOCATION IN SOURCE AND SINK TISSUES

Mature leaves that photosynthesize and produce a net surplus of sugar are referred to as source tissues and regions of net demand of photoassimilate for growth and storage are referred to as sink tissues. The pressure-flow model by Ernst Munch was proposed to explain the phloem transport mechanism and according to this model, phloem transport is driven by hydrostatic pressure gradients generated osmotically between source and sink organs (Taiz and Zeiger, 2006). The phloem transport mechanism is shown in Figure 1.1. It is important because phloem transport (both phloem loading and unloading) may be potentially manipulated by metabolic engineering for targeted resource allocation in plants. Metabolic engineering is targeted improvement of metabolic pathways for the production of metabolites by altering the regulation of biochemical pathways and cellular processes. Metabolic engineering can be done by the introduction or modification and/or optimization of pathways or enzymes in suitable cell types to allow them to produce novel compounds of interest.

Phloem loading is the accumulation of sugars from the mesophyll cells into the sieve element companion cell complex (SE-CCC). During phloem loading, sugars are accumulated in the SE-CCC and water moves in by osmosis thus generating a high-hydrostatic pressure in the phloem. There are two, well-characterized mechanisms of phloem loading in plants; apoplastic phloem loading and symplastic phloem loading (Taiz and Zeiger, 2006). In apoplastic phloem loading, the companion cells and the
sieve elements are cytoplasmically isolated, so the sugars from the mesophyll are first released into the cell wall space (apoplast) and are then loaded into the sieve elements via companion cells from the apoplast. The loading of sugars is an active process carried out by sugar-H⁺ symporters located in the plasma membrane of these cells for transport in to the SE-CCC, and is energized by the proton motive force generated by H⁺/ATPases (Taiz and Zeiger, 2006). In most plants that use apoplastic phloem loading, sucrose (Suc) is utilized as the main transport sugar. Raffinose family oligosaccharides (RFOs) such as raffinose (Raf), stachyose (Sta) and verbascose are prominent transport sugars in plants that use the symplastic phloem loading mechanism. The polymer trap model was proposed to explain the mechanism of symplastic phloem loaders (Turgeon, 1991). In the polymer trap mechanism, Suc diffuses into intermediary cells (specialized companion cells) from mesophyll cells through narrow and highly branched plasmodesmata. Inside intermediary cells, Suc is polymerized to RFOs which are thought to be too large to diffuse back to the mesophyll cells, and thus accumulate to increase the overall solute concentration in the SE-CCC and generate high hydrostatic pressure. The RFOs can be transported into the sieve elements and carried to the sink tissues in the translocation stream.

The RFO sugars such as Raf, Sta and verbascose are galactosyl derivatives of Suc and repeating galactosyl units in α.1-6 linkages. They are present in plants in various parts such as seeds, leaves, rhizomes, tubers, stems, and translocation pathways (Keller and Pharr, 1996). In the Cucurbitaceae family, Raf and Sta can be the main translocated phloem sugar. In addition to its role in phloem transport, RFOs especially Raf, act in plants as cryoprotectants thus aiding plants during cold stress
Galactinol (Gol) and Raf also have an important role in tolerance to temperature stress (Wienkoop, 2008). The RFOs are known to accumulate in storage tissue that might serve for carbon storage as well as stress protectant (Keller and Pharr, 1996). Indeed, in seeds it can be a very important source of carbon that can be readily metabolized in early germination periods (Downie and Bewley, 2000). It is also known that polymerization and metabolism of RFO can alter the vacuolar osmotic potential and hence may alter turgor pressure (Bachmann, 1995). The structures of common RFOs are shown in Figure 1.2. In the catabolic pathway, the galactosyl units of the RFOs are hydrolyzed by mostly alkaline $\alpha$-galactosidases to Suc and galactose (Gal) (Gao and Schaffer, 1999; Carmi, 2003).

Plant carbon partitioning can, in principle, be engineered to desired organs by altering hydrostatic pressure gradients between source leaves and target sinks. Targeted metabolic engineering with synthesizing alternate sugars (RFOs) in source leaves and catabolism in specific sink organs may favor increases in biomass allocation and also increase yield. The alkaline $\alpha$-galactosidases are most likely responsible for digesting RFOs in the cytoplasm and may influence our ability to manipulate RFO levels in engineered plants. Metabolic engineering can be used to generate RFOs at the inception of the phloem translocation stream of Arabidopsis which transports predominantly Suc. The manipulation of the RFO catabolic pathway to metabolize the RFOs can also broaden our knowledge of the fate of the RFOs throughout the plant. To realize this broader objective of metabolic engineering to manipulate source-sink relationships, we need to better understand the expression and activity of the anabolic and catabolic enzymes in the source and sink organs respectively. I hypothesize that
metabolic engineering of RFOs in targeted tissues can favor resource partitioning to increase harvest (Figure 1.3).

**Figure 1.1.** Schematic diagram of the flow of photoassimilates from source to sink by hydrostatically generated pressure gradients. The photoassimilates (sugars, water etc) are translocated via phloem, from the source leaves that have higher hydrostatic pressure to the sink tissues that have lower hydrostatic pressure (as the sugars are utilized for growth and water diffuses back into the xylem). The sieve element-companion cell complex is abbreviated as SE-CCC in the diagram.

**Figure 1.2.** Structure of Raffinose family oligosaccharides (RFOs). This image shows raffinose, a trisaccharide and stachyose, a tetrasaccharide, in relation to Suc and repeating galactosyl units in α1-6 linkages.
Figure 1.3: Schematic diagram showing the proposed carbon partitioning in source and sink tissues of the engineered plant tissues described in this thesis. High hydrostatic pressure will be generated in the source tissues because of engineered RFO biosynthesis. This promotes flow of more photoassimilates to the sink tissues which have low hydrostatic pressure. Although not pursued in this thesis, manipulation of α-galactosidase enzymes in desired recipient sinks may further target biomass to harvested organs.
CHAPTER 2

INVESTIGATING THE ROLE OF RAFFINOSE FAMILY OLIGOSACCHARIDES IN TRANSGENIC ARABIDOPSIS PLANTS

2.1. Introduction (RFO Biosynthesis)

Raffinose family oligosaccharides (RFOs) are composed of Suc and repeating galactosyl units in α.1-6 linkages. They are nearly ubiquitous among plants, but function in diverse roles depending on the species. RFOs are known to compose almost 50% of the phloem-translocated sugar in Cucurbitaceae and Lamiaceae (Bachmann and Keller, 1995). According to the “polymer trap” model of symplastic loading, Suc diffuses from the mesophyll into intermediary cells, where it is converted to RFOs. These sugars are too large to go back through the narrow plasmodesmata to the surrounding cells and so RFOs accumulate in the phloem (Turgeon, 1996). This symplastic phloem loading of the RFOs creates a high hydrostatic pressure in the source tissue to drive the flow of photoassimilates to the sink tissues.

The most common roles of RFOs in plants include desiccation protection during seed maturation; protection against various abiotic stresses, such as salt, drought, and cold stresses; long and short term carbohydrate storage (Winter, 2007); and phloem loading in species that utilize the polymer-trap mechanism (Keller and Pharr, 1996). The RFO pathway in Arabidopsis is induced during abiotic stress conditions like cold and oxidative stress. The common bugle (Ajuga reptans) is a frost hardy, perennial that has both the short-chain as well as the long-chain RFOs. It produces, translocates, and stores RFOs and can use them as antifreeze or antisalt stress solutes (Bachmann and Keller, 1995; Gilbert, 1997; Sprenger and Keller, 2000; Inan Haab and Keller, 2002).
In the biosynthetic pathway of RFOs, galactinol \(\text{Gol; } \alpha-D\text{-galactosyl}-(1-3)-1D\text{-myo-inositol}\) is synthesized by the addition of myo-inositol to uridyldiphosphate galactose (UDP-Gal) catalyzed by the enzyme galactinol synthase (GolS; UDP-galactose:myo-inositol-galactosyl transferase), and UDP is the byproduct of this reaction. This is also the committed step of the RFO biosynthetic pathway. In the subsequent steps of the pathway, raffinose \(\text{Raf; } \alpha-D\text{-Gal}-(1-6)\alpha-D\text{-Glc}-(1-2)\beta-D\text{-Fru}\) is produced by raffinose synthase (RafS; Galactinol:sucrose galactosyl transferase) by transfer of the galactosyl group from Gol to Suc (Lehle and Tanner, 1973); myo-inositol is the leaving group from this reaction. To produce the tetrasaccharide stachyose \(\text{Sta; } \alpha-D\text{-Gal}-(1-6)\alpha-D\text{-Gal}-(1-6)\alpha-D\text{-Glc}-(1-2)\beta-D\text{-Fru}\), another galactosyl is transferred from Gol to Raf by stachyose synthase (StaS; Galactinol:raffinose galactosyl transferase); myo-inositol is again the leaving group for this reaction. Likewise, other long-chain oligosaccharides can be made by this pathway by addition of more Gol to the subsequent oligosaccharides (Figure 2.1).

While the biosynthetic pathway of Raf and Sta is well established and known to be galactinol dependent, a galactinol-independent pathway responsible for the synthesis of long-chain RFOs is described (Bachmann, 1995; Inan Haab and Keller, 2002) in which galactosyl subunits are transferred between RFOs by galactosyl transferases (Peterbauer and Richter, 2001).

In this project, Arabidopsis was metabolically engineered to produce RFOs in the phloem, after the phloem loading step, specifically in the minor veins of mature leaves of the transgenic lines. Arabidopsis is a known apoplastic phloem loader and it uses sucrose transporters (SUTs) to load sugars in the phloem (Srivastava, 2008).
Arabidopsis produces RFOs that can be detected in the phloem but in trace amounts that have little effect on the hydrostatic pressure gradient between the source and the sink. In the engineered RFO plants, higher RFOs were produced in the source leaves in the phloem and detected in the translocation stream.

In this chapter- 1) the expression of the transgenes GALACTINOL SYNTHASE1 from *Cucumis melo* (*CmGas1*), RAFFINOSE SYNTHASE from *Cucumis sativa* (*CsRFS*) and STACHYOSE SYNTHASE1 from *Alonsoa meridionalis* (*AmSTS*) were analyzed in the homozygous transgenic Arabidopsis lines GRS47 and GRS63; 2) growth characteristics of the lines were analyzed; 3) the synthesis pattern of the RFOs were established in tissue culture and in soil; and 4) transport characteristics of the RFOs were analyzed.

### 2.2. Materials and Methods

#### 2.2.1. Plasmid Constructs

The pGPTV-Hyg-CmGas1p-CmGas1 construct to express *CmGas1*, encoding GALACTINOL SYNTHASE1, was previously constructed and described (Ayre, 2003). The plasmid pGPTV-Kan-CmGas1p-CsRFS was used to express RAFFINOSE SYNTHASE in the minor veins of transgenic Arabidopsis (Cao, 2013). The plasmid pGPTV-bar-MMVE1p-AmSTS was used to express STACHYOSE SYNTHASE1 in the minor veins of transgenic plants (Cao, 2013).

#### 2.2.2. Plant Materials and Growth Conditions of Plants in Soil

Plasmid vectors harboring appropriate DNA constructs were introduced to
Agrobacterium tumefaciens by electroporation (Sambrook, 2001). Wild type Arabidopsis Colombia-0 (Col-0) was transformed by Agrobacterium tumefaciens containing constructs by using the floral dip transformation procedure (Clough and Bent, 1998). The subsequent selections were carried out by a graduate student, Te Cao, in Dr Ayre’s laboratory. In order to select the transformation of all three genes, seeds were germinated on Murashige-Skoog (MS) medium with 1% sucrose containing hygromycin B (40 mg/L), kanamycin (100 mg/L) and glufosinate ammonium (10 mg/L, all from PhytoTechnology Laboratories, Shawnee Mission, KS). Homozygous lines (CmGAS1/CmGAS1; CsRFS/CsRFS; AmSTS1/AmSTS1) were resistant to all three antibiotics and did not show any segregation. The homozygous lines were named GRS63 and GRS47 (Cao, 2013). Seedlings of the homozygous lines were transferred to soil (Metromix, Sungro Horticulture, Agawam, MA) and grown in a growth chamber under appropriate conditions for different experiments. Seeds from mature plants were harvested manually and stored in microfuge tubes with small holes in the lids in sealed desiccators with desiccant for 7 days for drying, moved to -80 °C for 3 days to kill any insect eggs, returned to the desiccators for 1 day, and then placed in a sealed seed-storage chamber with desiccant for long term storage. Seeds of GRS47, GRS63 and WT were put out on soil and after two days cold treatment at 4°C, were grown under 14 h light/10 h dark cycles at 22°C. Plants were well spaced with one plant in each cell of a 36-cell growth tray (T.O. Plastics, Clearwater, MN), so as not to impact each other’s growth. The position and orientation of each flat was changed daily to compensate for any potential microclimates in the growth chamber (AR95L Percival Scientific, Peri, IA). These plants were then used in experiments for further analyses.
2.2.3. Real Time Quantitative PCR (RT-qPCR)

RNA was extracted from the rosettes of 22-day old GRS47, GRS63 and WT lines. For each time point and treatment, RNA was collected from two biological replicates, each consisting of leaves pooled from two plants. For RNA extraction, rosettes were harvested and frozen in liquid nitrogen. RNA was extracted by Trizol using manufacturer's protocol (Life Technologies, Grand Island, NY). The isolated RNA was treated with DNase and spectrophotometrically quantified at 260 nm and subsequently used for cDNA synthesis. cDNA synthesis was performed with Superscript III Reverse Transcriptase (Life Technologies), using oligo dT as the primer, as defined in the manufacturer's protocol. The reverse transcription reaction was carried out at 50°C for 30 min in a 25 μL reaction with 600 ng of the total RNA as template (as recommended by the manufacturer). For RT-qPCR, Sybr® Green PCR Master Mix (Life Technologies) was used with the following oligonucleotides using the manufacturer's protocol. The oligonucleotides used were, GolRTb_R (5′-tctcttttttccgtgtac-3′), GolRTb_F (5′-agcccattcctcccatttac-3′), RafRTb_R (5′-ccgaaatgccacccgatgaa-3′), RafRTb_F (5′-ggtgtggtgagatgcgagta-3′), StsRTa_R (5′-cttgatcctttgctccttcg-3′), StsRTa_F (5′-ccattttgctctcccgacta-3′), EF1α_R (5′-aggtccaccaacctgactg-3′), EF1α_F (5′-gagactcgtggtgcatctca-3′). The RT-qPCR was performed on a Eco qPCR system (Illumina, San Diego, CA) with denaturation at 95°C for 5 mins followed by 95°C for 10 s and annealing at 60°C for 30 s then, extension at 72°C for 35 s. This PCR cycle was repeated 40 times. Then, the relative expressions of the transcripts were calculated (Livak and Schmittgen, 2001).
2.2.4. Growth Characteristics

Seeds of GRS47, GRS63 and WT were grown in soil according to the growth conditions previously stated. 18 days after germination, plants were photographed. Rosette surface area (cm² per plant) was determined with ImageJ version 1.38x (Rasband, 2007). Twelve plants of each line were used to establish variation in this experiment. Plants were also monitored for flowering and documented daily as the percentage of plants with a visible inflorescence. In addition, the number of rosette leaves at flowering was also recorded.

The GRS47, GRS63 and WT plants were grown in tissue culture plates with MS medium to observe the root growth. 11-day old plants were photographed and root length was measured by using ImageJ software (Rasband, 2007). Twelve plants of each line were used to establish variation in this experiment.

2.2.5  Growing Plants in Tissue Culture Plates

GRS47, GRS63 and WT plants were grown for 11, 16 and 21-days under 14 h light/ 10 h dark cycles. The light intensity was 110-150 μmol photons m⁻² s⁻¹. MS modified Basal Medium with Gamborg vitamins (PhytoTechnology Laboratories) was made according to manufacturer’s protocol. The pH was adjusted to 5.8, and 4.8 g Gelrite gellan gum (Fisher Scientific, Fair Lawn, New Jersey) was added to solidify the medium. The medium, sterilized by autoclaving and cooled to 55°C, was poured in tissue culture plates to solidify. Approximately 50 Arabidopsis seeds of each line in a 2 mL microfuge tube were surface sterilized with chlorine gas for 4 hours in a sealed jar (Martinez-Zapater and Salinas, 1998). The sterilized seeds were distributed evenly on
plates containing MS medium with 1% Suc. The seeds were placed evenly on filter papers dyed black with India ink and dipped in MS medium, in regular intervals for better root growth. Parafilm (Cole-Parmer, Vernon Hills, IL) was used to seal the plates to allow aeration and to minimize fungal infection. The plates were placed vertically at 15° angle from vertical in the growth chamber redistributed for equal exposure to light inside the growth chamber. The plants were grown on MS medium with 1% sucrose for 7 days and then transferred to fresh tissue culture plates containing only MS medium for rest of the experiment period.

2.2.6. Sugar Extraction with MCW

The samples were collected after 2 h into the light period and the fresh weight was immediately determined. The age of the samples varied according to the sets of experiments performed. The samples were either frozen in liquid nitrogen or fresh tissue was used for Methanol- chloroform-water (MCW-12:5:3 ratio) extraction. Each sample was immediately immersed in ice-cold MCW extraction solution (five volumes per unit fresh weight) containing 10 μM lactose as internal standard and extracted for 15 mins in a 50°C water bath (Srivastava, 2008). Sugars from the tissues were extracted twice and extracts combined. Water was added to the combined extract (three parts water per five parts extract) to separate the aqueous and organic phases. Sugars were extracted in the aqueous phase. After centrifugation, the aqueous phase was collected and dried down to approximately 200 μL in a lyophilizer. To collect the neutral fraction containing sugars of interest, the concentrated extracts were passed through ion exchange columns composed of, from bottom to top, 250 μL of AG1-X8 anion-exchange resin
(Bio-Rad, Hercules, CA; Formate Form), 150 μL of polyvinylpolypyrrolidone (Sigma Aldrich, St. Louis, MO), and 250 μL of AG50W-X4 cation-exchange resin (Bio-Rad, Hydrogen Form) and the columns were washed with 1 mL degassed water. Sugars were separated and quantified using a CarboPac PA-20 or MA-1 column using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale CA). The PA20 column temperature was maintained at 30°C, while the sample tray was kept at 10°C and 50 mM NaOH eluent was used along with the quadruple waveform. Glucose (Glc) and Galactose (Gal) coelute as a single peak under these conditions. To separate the Glc and the Gal peaks the samples were run with a MA-1 column. Values were normalized against lactose which was used as the internal standard (Srivastava, 2008). Calculations and statistical analyses were done using Microsoft Excel. Six plants of each line were used to establish variation in this experiment.

2.2.7. Starch Assay of Plants

Quantitative starch analysis was performed on the residual tissues after sugar extractions with a starch assay kit according to the manufacturer’s instructions (Megazyme, Bray, Ireland; amyloglucosidase/α-amylase method). To remove chloroform from MCW treated leaves, 1 mL of 80% ethanol was added in 2 mL microfuge tubes, tubes were centrifuged and the supernatant was discarded. 100 μL of 80% ethanol was added and tissues dispersed by vortexing. To each tube, 1 mL of α-amylase solution made as per manufacturer’s protocol was added and the tubes incubated at 100°C in a dry bath for 6 minutes with stirring every 2 mins for better
mixing. Then, 50 μL of amyloglucosidase was added to the supernatant and the tubes were incubated at 50°C for 30 minutes. After centrifugation at 14,000 rpm for 5 mins, ~1 mL supernatant was transferred to a clean tube and 200 μL was transferred to glass test tubes, 2 mL of GOPOD Reagent (Glucose Determination Reagent including GOPOD Reagent Buffer and GOPOD Reagent Enzymes) was added and incubated at 50°C for 20 minutes. D-Glucose controls consisted of 0.1 mL of D-glucose standard solution (1 mg/mL) and 2 mL of GOPOD Reagent. Reagent blank solutions consist of 0.1 mL of water and 2 mL of GOPOD Reagent. The absorbance for each sample was read at 510 nm.

2.2.8. Phloem Exudation Analysis with EDTA

GRS63 and GRS47 and WT were grown in soil as stated above. The rosettes from 8, 12, 16, 20 and 24-day old plants were excised at the stem (hypocotyl region) and their fresh weights were measured. The hypocotyls were cut again under 5 mM EDTA (pH 6.0) and arranged into a tube (Coulter Counter, Pittsburgh, U.S.A.) containing 1 mL of 5 mM EDTA (with 10 μM lactose as internal standard) such that only the hypocotyls of the samples were submerged. The tubes were capped to maintain near 100% humidity and minimize the amount of solution drawn into the leaves by transpiration and xylem transport. Phloem exudates from the first 20 mins were discarded because there might be some contamination from the contents of cut cells. Subsequently, exudates from each of two one-hour periods were collected in labeled coulter counter tubes. The phloem exudates were aliquoted in 2 mL microfuge tubes and lyophilized to ~ 200 μL. The exudates then were passed through ion-exchange
columns and washed with 1 mL of degassed water. Sugars were separated and quantified using a CarboPac PA-20 column using HPAEC-PAD (Dionex) according to manufacturer’s protocol. Six plants of each line were used to establish variation in this experiment.

2.2.9. Photosynthetic Labeling with $^{14}$CO$_2$

GRS47, GRS63 as well as the WT plants were grown in tissue culture plates containing MS media as stated above in preparation for photosynthetic labeling with $^{14}$CO$_2$. The labeling chamber was placed below a 400-W metal halide lamp, and the leaves were labeled by mixing 5 μL of 1 μCi/mL NaH$^{14}$CO$_3$ with 15 μL of lactic acid in the barrel of a 1 mL syringe with a 22-gauge needle extending through the side of the chamber. Labeling was for 20 mins, and the excess $^{14}$CO$_2$ was withdrawn and passed over soda lime by a vacuum pump for 5 mins and the plants were left to photosynthesize in ambient air for 10 mins. The plants were taken out of the labeling chamber, cut, weighed and sugar was extracted with MCW as per the protocol stated above. The extracted sugar was put on a TLC plate in designated lanes for further analysis.

2.2.10. TLC Plate Scan of Labeled Plants

One-dimensional chromatography on silica gel TLC plates (Analtech, Newark, DE) was carried out in saturated tanks with TLC solvent (60mL chloroform: 70 mL acetic acid: 10 mL autoclaved water) - using 2 μL representing 25 μg of unlabeled standard sugars and 1-3 mg fresh weight (FWT) of labeled samples were spotted 2 cm from the
lower edge of the plate and at least 1 cm from the lateral border. The chromatograms were developed by the ascending technique with the mobile phase (Anand, 2012). Sugars were detected at 95°C for 20 mins using vanillin as a spraying reagent over the TLC plate and photographed. Then the TLC plate was put in a folder with autoradiography film and developed for 24 h and the development was repeated for 7 days. The X-ray plates were taken out and analyzed for labeled sugars. The plate was also put under a TLC scanner (Bioscan system 200 image scanner, Bioscan, Washington, DC) to detect labeled sugars in the plate. The identification and quantification of radiolabeled sugars were performed by radiometric scanning and co-migration with unlabeled commercial sugars, Glc, Gal, Fru, Suc, Lac, Gol, Raf and Sta.

2.3. Results

2.3.1. GRS47 and GRS63 Plants Produce More RFOs Compared to the WT Plants but Other Soluble Sugars and Starch Levels are Similar in All Lines

The homozygous transgenic RFO producing plants (GRS47, GRS63) were grown in soil and analyzed for RFO sugars and compared with the WT plants. GRS47 and GRS63 lines were independently transformed and chosen as homozygous RFO producing lines. The GRS47 line had 20-fold more Gol, 10-fold more Raf and 30-fold more Sta than the WT plants (Figure 2.2.B). The GRS63 line too had more RFOs compared to the WT but less than GRS47. The WT plants had negligible amounts of Gol, Raf and Sta as expected in the sugar extracts. In comparison, the other soluble sugars Glc, Fru and Suc, as well as starch, had levels similar in all three lines (Figure 2.2.A and C) showing that the increase in the amounts of RFOs are not at the expense of other sugars in the transgenic plants.
2.3.2. Transgene Transcript Levels Correlate with the RFO Levels

The transgene transcript abundance was measured for GRS47, GRS63 and WT lines. The rosettes from GRS47, GRS63 and WT lines were used to analyze the transcript levels of the RFO genes. The results show increased levels of \textit{CmGAS1}, \textit{CsRFS} and \textit{AmSTS} in the GRS47 line compared to GRS63 (Figure 2.3.A and B). \textit{CmGAS1} relative expression in GRS47 is almost 200% more than that of \textit{EF1α} (internal standard) whereas \textit{CmGAS1} expression in GRS63 is only 0.3% of \textit{EF1α}. GRS47 had 0.3% times the expression of \textit{CsRFS} and 0.2% times the expression of \textit{AmSTS} compared to the internal standard (Figure 2.3.C and D). As expected the WT does not show any expression of the genes but GRS47 line shows substantially more expression compared to GRS63 line (Figure 2.3). The higher levels of Gol substrate and possibly higher enzyme activities in GRS47 are likely responsible for promoting the higher levels of Raf and Sta product (Cao, 2013).

2.3.3. Growth Characteristic Analyses Show No Significant Phenotypic Differences among the Lines

The growth analyses were done to observe whether there were any phenotypic differences between the lines. The plants grown on media showed roots to be slightly longer roots in GRS47 compared to that of WT and GRS63, but the difference was not statistically significant (Figure 2.4.A). The rosette area analysis showed slightly less rosette area in GRS47 plants compared to WT and GRS63 plants but again, there was no statistical significance (Figure 2.4.B). The flowering-time analysis showed early flowering in WT and GRS63 in long day conditions (14 h light/10 h dark) compared to GRS47 (Figure 2.5.A). WT and GRS63 plants flowered around 19 days whereas
GRS47 plants flowered around 22 days. The number of leaves in WT and GRS63 lines was also less compared to that in GRS47 as their flowering time was earlier than GRS47 plants (Figure 2.5.B). But overall, none of the differences in growth characteristics were significantly different between the lines.

2.3.4. RFOs are Synthesized and Accumulated in the Transgenic Plants over a Time Period

Synthesis and accumulation pattern of RFOs in plants grown in soil were also analyzed. The plants were grown on soil for 9, 13, 17, 21 and 25-days and sugar was extracted from the aerial parts with MCW and analyzed as previously stated. The levels of Glc, Fru and Suc changed between time points but did not vary significantly between GRS47, GRS63 and WT plants (Figure 2.6.A, B and C). GRS47 plants showed more RFO content than GRS63 plants at all time points, which in turn, had more RFOs than WT plants (Figure 2.6.D, E and F). The plants showed a significant peak in RFO content at the 13th day but after that time point all the sugar contents decreased when calculated relative to the fresh weight of the plants. It was clear that the transgenic plants are synthesizing more RFOs compared to WT but the RFOs are not increasing at a steady rate as the transgenic plants are growing older. This experiment was repeated and a similar sugar peak in 12-day old plants was observed (Figure S1), thus showing that the substantial peak at 13 dpg was not an artifact.

2.3.5. RFOs are Synthesized and Accumulate in the Shoots and Roots of Transgenic Plants over a Time Period

The objective of this experiment was to observe the pattern of RFO accumulation
in shoots and roots of the transgenic plants over a time period in tissue culture. GRS47, GRS63 and WT lines were analyzed for sugar content in leaves and roots by HPAEC-PAD, which can detect sugars in very low quantities. Sugar content in the aerial part (rosettes) and the roots were measured separately and it was observed that RFOs increase in the transgenic plants with time but the 11-day old plants showed more sugar content in the transgenic lines (Figure 2.8.A and B). The levels of Glc, Fru and Suc were similar in all the 3 lines (Figure 2.7.A and B). The levels of sugar varied in different time points, most sugar accumulation was observed in the 11-day old plants.

2.3.6. RFO Levels Differ in the Phloem Exudates Over a Time Course

To measure the RFOs in phloem, a phloem exudation experiment was done. This experiment was also done to observe whether the soluble sugar content (Glc, Fru, Suc, as well as Gol, Raf and Sta) along the phloem varies over a time period. The plants were grown on soil and phloem exudates were collected at 12, 16, 20 and 24-days. The plants showed higher sugar in the exudates up until day 12 (Figure 2.9) after which there was a gradual decrease. As expected for Arabidopsis, Suc is the major transport sugar in both WT and transgenic plants. RFOs were negligible in exudates from WT but were present at low levels in the exudates of transgenic plants which show that the engineered sugars are phloem mobile. GRS47 plants showed almost 2-fold more Gol in their phloem exudates compared to that in the exudates of WT plants (Figure 2.9. A and B). The amount of Raf was 4-fold more in GRS47 than that in the exudates of WT plants (Figure 2.9.A and B). Sta also showed similar 4-fold increase in GRS47 compared to that in WT (Figure 2.9. A and B). The RFO sugars were also
detected in the phloem exudates of GRS63 (Figure 2.9. C), but at a lesser amount than that in GRS47 plants. From this experiment it is clear that the transgenic plants are synthesizing more RFOs compared to WT and it is also getting transported to the sink tissues via phloem.

2.3.7. Photosynthetic Labeling of Plants with $^{14}$CO$_2$ and TLC Plate Analysis Does Not Support High Levels of RFO Synthesis and Turnover in the Transgenic Plants

Photosynthetic labeling with $^{14}$CO$_2$ was conducted to observe the flux of photosynthate through the engineered RFO pathway. This experiment was done to observe the RFO synthesis activity in 17-day old WT and the transgenic plants grown in plates. Detection of labeled RFOs would signify its synthesis. Plants were exposed to labeled $^{14}$CO$_2$ and after 20 min exposure; sugars were extracted, resolved and determined by thin layer chromatography (Figure 2.11), and relative $^{14}$CO$_2$ incorporation into each sugar determined by autoradiography. Vanillin staining caused the sugars to appear as spots in the TLC plate. The autoradiography did not show any labeled spots for the unlabeled (standard) sugar lanes but there were strong spots on the sample lanes, specifically for Suc and Glc (Figure 2.10). Labeling of Raf and Gol (co-migrating) were also detected but at much lower intensity, and only in GRS47 extracts.

To further quantify the labeling in plant extracts, the TLC plate was subjected to radiometric scanning and the labeled extracts co-migrated with the commercial sugar standards. Among the labeled extracts, Suc was the most labeled sugar with as much as 80% of $^{14}$C labeling, as expected since it is the first non-phosphorylated product of photosynthesis and hexose sugars showed as much as 30% of $^{14}$C labeling (Table 2.1). But the scan also showed some areas with labeling that corresponds to the RFO values
but it was much less than that of the Suc labeling, the highest being 2% of the total labeling. These results suggest that despite the high concentrations of RFO found in 17-day old plants, very little was created from the photoassimilate during the labeling period. This suggests that the flux rate through the engineered pathway was very low, and implies RFOs in rosettes accumulate over a longer time frame.

2.4. Discussion

_CmGAS1, CsRfS_ and _AmStS_ driven by minor-vein and companion-cell specific promoters were used to metabolically engineer the RFO pathway in the phloem of GRS47 and GRS63 plants. The sugar analysis of the transgenic plants showed RFO accumulating to as much as 50% of the soluble carbohydrate without a decrease in Suc, Glc, Fru or starch levels (Figure 2.2). Although RFO accumulated to 50% of total soluble sugars (~1.6 nmoles mg\(^{-1}\) FWT in GRS47), this is a relatively small amount when compared to the total non-structural carbohydrate content in the leaves of a plant: the starch level after 2 h into light period was similar between the lines ~7 nmoles mg\(^{-1}\) FWT. The normal cycling of carbohydrate pool is ~22 fold more than the RFOs found in the transgenic lines.

The expression levels of the _CmGAS1, CsRfS_ and _AmSTs_ genes in the transgenic as well as the WT lines were measured. These genes were not detected in WT and show lower expression in GRS63 relative to GRS47 lines (Figure 2.3.A and B). _CmGAS1_ has the strongest expression followed by that of the _CsRfS_, whereas the _AmSTs_ has the lowest expression in GRS47. The expression pattern is the same for all the genes in GRS63 but the expression level is less compared to GRS47 line (Figure
2.3). The higher levels of Gol substrate and possibly higher enzyme activities in GRS47 are likely responsible for promoting the higher levels of Raf and Sta product in this line. These results are consistent with the levels of RFO sugars observed in the GRS47 and GRS63 lines.

The rosette area, flowering time, and number of leaves at flowering as well as the root length did not vary significantly between the lines. Although the difference was not statistically significant, it was observed that the root length of GRS47 plants were slightly longer compared to GRS63 and WT plants (Figure 2.4.A) but its rosette area was slightly less compared to GRS63 and WT plants (Figure 2.4.B). The flowering time analysis of WT, GRS47 and GRS63 lines show that the flowering time of WT and GRS63 is similar whereas the GRS47 is flowering a little later than WT (Figure 2.5.A).

The time course experiments with sugar extraction (both in MS media and soil) show a change in the pattern of RFO accumulation in WT and GRS47 and GRS63 lines but the RFOs do not increase steadily as the plant grows but shows a peak at ~12 days (Figures 2.6, 2.7 and 2.8). Reasons for the peak are not clear but the peak was reproducible (Figure S1).

Since roots could not be analyzed in the soil experiment, a tissue culture experiment was done and similar results were obtained. While growing plants in the tissue culture experiment, one of the problems faced was the irregular growth of the roots over the filter paper. This was resolved by dipping the dyed filter paper in MS media (without Suc) so that the roots are better adhered to the paper and it also made it easier to transfer plants from one plate to another.
In the experimental results of phloem exudation, RFO sugars were detected in the exudates showing that the RFOs are transported in the phloem from the source to the sink tissues (Figure 2.9). Exudation rates of Glc, Fru and especially Suc in the transgenic lines were greater than those of WT. RFOs were very low but high Glc and Fru in the exudates could be due to invertase in the phloem. This experiment also showed a similar pattern of difference in RFO amounts in the exudates at different time points. At the 24th day there was less sugar in the phloem exudates possibly because the inflorescences were forming and acting as stronger sinks.

To further verify the synthesis of the RFOs, photosynthetic labeling with $^{14}$CO$_2$ was done. The developed autoradiograph showed strong labeling of Suc as expected (Figure 2.10) but the labeling of RFO sugars was very faint. The TLC plate scan also showed areas of labeled RFOs in the plate (Figure 2.11) but as with the autoradiograph they were at very low rates: the highest incorporation was only 2% of the total, whereas hexose sugars had as much as 30% of the total labeling and Suc had as much as 80% labeling. The low specific activity of the RFOs suggests that RFO synthesis and turnover are low in the transgenic plants.

GRS47 and GRS63 lines showed low rates of RFO synthesis and accumulation was slower than the growth rate of the plants: that is, RFO levels dropped gradually as fresh weight increased, which might be due to increased α-galactosidase activity in older plants. GRS47 and GRS63 lines have low amounts of RFOs in the transport stream that is unlikely to affect the osmotic gradient between source and sink tissues. There might be transport of the RFOs, to the mesophyll tissues, where it is sequestered and accumulated in inactive pools (i.e. vacuoles) over time. The metabolic engineering
strategy needs to be reassessed to be able to generate more RFOs and in turn manipulate resource partitioning between the source and the sink in the plants.

Figure 2.1: Biosynthetic pathway of RFOs in plants
Figure 2.2: Sugar components in the 22-day old rosettes of WT, GRS47 and GRS63 plants. (A) Glc/Gal, Fru and Suc (B) Gol, Raf and Sta and (C) Starch in the indicated lines. Variation is expressed as SD; n=6 sibling plants.
Figure 2.3: Transgene transcript abundance as determined by RT-qPCR, relative to EF1α expression. The expression levels of CmGAS1 in (A) WT and GRS47 and (B) WT and GRS63 (C) the expression levels of CsRFS in WT, GRS47 and GRS63 lines (D) the expression levels of AmSTS in the WT, GRS47, GRS63 lines. Variation is expressed as SD; n=6 sibling plants.
Figure 2.4: Growth characteristics analysis of WT, GRS47 and GRS63 lines. (A) root length analysis (B) rosette area analysis in the indicated lines. Variation is expressed as SD; n=12 sibling plants.

Figure 2.5: Flowering time analysis of WT, GRS47 and GRS63 lines (A) flowering time analysis and (B) number of leaves at flowering in the indicated lines. Variation is expressed as SD; n=12 sibling plants.
Figure 2.6: RFO sugar analysis in a time course experiment in soil (9, 13, 17, 21 and 25 dpg) with WT, GRS47 and GRS63 lines. (A) Glc/Gal, (B) Fru, (C) Suc, (D) Gol, (E) Raf, and (F) Sta in the indicated lines. Variation is expressed as SD; n=6 sibling plants. See supplement figure (S1) for a similar experiment conducted after a six month period.
Figure 2.7: Sugar analyses of (A) rosettes and (B) roots in a time course experiment (11, 16 and 21 dpg) in MS-media plates between WT, GRS47 and GRS63 lines. Glc/Gal, Fru and Suc levels are compared between the lines as well as between roots and rosettes. Variation is expressed as SD; n=6 sibling plants.
Figure 2.8: RFO sugar analysis of (A) rosettes and (B) roots in a time course experiment (11, 16 and 21 dpg) in MS-media plates with WT, GRS47 and GRS63 lines. Gol, Raf and Sta levels are compared between the lines as well as between roots and rosettes. Variation is expressed as SD; n=6 sibling plants.
Figure 2.9: Sugar analysis in phloem exudates in a time course experiment (12, 16, 20 and 24 dpg) in (A) WT, (B) GRS47 and (C) GRS63 lines. Variation is expressed as SD; n=6 sibling plants.
Figure 2.10: Image of labeled sugars in the autoradiograph in the photosynthetic labeling experiment with WT, GRS47 and GRS63 plants.

Figure 2.11: Image of the TLC plate with \(^{14}\text{C}\) labeled and unlabeled standard sugars. The TLC plate was stained with vanillin to visualize the spots and scanned for labeled sugars with extracts from WT, GRS47 and GRS63 lines.
Table 2.1: Radiometric scanning with representative extracts on the TLC plate.

<table>
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<th>Region:</th>
<th>Region CPM</th>
<th>% of Total</th>
<th>Rf</th>
<th>Possible Sugars</th>
<th>Std</th>
<th>Rf</th>
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<tbody>
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<td>1.61</td>
<td>0.121</td>
<td>Origin</td>
<td>Sugars Values:</td>
<td></td>
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<td>0.479</td>
<td>Sucrose</td>
<td>Raf/Gol</td>
<td>0.251</td>
</tr>
<tr>
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<td>13.02</td>
<td>0.574</td>
<td>Hexose</td>
<td>Lactose</td>
<td>0.311</td>
</tr>
<tr>
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CHAPTER 3
LINKING RFO BIOSYNTHESIS WITH THE MYO-INOSITOL PATHWAY

3.1. Introduction

In higher organisms, myo-inositol has been known to be incorporated into various pathways, as an example, the sphingolipid signaling pathway and the myo-inositol phosphate pathway. In turn, it can impact regulation of gene expression, stress tolerance, phosphorus storage and oligosaccharide synthesis (Donahue, 2010). Thus, myo-inositol synthesis impacts metabolites involved in many different and critical plant biochemical pathways.

Myo-inositol 1-phosphate synthase1 (MIPS1) is the enzyme in the rate-limiting step of myo-inositol synthesis and has a larger overall impact on myoinositol levels in plants compared to the family members MIPS2 and MIPS3. MIPS1 is required for proper growth and development in plants as well as suppression of spontaneous cell death. It has been observed that decreased myo-inositol leading to decreased phosphatidylinositol levels in mips1 mutants correlate with elevated levels of a family of waxy lipid molecules made up sphingosine and a fatty acid known as ceramides in the plants. Ceramides are involved in programmed eventually leading to cell death (Donahue, 2010). The expression of MIPS1 is mostly constitutive and higher in all types of tissues compared to MIPS2 and MIPS3 except in roots and seeds. The higher overall expression of MIPS1 in almost all cell types suggests that it controls most of the myo-inositol synthesis in plants. It was also reported that the levels of Gol decreased in mips1 mutants (Donahue, 2010). Thus, the role of myo-inositol in galactinol synthesis was explored to link the biosynthesis of myo-inositol to the RFO biosynthetic pathway.
To increase the amounts of RFO in the transgenic lines, the *MIPS1* overexpressing plants (with *MIPS1* driven by 35S promoter) were obtained as a gift from Dr Glenda Gillaspy (Virginia Tech, VA) and had almost 4.5-fold more myo-inositol compared to WT plants (Donahue, 2010). The *MIPS1* plants were crossed with the GRS47 and GRS63 plants. This rationale was that the overproduction of myo-inositol may drive greater biosynthesis of RFOs (Figure 3.1) in the crossed MIPS1 x GRS47 and MIPS1 x GRS63 plants.

The biosynthetic pathway linking the synthesis of RFOs and myo-inositol is shown in Figure 3.1. The rate-limiting step of myo-inositol synthesis is catalyzed by L-myoinositol 1-phosphate synthase (*MIPS1*) (Loewus, 1980, 1984). This reaction is followed by dephosphorylation of 1D-myoinositol 6-phosphate to myo-inositol, which is catalyzed by the myo-inositol monophosphatase (*IMP*). These two reactions together are known as the Loewus pathway, in plants it is the only known route for myo-inositol synthesis.

In this chapter- 1) transgenic plants over expressing the upstream genes myo-inositol 1-phosphate synthase1 (*MIPS1*) were crossed with the GRS47 and GRS63 lines; and 2) the crossed RFO and MIPS1 (MIPS1 x GRS47 and MIPS1 x GRS63) lines were analyzed for RFO sugar composition and growth characteristics.

### 3.2. Materials and Methods

#### 3.2.1. Crossing RFO Plants with the MIPS1 Plants

GRS47, GRS63 and MIPS1 (Donahue, 2010) were grown in soil in the growth chambers with conditions stated previously. The plants were monitored for flowering...
and when the inflorescence was approximately 15 cm long, they were crossed. GRS47 and GRS63 plants were used as the maternal plants and the MIPS1 plants were used as pollen donors. The crossed flowers were marked with colored tape and other flowers were cut off to minimize chances of contamination of crossed and non-crossed seeds. Seeds were collected from the crossed siliques only. The plants grown from these seeds were genotyped to confirm crossed MIPS1 x GRS47 and MIPS1 x GRS63 plants. Genotyping was done with mips1-2 oligonucleotides (Donahue, 2010) to confirm the crossing between the lines using Phire Plant Direct PCR kit (Thermo Scientific, Sunnyvale, CA) according to the manufacturer’s protocol. Touchdown PCR was done using the following PCR conditions: denaturation at 98°C for 5 mins followed by another short denaturation at 98°C for 5 s and annealing at 72°C for 15 s then extension at 72°C for 25 s. This was done for 12 cycles with a 1°C drop in the annealing temperature in each cycle. It was followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 65°C for 15 s, and extension at 72°C for 25 s and a final extension at 72°C for 30 s.

3.2.2. Growth Characteristics Analysis of Crossed and WT Plants

Flowering time, number of leaves at flowering for all the lines were analyzed according to previously stated protocol.

3.2.3. Sugar and Starch Analysis of Crossed and WT Plants

The sugars from all crossed and WT lines were extracted with MCW and analyzed in HPAEC-PAD with PA-20 and MA-1 columns for separation of peaks.
according to the previously stated protocol. Starch analysis was also done according to the protocol described above.

3.3. Results

3.3.1. RFO Transgenic Plants were Crossed with MIPS1 Transgenic Plants to Generate MIPS1 x GRS47 and MIPS1 x GRS63 Plants

The plants were crossed, harvested and genotyped to show successful crossing (Figure 3.2). The successfully crossed plants expressed the MIPS1 gene and were designated MIPS1 x GRS47 and MIPS1 x GRS63. The presence of genomic MIPS gene (~1800bp band, Figure 3.2) was observed in all the lines whereas the MIPS1 cDNA was observed only in the crossed lines (~1539bp band, Figure 3.2) (Donahue, 2010). Another band (~1700bp, Figure 3.2) was also observed in the MIPS and the crossed lines which could be due to non-specific binding of the primers. Since each gene was segregating independently, crossed created hemizygous lines, which were used rather than homozygous lines. The crossed lines (MIPS1 x GRS47 and MIPS1 x GRS63) were hemizygous for all the 4 genes (CmGAS1, CsRFS, AmSTS and MIPS1) in the F1 generation and were used for further analysis.

3.3.2. Growth Analysis Does Not Show Any Significant Difference Between the MIPS1 x GRS47, MIPS1 x GRS63 and WT Plants

The crossed F1 plants were grown in soil to analyze their growth characteristics. The flowering time did not vary significantly between the crossed lines, WT, GRS47, GRS63 and the MIPS1 lines (Figure 3.3.A). But, it was observed that the flowering time of the MIPS1 x GRS47 and MIPS1 x GRS63 were similar to that of WT but they
flowered early compared to the MIPS1 or the GRS47 lines, even though the difference is not statistically significant (p > 0.5 in t-test). The number of leaves at flowering showed the crossed lines had a similar number of leaves as the WT plants (Figure 3.3.B).

3.3.3. Sugar and Starch Analysis Show Higher Myo-inositol But Not High RFO Levels in the MIPS1 x GRS47 and MIPS1 x GRS63 Plants

Sugar analysis with MCW showed more myo-inositol in the crossed plants but not significant increases in their RFO content (Figure 3.4). The sugar analysis was done by HPAEC-PAD with both the PA20 column and the MA1 column: myo-inositol and Gol were resolved with the MA-1 column for better separation of peaks while the other sugars were resolved with the PA-20 column. The Glc, Fru, Suc levels of the MIPS1 and MIPS1 x GRS47, MIPS1 x GRS63 lines were similar but somewhat less compared to that in the WT, GRS47 and GRS63 lines (Figure 3.4.A). The MIPS1 plants have almost 4-fold more myo-inositol than GRS47 and GRS63 plants (Figure 3.4B). The amounts of myo-inositol was highest in the MIPS1 plants followed by MIPS1 x GRS47 and MIPS1 x GRS63 plants but the amounts of RFO sugars did not increase in the MIPS1 x GRS47 compared to the GRS47 line (Figure 3.4.B). The MIPS1 x GRS63 line showed more RFOs (Gol, Raf, and Sta) compared to the GRS63 line, though other soluble sugars (Glc, Fru, Suc) did not vary significantly (Figure 3.4.A and B). The level of starch in all the lines was also similar (Figure 3.4.C). The overall increase in the quantity of myo-inositol did not increase in the RFO levels in the crossed lines.
3.4. Discussion

The MIPS1 lines are over expressing *MIPS1* that increases the levels of myo-inositol in the transgenic plants. Since myo-inositol is upstream in the RFO biosynthetic pathway (Figure 3.1), the RFO plants were crossed with the MIPS1 plants (Figure 3.2). Though the crossed plants had more myo-inositol, they did not show increase in the RFOs, probably because of the low expression level of the downstream genes (*CsRFS* and *AmSTS*) that produces Raf and Sta in the RFO biosynthetic pathway. The RFO genes and MIPS1 could be separated spatially, which could be a reason of low RFO levels in the crossed lines. Also, increases in only myo-inositol as a substrate and not UDP-Gal, might be another limiting factor in the biosynthetic pathway of the RFOs. Increasing the availability of UDP-Gal may enhance the overall RFO produced in the crossed plants. Analysis of the crossed lines showed that the other sugars (Glc, Fru and Suc) did not vary significantly between the lines (Figure 3.4). Also, the increased myo-inositol did not affect the phenotype of the plants nor did it affect the flowering time of the crossed lines (Figure 3.3). The MIPS1 x GRS47 and the MIPS1 x GRS63 lines flowered a little earlier but the difference was not statistically significant.

The committed step of the RFO biosynthetic pathway is catalyzed by galactinol synthase (GolS) which synthesizes Gol from UDP-D-galactose and myo-inositol (Keller and Pharr, 1996), so insufficient GolS activity even in the presence of higher myo-inositol will not enhance the downstream products (Karner, U.*et al*, 2004). There is complex regulation of downstream pathways and processes impacted by myo-inositol synthesis, including cross talk with other pathways resulting in various types of
regulation including feedback regulations which might also impact the RFO biosynthetic pathway.

**Figure 3.1**: Biosynthetic pathway linking the synthesis of RFOs and myo-inositol. D-glucose-6-phosphate is converted to 1D-myoinositol-6-phosphate by *myo-inositol 1-phosphate synthase* (*MIPS1*). The subsequent dephosphorylation step is catalyzed by *inositol mono phosphatase* (*IMP*) to produce myo-inositol. Myo-inositol can be used in various pathways including the first step of RFO biosynthesis to produce Gol, along with UDP-Gal.

**Figure 3.2**: PCR genotyping image of WT, GRS47, GRS63, MIPS1, MIPS1 x GRS47 and MIPS1 x GRS63 lines. Negative control reaction is represented as NC in the image. Expected bands: WT=1800 bp and MIPS1=1539 bp.
Figure 3.3: Flowering time analysis of WT, GRS47, GRS63, MIPS1, MIPS1 x GRS47 and MIPS1 x GRS63 lines. (A) flowering time analysis and (B) number of leaves at flowering in the indicated lines. Variation is expressed as SD; n=6 sibling plants.
Figure 3.4: Sugar components in the WT, GRS47, GRS63, MIPS1, MIPS1 x GRS47 and MIPS1 x GRS63 lines. (A) Glc, Fru, Suc and (B) Myo-inositol, Gol, Raf, Sta and (C) Starch levels in the indicated lines. Variation is expressed as SD; n=6 sibling plants.
CHAPTER 4

INVESTIGATING THE ROLE OF \( \alpha \)-GALACTOSIDASES IN THE DEGRADATION OF RAFFINOSE FAMILY OLIGOSACCHARIDES

4.1. Introduction (RFO Degradation)

The alkaline \( \alpha \)-galactosidases (\( AtSIP1 \) and \( AtSIP2 \) in Arabidopsis) are most likely responsible for digesting RFOs in the cytoplasm and may influence our ability to manipulate RFO levels in engineered plants. Alkaline and acidic \( \alpha \)-galactosidases are homologous to seed imbibition proteins (SIPs), and are predominately expressed during germination (Carmi, 2003, Dai, 2006). It has been previously reported that in cucurbits the metabolism of the translocated RFOs are caused by \( \alpha \)-galactosidases that are active at neutral or slightly alkaline pH, similar to that of melon (Gao, 1999).

RFO synthesis is reasonably well described, but our understanding of the degradation is underdeveloped. RFOs are digested by \( \alpha \)-galactosidases to Suc and Gal. Gal is metabolized to Gal-1-P by galactokinase. Gal-1-P can be digested by two different pathways, the Lelior pathway and an alternate pathway used by plants (Figure 4.1). In the Lelior pathway, Glc-1-P is produced from Gal-1-P by hexose-1-P uridylytransferase with UDP transfer from UDP-Glc to Gal-1-P. However in the alternate plant pathway, UDP-Gal and PPi are produced from Gal-1-P and UTP by a UDP-galactose pyrophosphorylase enzyme. Then, UDP-Gal is converted to UDP-Glc, by UDP-4-glucose epimerase (Keller and Pharr, 1996). The Suc can be further digested to Glc and Fru by invertase and to Fru and UDP-Glc by sucrose synthase. The monosaccharides (Glc, Fru and UDP-Glc) can be used in other metabolic pathways (Figure 4.1).
The α-galactosidase gene is known to impact the freezing tolerance in petunia and is expected to do the same in other crops (Pennycooke, 2003). The expression of the AtSIP1 gene increases during abiotic stresses like osmotic and heat stress, especially in the roots. High expression of AtSIP1 is also seen in roots during selenium stress (Van Hoewky, 2008). It is mostly expressed in the siliques and imbibing seeds and roots (Schimdt, 2005). The expression levels of AtSIP2 on the other hand could be seen in rosette leaves, dry seeds, as well as flowering petals (Winter, 2007). In abiotic stress, especially in osmotic stress, AtSIP2 expression is very high during the first 24 hours.

We propose that biomass can be targeted to desired organs by engineering RFOs in the phloem of source leaves and engineering their catabolism in specific sink organs (Figure 1.3). To realize this broader objective, we need to better understand the expression and activity of AtSIP1 and AtSIP2 in the endogenous catabolic pathway. We are therefore characterizing the RFO metabolism in single and double knockout mutations of AtSIP1 and AtSIP2. In my project: 1) morphological and physiological traits of the homozygous α-galactosidases single knockout plants (Atsip1 and Atsip2) were observed and analyzed; 2) α-galactosidase double knockout plants (Atsip1/2) were generated by crossing the α-galactosidase single knockout lines; 3) homozygous α-galactosidases double knockout plants (Atsip1/2) were selected; and 4) RFOs in the seeds of the transgenic plants as well as in the full grown plants were analyzed.
4.2. Materials and Methods

4.2.1. Plant Materials

The plant materials were identified in the SALK database and obtained from ABRC to select WT$_{SIP1}$ (wild type AtSIP1- At1G55470), Atsip1 (AtSIP1 knockout), WT$_{SIP2}$ (wild type AtSIP2- At3G57520) and Atsip2 (AtSIP2 knockout), plants. The plant materials received from ABRC are listed as follows: SALK_090247, CS851185, SALK_095632 were the Atsip1 mutant segregating lines and SALK_113663, CS834975, CS366658 and CS26474 were the Atsip2 mutant segregating lines. All of the seeds were of Colombia-0 ecotype except CS26474 which was Landsberg-Erecta ecotype. The seeds from these lines were planted in soil and grown as previously described.

4.2.2. Genotyping the SALK Lines

The seedlings were genotyped and WT$_{SIP1}$, Atsip1 (CS851185), WT$_{SIP2}$ and Atsip2 (CS366658) lines were identified and used for further analysis. The genotyping was done with the oligonucleotides: SIP1_R (5’-agtaacaatgaccgttggtgc-3’), SIP1_F (5’-atctggcatctgaacacaacc-3’), SIP2_R (5’-agtcctgacacttgcgtatcc-3’), SIP2_F (5’-gataaggcggtgaaactagc-3’), LB_SIP1 (5’-aacgtccgcaatgtttatagttg-3’) and LB_SIP2 (5’-atatggccatcttatagctgtgc-3’). The Phire Plant Direct PCR kit (Thermo Scientific, Sunnyvale, CA) was used according to manufacturer’s protocol and touchdown PCR was done using the following PCR conditions: Denaturation at 98°C for 5 mins followed by short denaturation at 98°C for 5 s and annealing at 65°C for 15 s, and extension at 72°C for 25 s. This was done for 12 cycles with a drop of 1°C in the annealing
temperature each cycle. It was followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 65°C for 15 s, and extension at 72°C for 25 s, and a final extension at 72°C for 1 min.

4.2.3. Crossing of \textit{Atsip1} and \textit{Atsip2} Plants and Selection of \textit{Atsip1/2} Plants

\textit{Atsip1} and \textit{Atsip2} as well as the respective WT plants were grown in soil according to the previously stated protocol. The plants were monitored for flowering, and when the inflorescences were approximately 15 cm long, crosses were made. In one set of crosses, the \textit{Atsip1} plants were used as mother plants and the \textit{Atsip2} plants were used as pollen donors and in another set, the \textit{Atsip2} plants were used as mother plants and the \textit{Atsip1} plants were used as pollen donors. The plants grown from these seeds were genotyped with the above listed oligonucleotides and PCR conditions to confirm successful crossing of \textit{Atsip1} with \textit{Atsip2} plants. The F1 generations of seeds were heterozygous for \textit{AtSIP1} and \textit{AtSIP2}. The F1 seeds were then taken to the F2 generation and were PCR genotyped plants with homozygous knockouts for both genes and were designated \textit{Atsip1/2}. These plants were then used in experiments for further analyses.

4.2.4. RNA Extraction and Semi-quantitative RT-PCR

Total RNA was isolated from the rosette using Trizol (Life technologies), according to the manufacturer’s instructions. Total RNA samples were treated with RNase-free DNasel and purified again with Trizol. A total of 600 ng of total RNA was reverse transcribed in the presence of RNase-OUT RNAase inhibitor (Promega
Corporation, Madison, WI) with oligo dT primers and SuperScript III reverse transcriptase (Life technologies,) according to the manufacturer’s protocol. For semiquantitative PCR, 1 μL of cDNA was amplified with 200 μM of each dNTP (dATP, dTTP, dCTP, dGTP) and 10 μM of each oligonucleotide and REDTaq Genomic DNA Polymerase (Sigma Aldrich, St. Louis, MO) and supplied buffer in a 25 μL reaction. Transcript abundance was relative to ubiquitin10 (UbQ), using oligonucleotides UBQ1 and UBQ2. (Weigel and Glazebrook, 2002). The oligonucleotides used were: UBQ1 (5’-gatctttgccggaaaacaattggaggatggt-3’), UBQ2 (5’- cgacttgtcattagaagaagagataacagg-3’), SIP1_Rev (5’-cacaatggctctccagaat-3’), SIP1_Fwd (5’-ctgggaccatttctggttgt-3’), SIP2_Rev (5’-gaacatttcctccctcgtca-3’) and SIP2_Fwd (5’-cgttgcacaataggacct-3’). The PCR was done with denaturation at 94°C for 5 mins followed by short denaturation at 94°C for 30 s and annealing 60°C for 45 s, then elongation at 72°C for 60 s. The PCR cycle was repeated 40 times.

4.2.5. Seed Germination Analysis in SIP Lines

The seeds of WT<sub>SIP1</sub>, Atsip1, WT<sub>SIP2</sub>, Atsip2 and Atsip1/2 lines were harvested at the same time. These seeds were put out on tissue culture plates with MS medium as per the protocol previously stated. The plates were observed every day for 3 days after putting in the growth chamber and the seed germination in each plate was observed and recorded for analysis.

4.2.6. Sugar Analysis during Seed Germination

Approximately 50 seeds of WT<sub>SIP1</sub>, Atsip1, WT<sub>SIP2</sub>, Atsip2 and Atsip1/2 lines were harvested at the same time. These seeds were put out on tissue culture plates with MS medium as per the protocol previously stated. The plates were observed every day for 3 days after putting in the growth chamber and the seed germination in each plate was observed and recorded for analysis.
put in 2 mL microfuge tubes with a hole on the lid. 50 μL of autoclaved water was added to each tube to aid in seed germination. These tubes were kept in the cold chamber at 4°C for approximately 48 h and then put in growth chamber with optimal light conditions for 5 h. The tubes were then covered with aluminum foil and kept in dark to prevent photosynthesis. Sugar extraction from the seeds was done with MCW at 0 h (after the 5 hr light treatment), 24 h, 48 h and 72 h as per previously stated protocol. The sugar extract was analyzed for different sugar components in the HPAEC-PAD using PA20 column.

4.2.7. Growth Characteristics Analysis

Seeds of all the lines were put out on soil and grown in growth chambers as previously stated. The plants were monitored for rosette area, flowering time and number of leaves at flowering according to the protocols previously stated. The plants were grown in MS medium in tissue culture plates to observe root length as per the protocols previously described.

4.2.8. RFO Sugar Analysis

The WT_{SIP1}, Atsip1, WT_{SIP2}, Atsip2 and Atsip1/2 plants were grown in soil and sugar was extracted with MCW using the previously stated protocol and quantified using a CarboPac PA-20 column using HPAEC-PAD as per protocol described before. Six plants of each line were used to establish variation in this experiment.
4.3. Results

4.3.1. *Atsip1* and *Atsip2* Lines were Genotyped and Selected

To identify homozygous *AtSIP1* (At1G55470) and *AtSIP2* (At3G57520) knockout lines, the plant materials were ordered from the SALK database. These were segregating lines which were genotyped to identify the WT, heterozygous and homozygous knockout lines. The CS851185 (*Atsip1*) and CS366658 (*Atsip2*) lines were chosen because of the T-DNA insertion in the exons were likely to be the most effective knockout of the *AtSIP1* and *AtSIP2* genes. The PCR bands resulting from genotyping were cloned into PGEM-T Easy vector according to manufacturer’s protocol (Promega) and sequenced to confirm the insertion of the T-DNA. The *Atsip1* line had an insertion in the 2\(^{nd}\) exon (Figure 4.2) whereas the *Atsip2* line had an insertion in the 5\(^{th}\) exon (Figure 4.4). The T-DNA insertion bands of *Atsip1* and the *Atsip2* lines can be clearly identified in the PCR genotyping images (Figure 4.3 and 4.5 respectively).

4.3.2. *Atsip1* and *Atsip2* were Crossed to Generate *Atsip1/2* Double Knockout Line

The homozygous *Atsip1* plants and the homozygous *Atsip2* plants were crossed to obtain double knockout lines for both *AtSIP1* and *AtSIP2* genes. The seeds obtained from the crossed lines were put out and the seedlings were genotyped. The F1 generations of crossed plants were all heterozygous for *Atsip1* and *Atsip2* genes. The segregating F2 generation seedlings were genotyped and ~4% of the plants were homozygous knockout for both *AtSIP1* and *AtSIP2* and were designated *Atsip1/2*. The selected line when genotyped, showed T-DNA insertion bands of both *Atsip1* and *Atsip2*.
lines (Figure 4.6) and no bands corresponding to the intact WT gene. This line was grown in soil for further experiments and analysis.

4.3.3. Generation of *Atsip1, Atsip2 and Atsip1/2* Lines were Confirmed by Semi-Quantitative RT-PCR

The objective of this experiment was to verify the absence of *AtSIP1* and *AtSIP2* transcript in the homozygous *Atsip1/2* as well as *Atsip1* and *Atsip2* lines. RNA was extracted from the rosettes of WT*$_{SIP1}$, Atsip1, WT*$_{SIP2}$, Atsip2* and *Atsip1/2* lines and semi quantitative RT-PCR showed no expression of the *AtSIP1* and *AtSIP2* genes in the double knockout lines. The *Atsip1* line showed a faint band (Figure 4.7.A) in the gel image corresponding to the *AtSIP1* band which most likely was a overflow from the adjacent WT well but no band for *AtSIP2* in the *Atsip2* line was observed (Figure 4.7.B). The *Atsip1/2* line did not show any *AtSIP1* or *AtSIP2* transcript expression in the agarose gel electrophoresis image thus showing the absence of both the genes (Figure 4.7A and B). Ubiquitin (UbQ) was used as an internal control to show that the PCR was working effectively.

4.3.4. The Pattern of Seed Germination Shows Delay in the *Atsip1/2* Line

The high expression of *AtSIP1* and *AtSIP2* was observed during seed germination in plants. Inhibiting $\alpha$-galactosidases activity is known to delay in seed germination (Carmi, 2003). Therefore the absence of both *AtSIP1* and *AtSIP2* expression in the *Atsip1/2* line may result in reduced RFO degradation during seed germination as both the $\alpha$-galactosidases responsible for metabolizing the RFOs are absent in this line. To test for the evidence of reduced RFO degradation during
germination, seed germination pattern between the same aged seeds of different SIP lines were observed. The observation was taken every 24 h for 3 days and the data was recorded and analyzed. The single and the double knockout lines germinated later than the WT plants in initial period of germination (Figure 4.8). The Atsip1 (86% germination), Atsip2 (83% germination) as well as the Atsip1/2 (77% germination) have slower germination rates compared to the respective WT plants in the first 48 h during seed germination. However, the single knockout lines, germination percentage was equal to that of their WT counterparts at 72 h, whereas the double knockout line germination rate was not. This data shows that the absence of RFO degrading genes have greater effect when both the AtSIP1 and the AtSIP2 were absent.

4.3.5. RFO Degradation During Seed Germination Analysis Shows Higher RFO in Atsip1/2 Line

The seed germination percentage shows a distinct difference between the SIP lines, so, sugar extraction with MCW was done to quantify the sugars in the germinating same aged seeds. The WT<sub>SIP1</sub>, Atsip1, WT<sub>SIP2</sub>, Atsip2 and Atsip1/2 lines were analyzed for RFO levels during the first 72 h of seed germination and it was observed that the Atsip1, Atsip2 and the Atsip1/2 had more RFOs in the seeds compared to their WT seeds (Figure 4.9.B, D, F and H). At 0 h into the experiment all the lines have similar amounts of RFOs in the seeds (Figure 4.9.B). But after that, especially in the first 48 h of germination, it was seen that the WT<sub>SIP1</sub> and WT<sub>SIP2</sub> plants had less RFOs, presumably because of the α-galactosidase (AtSIP1 and AtSIP2) activity, compared to the single and double knockout SIP lines (Figure 4.9.F). The level of RFOs are highest in the Atsip1/2 line showing that the absence of both the AtSIP1 and AtSIP2 genes have
greater effect on RFO levels than the single knockout lines (Figure 4.9.B, D, F and H).
The levels of other sugars (Glc/Gal, Fru and Suc) did not vary between the lines (Figure 4.9.A, C, E and G).

4.3.6. Growth Characteristic Analysis Does Not Show Significant Differences in the Phenotype between the SIP Lines

The WT Sip1, Atsip1, WTSip2, Atsip2 and Atsip1/2 lines were analyzed for growth characteristics as well. Growth analyses were done to observe for any phenotypic differences between the lines. None of the lines showed any statistically significant difference for the growth characteristics measured. Plants grown on media showed slightly longer roots in lines Atsip1, Atsip2 and Atsip1/2 compared to that of the WTSip1 and WTSip2 lines, but the differences were not significant (Figure 4.10.A). The rosette area analysis showed slightly less rosette area in Atsip1, Atsip2 and Atsip1/2 plants compared to their respective the WT Sip1 and WTSip2 lines but again the differences were not significant between the lines (Figure 4.10.B).

The flowering time analysis shows an early flowering phenotype in the WTSip1 and WTSip2 in long day conditions (14 h light/ 10 h dark) compared to the Atsip1, Atsip2 and Atsip1/2 plants (Figure 4.11.A). The number of leaves at flowering followed similar pattern as the flowering time among the lines, so less leaves in WT lines compared to the knockout lines (Figure 4.11.B). The overall growth characteristics analysis shows that the SIP lines (WTs, single knockouts and the double knockout) do not have significant difference between them, so the initial delay in seed germination in the knockout lines do not seem to have an effect on the overall growth of the plants.
4.3.7. Sugar Analysis Show Higher RFO in *Atsip1/2* Line

The WT<sub>SIP1</sub>, *Atsip1*, WT<sub>SIP2</sub>, *Atsip2* and *Atsip1/2* lines were analyzed for difference in sugar, especially RFO sugar, levels in 21-day old seedlings. The lines show similar amounts of soluble sugars (Glc, Fru and Suc) though there is less in the single knockout lines (*Atsip1*, *Atsip2*) compared to their respective wild types (WT<sub>SIP1</sub> and WT<sub>SIP2</sub>) (Figure 4.12.A). The level of starch also did not vary significantly between the lines (Figure 4.12.C). But, the results also show 3-10 fold more RFOs (Gol, Raf, Sta) in the *Atsip1/2* line compared to other lines (Figure 4.12.B). The *Atsip1* and the *Atsip2* lines also showed 1-2 folds more RFO sugars compared to their respective WT lines but the difference was not statistically significant. The presence of more RFOs in the *Atsip1/2* line implies that alkaline α-galactosidases are required for RFO degradation and that RFO sugars accumulate in the absence of this activity.

4.4. Discussion

The WT<sub>SIP1</sub>, *Atsip1*, WT<sub>SIP2</sub>, *Atsip2* lines were selected as a way to test the role of α-galactosidases activity on the RFO content of the plants through its catabolic pathway. Genotyping of CS851185 lines showed segregation for the *AtSIP1* gene and led to WT<sub>SIP1</sub> as well as *Atsip1* lines. Cloning and sequencing showed the insertion of the T-DNA in the 2<sup>nd</sup> exon (Figure 4.2 and 4.3). Genotyping of CS366658 line similarly showed segregation of *AtSIP2* gene and was used to identify WT<sub>SIP2</sub> as well as *Atsip2* lines. Cloning and sequencing showed the insertion of the T-DNA in the 5<sup>th</sup> exon (Figure 4.4 and 4.5). The *Atsip1* and the *Atsip2* lines were crossed and the homozygous double knockout *Atsip1/2* line was selected among the F2 progeny (Figure 4.6). The absence
of both AtSIP1 and AtSIP2 transcript expression in the Atsip1/2 line shows that it is a homozygous double knockout for both the genes (Figure 4.7A and B). WT_{SIP1}, WT_{SIP2} both were used as WTs for Atsip1 and Atsip2 respectively because the single knockout lines were chosen from different batches of seeds and so were compared to their respective WTs, even though both were Col-0 ecotype.

The alkaline α-galactosidases are previously known to act mostly in the seeds during germination. As expected, the seed germination percentage and the RFO degradation observations during seed germination showed marked difference among the SIP lines. There were visible differences in seed germination percentage in the Atsip1 and Atsip2 lines compared to their respective wild type plants but seed germination in the Atsip1/2 line was most reduced (Figure 4.8). Also, as expected, the Atsip1/2 line showed higher amounts of RFOs in the seeds compared to the WT and the single knockout lines at 24, 48 as well as 72 h (Figure 4.9). This data also correlates with the seed germination pattern. Thus it seems that the absence of alkaline α-galactosidases, or alternatively the presence of more RFOs in the seeds, cause delays its germination during the period of early seed germination.

The plants were analyzed for root length, rosette area, flowering time as well as number of leaves at flowering and it was observed that the absence of the AtSIP1 and AtSIP2 did not affect the overall growth and phenotype of the plants except during seed germination. There were minor differences like longer root length, smaller rosette area and delayed flowering in the Atsip1/2 line but all these differences were not statistically significant. It is surmised that the absence of the alkaline α-galactosidases do not have any visible or significant phenotypic effect on older plants. This is probably because the
AtSIP1 and AtSIP2 are mostly expressed in the seeds and are known to function during seed germination period and during mostly abiotic stress conditions (Gilbert, 1997). RFOs accumulate in plants under various stress conditions and the α-galactosidases are known to be active during the subsequent RFO degradation after the stress is eliminated (Van Hoewyk, 2008). The sugar extractions from the Atsip1/2 line show more RFOs in these plants as well (Figure 4.12.B). The WT_SIP1 and Atsip1 lines did not show much difference in the soluble sugar components (Glc, Fru and Suc) or the RFO sugar components (Gol, Raf and Sta). A similar trend was observed in the WT_SIP2 and Atsip2 lines. In Atsip1/2 there was not much difference in Glc, Fru and Suc levels from the other lines (Figure 4.12A), but it had almost 10-fold more Gol, 3-fold more Raf and 3-fold more Sta compared to the WT line. It is thus concluded that absence of both alkaline α-galactosidases maybe more effective in promoting the accumulation of RFOs in mature plants. It is noteworthy that more RFOs do not result in a visible phenotype in these Atsip1/2 plants, consistent with similar observations in the RFO producing transgenic Arabidopsis lines (GRS47 and GRS63). The seeds of the Atsip1/2 line need to be analyzed more for any phenotypic difference. These experiments with Atsip1/2 plants argue that combining RFO synthesis with reduced α-galactosidases activity would be a worthwhile future endeavor to more effectively modulate RFO anabolism and catabolism in specific target organs.
**Figure 4.1:** Catabolic pathway of RFOs showing the Lelior pathway and the alternate pathway used by plants.

**Figure 4.2:** Schematic diagram of α-galactosidase knockout Atsip1 line showing the T-DNA insertion in the 2nd exon. The black arrows indicate positions of gene specific primers, orange arrow indicates the position of T-DNA primers and green arrows indicate position of RT-PCR primers. The schematic diagram is not to scale.
**Figure 4.3:** PCR genotyping image of Atsip1 line Lane 1=DNA ladder, Lane 2= WT\textsubscript{SIP1} DNA probed with SIP1 and LB\_SIP1 oligos (962 bp); Lane 3=Atsip1 DNA probed with SIP1 and LB\_SIP1 oligos (600 bp).

**Figure 4.4:** Schematic diagram of α-galactosidase knockout Atsip2 line showing the T-DNA insertion in the 5\textsuperscript{th} exon. The black arrows indicate positions of gene specific primers, orange arrow indicates the position of T-DNA primers and green arrows indicate position of RT-PCR primers. The schematic diagram is not to scale.
Figure 4.5: PCR genotyping image of Atsip2 line. Lane 1=DNA ladder, Lane 2=WT<sub>SIP2</sub> DNA probed with SIP2 and LB_SIP2 oligos (1014 bp); Lane 3=Atsip2 DNA probed with SIP2 and LB_SIP2 oligos (500 bp).

Figure 4.6: PCR genotyping image Atsip1/2 line. Expected bands: Lane 1=DNA ladder, Lane 2=Atsip1/2 DNA probed with SIP1 and LB_SIP1 oligos (600 bp); Lane 3=Atsip1/2 DNA probed with SIP2 and LB_SIP2 oligos (500 bp). Both the Atsip1 and Atsip2 T-DNA insertion bands were observed in the Atsip1/2 line.
**Figure 4.7**: RT-PCR images of WT$_{SIP1}$, Atsip1, WT$_{SIP2}$, Atsip2 and Atsip1/2 lines. UBQ10 (UbQ) was used as an internal control. The Atsip1/2 line did not show the expression of AtSIP1 (A) as well as the AtSIP2 (B) gene.

**Figure 4.8**: Seed germination percentage in WT$_{SIP1}$, Atsip1, WT$_{SIP2}$, Atsip2, and Atsip1/2 lines.
Figure 4.9: Sugar degradation in WT_{SIP1}, At_{sip1}, WT_{SIP2}, At_{sip2} and At_{sip1/2} lines at (A, B) 0 h, (C, D) 24 h, (E, F) 48 h and (G, H) 72 h after seed germination. A, C, E and G shows Glc, Fru and Suc measurements and B, D, F and H shows Gol, Raf and Sta measurements in the indicated lines. Variation is expressed as SD; n=6 sibling plants.
Figure 4.10: Growth characteristics analysis in WT<sub>SIP1</sub>, Atsip1, WT<sub>SIP2</sub>, Atsip2 and Atsip1/2 lines. (A) root length analysis and (B) rosette area analysis of the indicated lines. Variation is expressed as SD; n=12 sibling plants.

Figure 4.11: Flowering time analysis of WT<sub>SIP1</sub>, Atsip1, WT<sub>SIP2</sub>, Atsip2 and Atsip1/2 lines (A) flowering time analysis and (B) number of leaves at flowering analysis of the indicated lines. Variation is expressed as SD; n=12 sibling plants.
Figure 4.12: Sugar component analysis in WT$_{SIP1}$, Atsip1, WT$_{SIP2}$, Atsip2 and Atsip1/2 lines. (A) soluble sugars (Glc, Fru and Suc) in the indicated lines. (B) RFO sugars (Gol, Raf and Sta) in the indicated lines and (C) Starch in the indicated lines. Variation is expressed as SD; n=6 sibling plants.
CHAPTER 5
BROAD IMPACT OF METABOLICALLY ENGINEERED PLANTS IN CROP YIELD BY MANIPULATING THE FLOW OF BIOMASS

Traditional methods like pruning and breeding have historically been used in crop production to divert photoassimilates to harvested organs, but molecular biotechnology is now poised to increase yield by manipulating resource partitioning. The broader objectives of this project aim at increasing yields of specific sink tissues by altering the flow of biomass. We proposed that biomass can be targeted to desired organs by engineering RFOs in the phloem of source leaves and engineering their catabolism in specific sink organs. Plant lines with more novel sugars can be engineered with increase in its biosynthetic genes as well as manipulating the catabolic pathway by decreasing its metabolism.

There are systems-based frameworks for exploration of global phenotypic effects of knocking out genes, insertion of genes as well as up regulation/down regulation of gene expression. There is a genome-scale metabolic network model (AraGEM) for primary metabolism for a compartmentalized plant cell based on the Arabidopsis genome (Dal’Molin, 2010). This network can validate through the simulation of plant metabolic functions by help of in silico metabolic flux model of plant metabolism. This resource might be valuable for further development of metabolic engineering study.

For future research, the RFO producing plants (GRS47, GRS63) can be crossed with the \(\alpha\)-galactosidase knockout lines (Atsip1/2) to make lines that produce and accumulate more RFOs. Thus introducing an engineered RFO pathway into these plants will illuminate if endogenous alkaline \(\alpha\)-galactosidases influence our ability to
control RFO accumulation and distribution through metabolic engineering. The line produced will have an enhanced biosynthetic pathway of RFOs as well as absence of the α-galactosidase genes to prevent its metabolism, and thus should result in greater accumulation of RFOs in the plants. The resulting lines can then be engineered with sink specific expression of α-galactosidases. The resulting lines might potentially have greater hydrostatic pressure gradient between the engineered sink and the source tissues causing higher amounts of photosynthate flowing towards the engineered sink. The engineered sink is proposed to have higher biomass.

There is an ever growing need for greater yield worldwide. This can be manipulated in plants for greater yield in specific sink tissues without any external or chemical (fertilizer) application. In another important aspect, the role of RFOs in plants during stress is well documented. The understanding and manipulation of the enzymatic mechanism responsible for both RFO accumulation as well as degradation may be effective in the production of hardier as well as stress resistant crop plants (Pennycooke, 2003). By increasing the hydrostatic pressure gradients between source organs like leaves and target sinks, directed nutrient transport to favor specific target organs may occur this would lead to increased yield of harvested products by a unique approach.
Figure 5.1: Sugar analysis of rosettes in a time course experiment in soil (8, 12, 16, 20 and 24 dpg) of GRS47, GRS63 and WT lines (June 2012). (A) Glc/Gal, (B) Fru, (C) Suc, (D) Gol, (E) Raf and (F) Sta in different lines. Variation is expressed as SD; n=6 sibling plants.
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