METABOLIC ENGINEERING OF RAFFINOSE-FAMILY OLIGOSACCHARIDES IN THE PHLOEM REVEALS ALTERATIONS IN PATTERNS OF CARBON PARTITIONING AND ENHANCES RESISTANCE TO GREEN PEACH APHID

Te Cao

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Phloem transport is along hydrostatic pressure gradients generated by differences in solute concentration between source and sink tissues. Numerous species accumulate raffinose-family oligosaccharides (RFOs) in the phloem of mature leaves to accentuate the pressure gradient between source and sinks. In this study, metabolic engineering was used to generate RFOs at the inception of the translocation stream of *Arabidopsis thaliana*, which transports predominantly sucrose. To do this, three genes, *GALACTINOL SYNTHASE*, *RAFFINOSE SYNTHASE* and *STACHYOSE SYNTHASE*, were expressed from promoters specific to the companion cells of minor veins. Two transgenic lines homozygous for all three genes (GRS63 and GRS47) were selected for further analysis. Sugars were extracted and quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and 21-day old plants of both lines had levels of galactinol, raffinose, and stachyose approaching 50% of total soluble sugar. All three exotic sugars were also identified in phloem exudates from excised leaves of transgenic plants whereas levels were negligible in exudates from wild type leaves. Differences in starch accumulation or degradation between wild type and GRS63 and GRS47 lines were not observed. Similarly, there were no differences in vegetative growth between wild type and engineered plants, but engineered plants flowered earlier. Finally, since the sugar composition of the phloem translocation stream is altered in these plants, we tested for aphid feeding. When green peach aphids were given a choice between WT and transgenic plants, WT plants were preferred. When aphids were reared on only WT or only

transgenic plants, aphid fecundity was reduced on the transgenic plants. When aphids were fed on artificial media with and without RFOs, aphid reproduction did not show differences, suggesting the aphid resistance is not a direct effect of the exotic sugars.

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CHAPTER 1

INTRODUCTION

Photosynthesis converts solar energy into chemical energy. In this process, light energy drives the synthesis of carbohydrates from carbon dioxide and water with oxygen as the byproduct. These carbohydrates can be stored or transported and used for biosynthesis or as an energy source during respiration. Mature leaves are the most active photosynthetic tissue in most plants. Two vascular systems - the xylem and the phloem- extend throughout the plant body: The xylem transports water and minerals from the soil to photosynthetic leaves of the plant, and the phloem transports water, minerals, and organic solutes from mature leaves to the sink tissues.

The phloem is the vascular tissue that translocates the carbohydrates generated by photosynthesis from mature leaves to regions of growth or storage. Since it is responsible for distributing biomass, the phloem is an important determinant of plant productivity. Phloem tissue contains parenchyma cells, sieve elements, and companion cells. Parenchyma cells store starch, protein, oils and water for plants. Sieve elements are the conducting cells that allow movement of sugars and other organic materials throughout the plant. Mature sieve elements lack nearly all organelles and are connected end to end to allow transport between cells (Taiz and Zeiger, 2006). Companion cells are cytoplasmically dense and are connected to sieve elements by plasmodesmata-pore units to form the sieve element-companion cell complex (SE-CCC) (Haritatos et al., 2000). These two cells are derived from the same mother cell. Figure 1 illustrates the anatomy of a sieve element and companion cell in the SE-CCC.

Mature leaves are referred to as *source* tissues and regions of growth and storage are termed *sink* tissues. The pressure-flow model was proposed (Ernst Munch in 1930) to explain phloem transport mechanism. According to the pressure-flow model, phloem transport is driven

by hydrostatic pressure gradients generated osmotically between source and sink. Figure 1 shows an illustration of osmotically-generated-pressure flow. In mature leaves, sugars produced from CO_2 and light energy accumulate in the phloem and water enters the phloem osmotically to create a high turgor pressure. In sink tissues, the hydrostatic pressure is reduced because of the loss of solutes for growth and metabolism. The hydrostatic pressure gradient results in bulk flow of water and dissolved solutes from source to sink tissues. The degree of the pressure gradient controls nutrient distribution (Buchanan, Gruissem and Jones, 2000).

1.1 Phloem Loading

The accumulation of sugars from the mesophyll cells into the SE-CCC is called phloem loading; the distribution of sugars from sieve elements to flanking tissues in sink organs is called phloem unloading. During phloem loading, energy is expended to accumulate sugar in the SE-CCC, water moves in by osmosis and high-hydrostatic pressures are consequently generated in the phloem. Two mechanisms, apoplastic loading and symplastic loading, are well-characterized (Taiz and Zeiger, 2006). Recently, passive flux of sucrose or sugar alcohols from mesophyll cells to the sieve elements of minor veins has been found to be a transport mechanism in some plants, especially woody plants (Rennie and Turgeon, 2009).

1.1.1 Apoplastic Phloem Loading

In apoplastic phloem loading, sugars from the mesophyll are first released into the cell wall space (apoplast), and are then loaded into the sieve elements and companion cells from the apoplast. The loading of sugars is an active process carried out by sugar-proton symporters located in the plasma membrane of these cells. Sugar-proton symporters use the proton motive force generated by H^+/ATPases to move sugars into the SE-CCC against a concentration gradient

(Taiz and Zeiger, 2006). In most plants that use apoplastic phloem loading, sucrose is utilized as the main transport sugar. Sucrose-H^+ symporters (sucrose transporters) which mediate the transport of sucrose from apoplast into the SE-CCC, have been found in many plants. Figure 2 illustrates the mechanism of apoplastic phloem loading. Sucrose transporters have important regulatory roles in the distribution and partitioning of assimilates in plants. To date, five sucrose transporter families have been identified and characterized for a variety of plants (Fan, et al., 2009).

1.1.2 Symplastic Phloem Loading

Sucrose derivatives such as raffinose, stachyose 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 loading (Turgeon, 1996). In the polymer trap mechanism (Figure 3), sucrose diffuses into intermediary cells (specialized companion cells) from mesophyll cells through narrow and highly branched plasmodesmata. The high number of plasmodesmata that connect intermediary cells to surrounding mesophyll cells is a distinguishing feature of intermediary cells (Oparka and Turgeon, 1999). Inside intermediary cells, sucrose is polymerized to raffinose family oligosaccharides (RFOs). This conversion from sucrose to RFOs reduces the concentration of sucrose to maintain the sucrose 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 larger size and consequently accumulate to increase the overall solute concentration, and generate hydrostatic pressure. RFOs then enter the sieve elements through the wider plasmodesmata-pore units that connect these two cells (Turgeon and Ayre, 2005).

1.2 Raffinose Family Oligosaccharides (RFOs):

The occurrence of RFOs in plants is widespread and their physiological functions include carbon transport, carbon storage, and protection against various stresses, such as salt, drought, and cold stresses (Keller and Pharr, 1996). RFOs are α-galactosylated derivatives of sucrose, which differ in the number of galactosyl moieties. Raffinose is a trisaccharide (αD-Gal-(1-6)-αD-Glc-(1-2)-βD-Fru), stachyose is a tetrasaccharides (αD-Gal-(1-6)- αD-Gal-(1-6)-αD-Glc-(1-2)-βD-Fru), and verbascose is a pentasaccharide (αD-Gal-(1-6)- αD-Gal-(1-6)- αD-Gal-(1-6)-αD-Glc-(1-2)-βD-Fru). RFOs are naturally found in many seeds, numerous vegetables (green beans, soybeans, *etc*.) (Nakakuki, 2002) and numerous trees and herbs (*Catalpa speciosa*, *Coleus blumei*, *etc*.) (Turgeon, 2004). Figure 4 shows the chemical structure of RFOs. RFOs are the main transport sugars in symplastic phloem loading, with stachyose being the most dominating. Their synthesis is proposed to be as part of the polymer trap mechanism for symplasmic phloem loading (Turgeon, 1996).

1.2.1 Biosynthesis of RFOs

RFOs are synthesized from sucrose by the sequential addition of galactose moieties by α-galactosyltransferases. The biosynthetic pathway of the RFOs is well-characterized (Keller and Pharr, 1996). The three-step reaction leading to the tetrasaccharide stachyose is illustrated in Figure 5. The committed step of RFOs synthesis is catalyzed by galactinol synthase (GolS; UDP-galactose:myo-inositol-galactosyl transferase; EC 2.4.1.123). Galactinol (α-D-galactosyl-(1-3)-1D-myo-inositol) is generated from myo-inositol and UDP-galactose, with UDP as the leaving group. In the remaining steps of RFOs biosynthesis, galactinol serves as the donor of the galactosyl moiety. Raffinose (Raf; α-D-Gal-(1-6)-α-D-Glc-(1-2)-β-D-Fru) is synthesized from sucrose and galactinol by raffinose synthase (RafS; Galactinol:sucrose galactosyl transferase; EC

2.4.1.82) and myo-inositol is produced as the leaving group; Stachyose (Sta; α-D-Gal-(1-6)-α-D-Gal-(1-6)-α-D-Glc-(1-2)-β-D-Fru) is produced from raffinose and galactinol by stachyose synthase (StaS; Galactinol:raffinose galactosyl transferase; EC 2.4.1.67) and myo-inositol is produced as the leaving group. Further extensions of the chain of galactosyls lead to generation of other members of RFOs.

1.2.2 Catabolism of RFOs

The distribution of sugars from sieve elements in sink tissues is called phloem unloading. Generally, sugars move from sieve elements to the surrounding post-phloem tissues through plasmodesmata (Lalonde et al., 2003). RFO catabolism in plants has received relatively little attention. Figure 6 illustrates possible catabolic pathways of RFOs. RFOs are digested by α-galactosidases (αGal; EC 3.2.1.22) to generate sucrose and galactose. Sucrose may be digested to fructose and glucose by invertase or to fructose and UDP-Glucose by sucrose synthase. Fructose, glucose and UDP-Glucose can then readily enter other metabolic pathways. For digestion of galactose, ATP is used to convert galactose to Galactose-1-P by galactokinase (EC 2.7.1.6). Galactose-1-P can be digested by two different pathways, one of which is the Lelior pathway. In the Lelior pathway, which is used in many organisms, hexose-1-P uridylytransferase (EC 2.7.7.12) catalyzes Galactose-1-P to form Glucose-1-P with UDP transfer from UDP-Glucose to Galactose-1-P. However, plants use another way to digest Galactose-1-P. Pyrophosphorylase (EC 2.7.7.64) is used to form UDP-Galactose and PPi from Galactose-1-P and UTP. UDP-Galactose is converted to UDP-Glucose, which readily enters metabolism, by UDP-4-Glucose epimerase (EC 5.1.3.2) (Keller and Pharr, 1996).

1.2.3 Physiological Roles of RFOs

The occurrence of RFOs in plants is nearly ubiquitous and is implicated in carbon transport. In over 500 species, belonging to almost 100 families, sucrose is found in phloem exudates, and RFOs were in two-thirds of these. In some species tested, RFOs are the main transport sugars and their synthesis was proposed to be as part of the polymer trap mechanism for symplasmic phloem loading (Turgeon, 1996). RFOs were proposed to play a role in reducing solute leakage during long-distance transport (Ayre et al., 2003).

Plants are frequently exposed to environmental stresses in both natural and agricultural conditions. Stress tolerance is the plant's fitness to cope with an unfavorable environment. RFOs play important roles in tolerance to different stresses, including salt, drought, and cold stresses. Raffinose in particular, and to a lesser extent stachyose, accumulate during seed development and is thought to play roles in desiccation tolerance of seeds (Bailly et al., 2001). Galactinol synthase in numerous species is stress-inducible and is proposed to play a key role in the accumulation of galactinol and raffinose during abiotic stress, and galactinol and raffinose may function as osmoprotectants in drought-stress tolerance of plants (Taji et al., 2002). Sucrose and RFOs, particularly raffinose, functions as compatible solutes during drought stress in the leaves of the resurrection plant *Xerophvta viscose* (Peters et al., 2007). The accumulation of raffinose was associated with increased freezing tolerance of the leaves (Pennycooke et al., 2003). Although ostensibly beneficial, raffinose is not essential for basic freezing tolerance for cold acclimation of *Arabidopsis thaliana* (Zuther et al., 2004). Recently, RFOs have been suggested to play a protective role in the natural frost tolerance of the evergreen labiate *Ajuga reptans* (Peters et al., 2009). In addition, Galactinol and RFOs were found to protect plant cells from oxidative stress and may scavenge hydroxyl radicals as novel intracellular antioxidants in plants

under several types of stress (Nishizawa et al., 2008). Galactinol has been suggested to be a signaling component of the induced systemic resistance caused by pathogens (Kim et al., 2008).

RFOs have been found in different plant organs, such as leaves, stems, tubers, bulbs, fruit, and seeds and may function as long- or short-term storage reserves. RFOs accumulate to serve the dual purposes of carbon storage and stress tolerance in most plants (Kellar and Pharr, 1996). In seeds, RFOs are one of the most prominent soluble sugars. RFOs may be an essential source of rapidly metabolizable carbon in early germination (Downie and Bewley, 2000).

1.3 Objectives

The main goal of this project for our lab is to manipulate the hydrostatic pressure gradients that control phloem transport between source and sink tissues to favor biomass distribution to organs that are harvested. Brian Ayre proposed that plant carbon partitioning can be targeted to desired organs by altering hydrostatic pressure gradients between source leaves and desired sinks. To test this hypothesis, metabolic engineering was used to generate RFOs in the phloem of mature leaves of *Arabidopsis thaliana* to increase hydrostatic pressure in source leaves. Simultaneously, in order to decrease hydrostatic pressure in sink tissues, metabolic engineering will be employed to degrade RFOs in specific recipient tissues. By increasing the hydrostatic pressure gradients between source leaves and target sinks, directed nutrient transport to favor specific target organs may occur (Figure 7).

Three genes, *GALACTINOL SYNTHASE*, *RAFFINOSE SYNTHASE* and *STACHYOSE SYNTHASE*, were expressed to make RFOs in the companion cells of minor veins. Companion-cell-specific promoters were used to express the three genes in the companion cells. *GALACTINOL SYNTHASE* (*CmGAS1)* from melon (*Cucumis melo*) and the *RAFFINOSE SYNTHASE* (*CsRFS*) from cucumber (*Cucumis sativus*) were expressed from the *CmGAS1*

promoter, which confers gene expression to the minor veins (Haritatos et al., 2000; Ayre et al., 2003). *STACHYSOSE SYNTHASE* (*AmSTS1*) from Alonsoa (*Alonsoa meridionalis*) was expressed from the *Mature Minor Vein Element1* (*MMVE1*) promoter, which was identified as being specific to companion cells (McGarry et al., 2008). Previous students transformed Arabidopsis plants with the three biosynthetic genes. Each had a different marker for selection of transgenic plants.

My objectives in this work were to 1) select transgenic homozygous plants for the three genes required for production of stachyose; 2) quantify RFOs with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD); 3) analyze carbon distribution and mobilization; 4) analyze vegetative growth rate and transition to reproductive growth; 5) test the impact of engineered RFOs on phloem-feeding aphids.

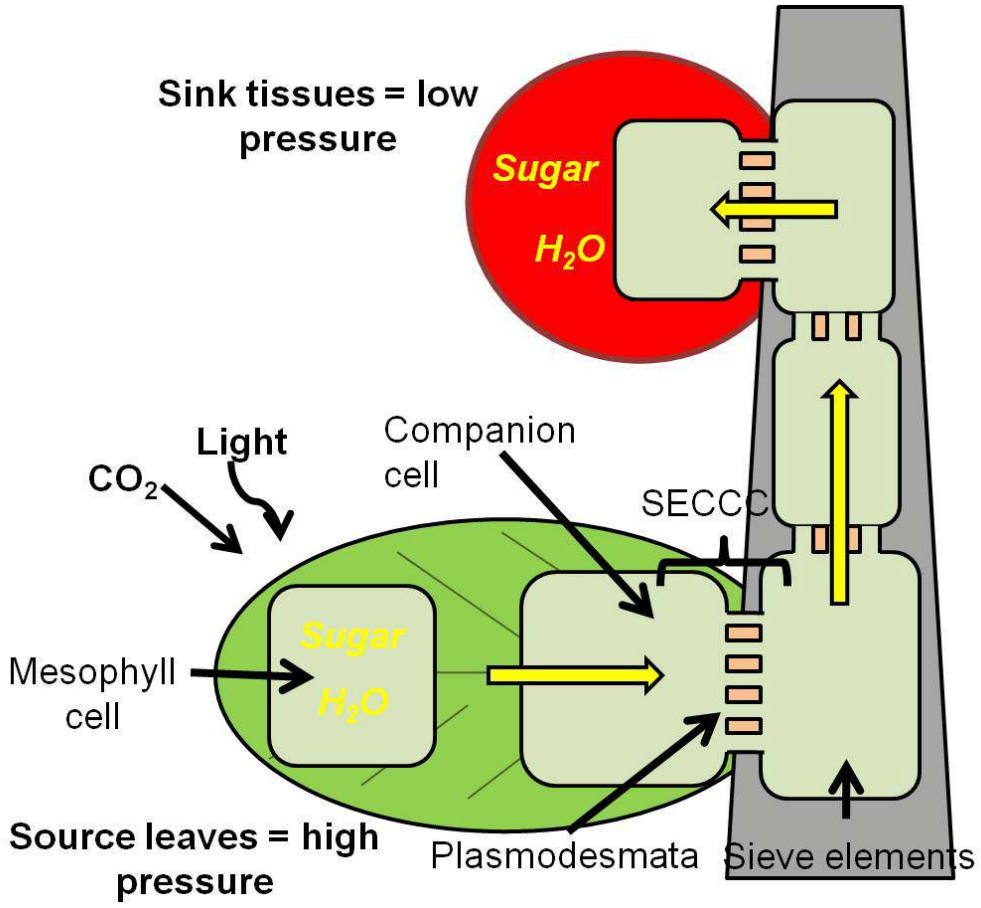


Figure 1. Overview of phloem anatomy and osmotically-generated-pressure flow between source and sink tissues.

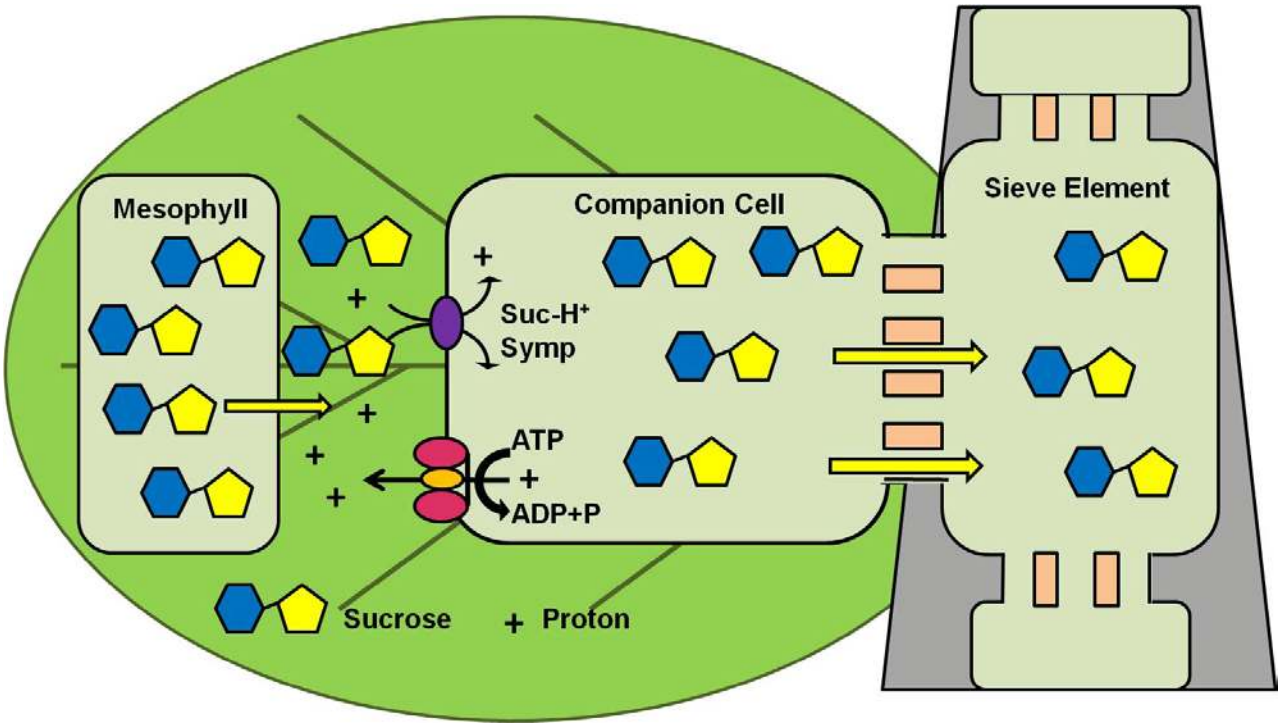


Figure 2. Apoplastic phloem loading mediated by sucrose-proton symporters.

Suc-H^+ Symp is sucrose-proton symporters.

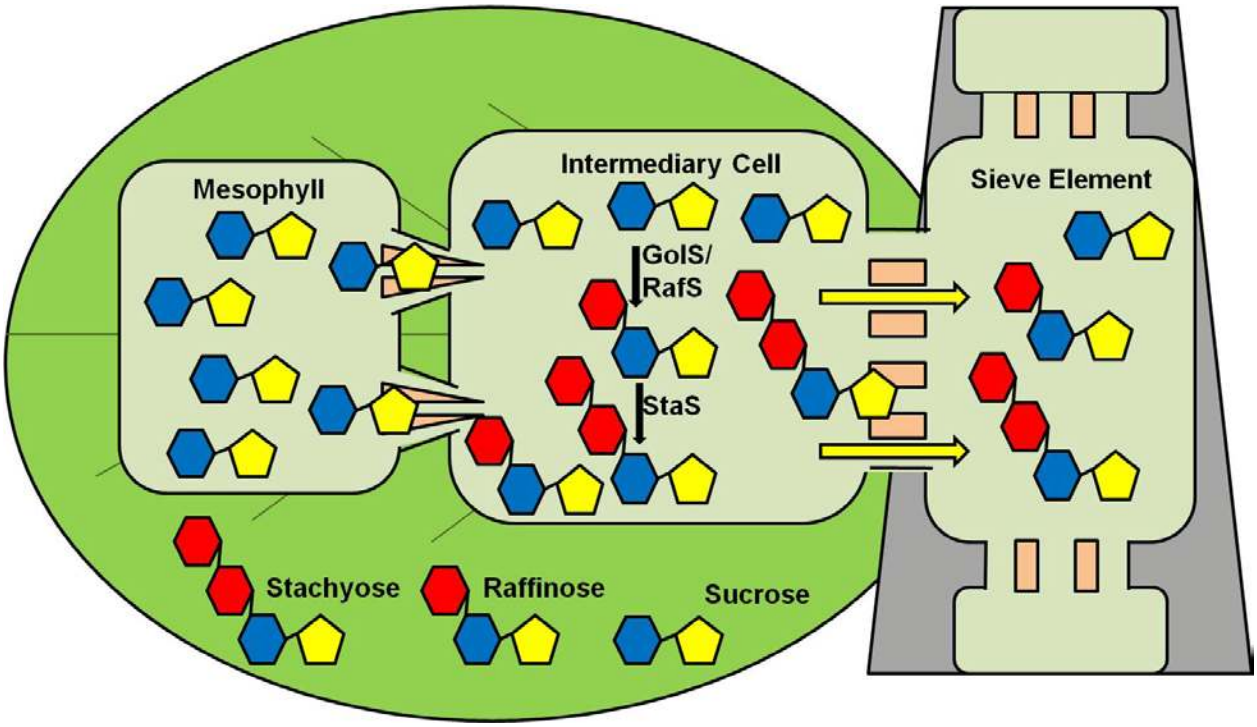


Figure 3. The polymer trap model for symplastic phloem loading. GolS is galactinol synthase. RafS is raffinose synthase. StaS is stachyose synthase.

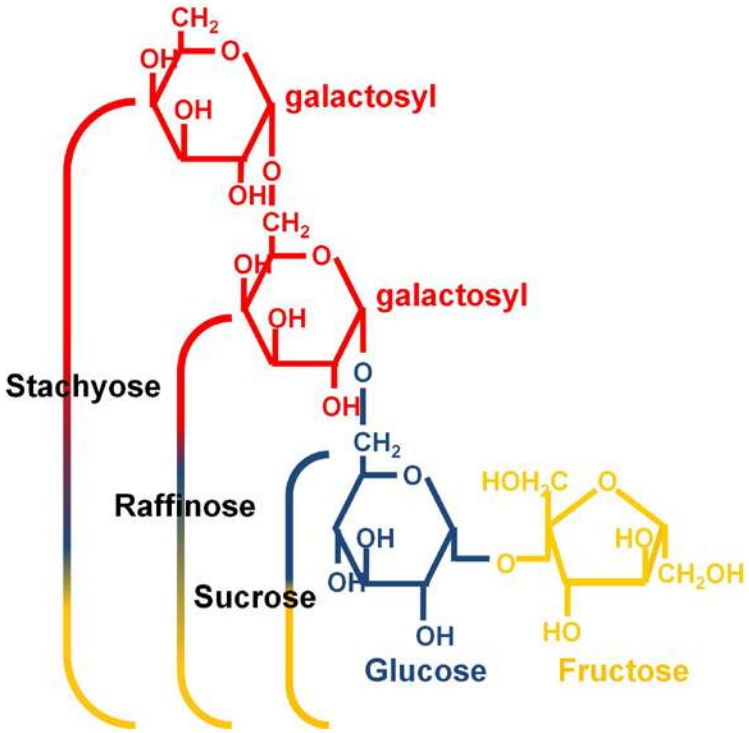


Figure 4. Chemical structure of RFOs.

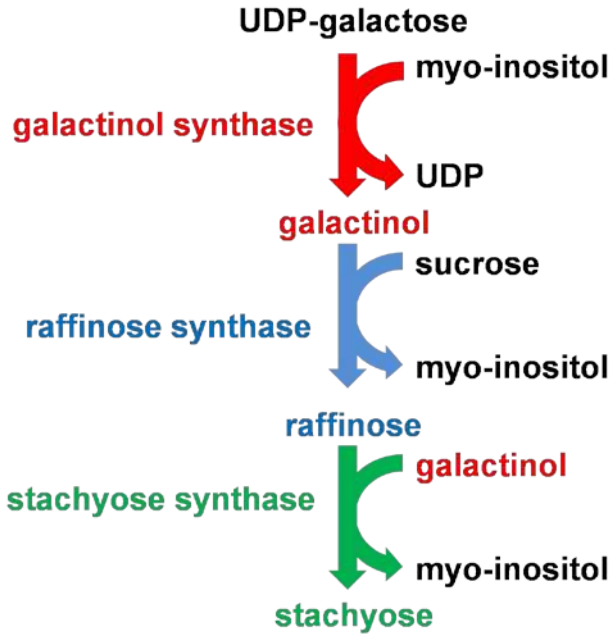


Figure 5. Biosynthetic pathway of RFOs in plants.

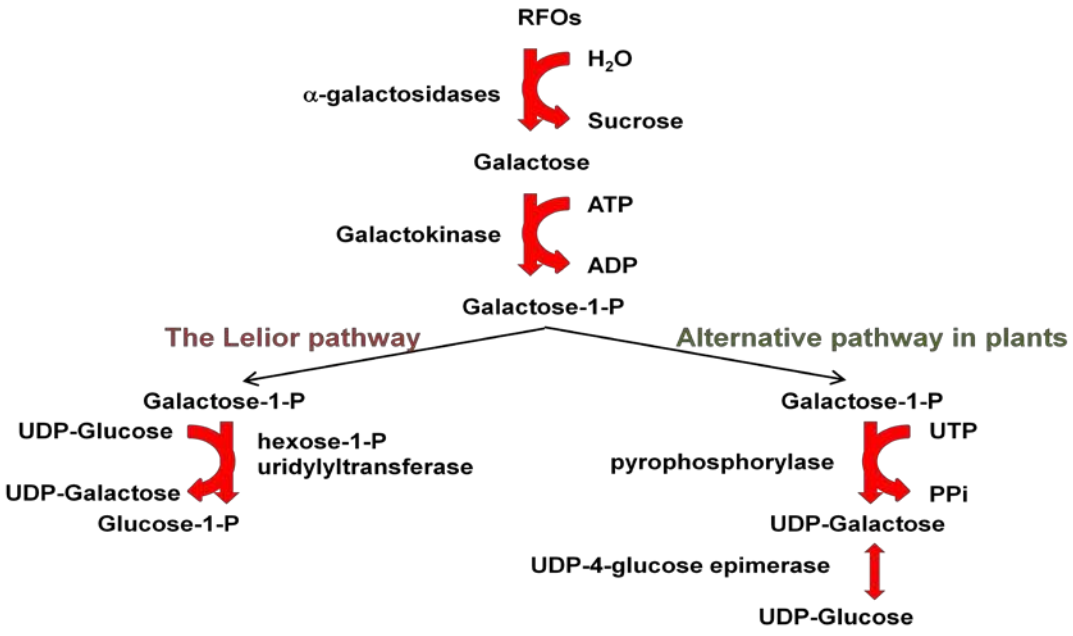


Figure 6. Catabolic pathway of RFOs in plants.

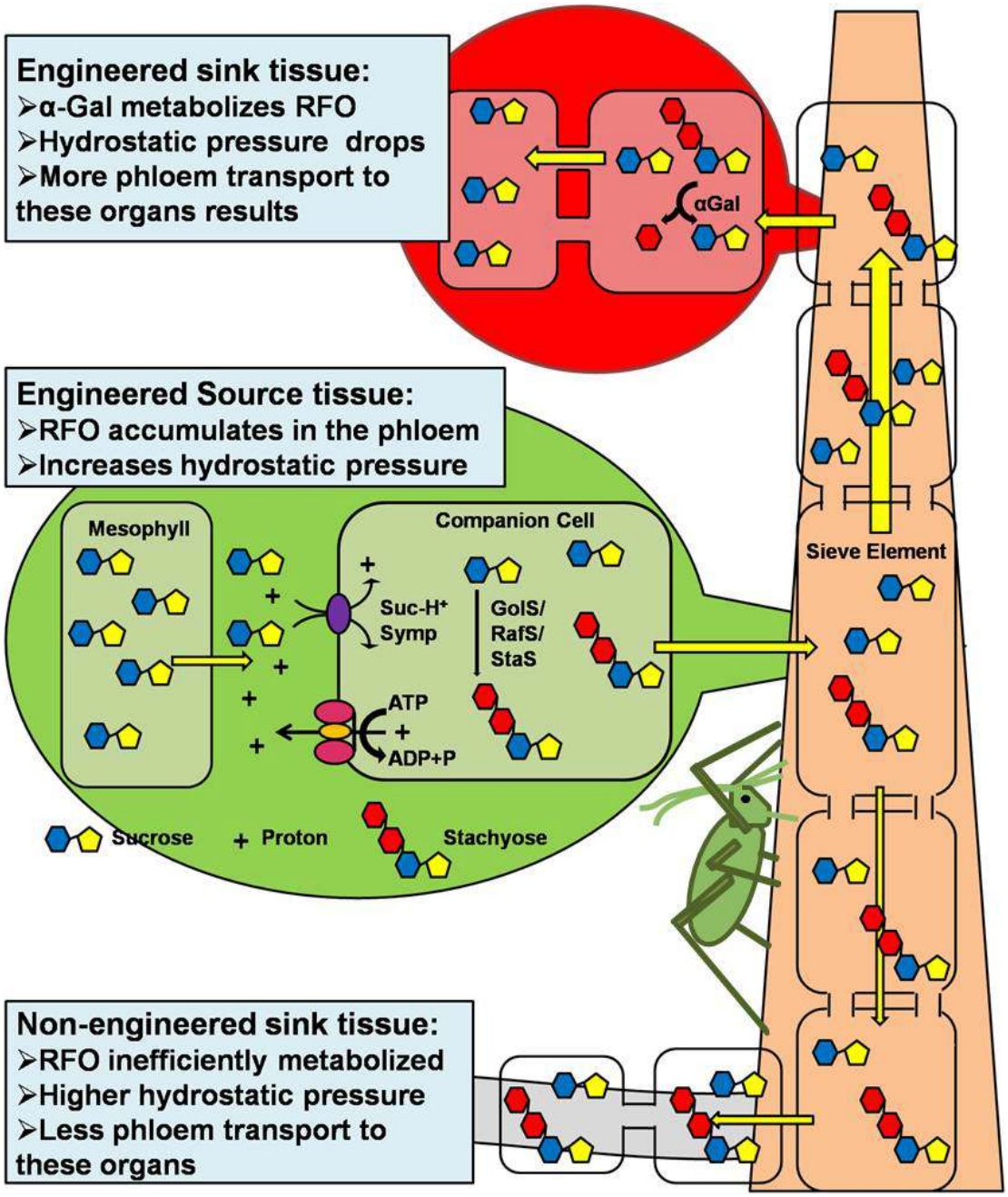


Figure 7. Proposed metabolic engineering strategy in source and sinks to target phloem transport to specific tissues. Sucrose loading from the apoplast with Suc/H+ symporters is converted to RFO in companion cells of source leaves (middle green leaf). RFO are efficiently metabolized in engineered target sink tissues, reducing the solute concentration and promoting phloem transport (top: red fruit). Tissues not engineered for RFO degradation accumulate RFO, inhibiting phloem transport (bottom: gray root). This thesis composes a portion of these large objectives and tested the impact of engineered RFOs on phloem-feeding aphids.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plasmid Construction

The pGPTV-Hyg-CmGAS1p-CmGAS1 construct to express *CmGAS1*, encoding galactinol synthase, was previously described (Ayre et al.,2003) and consists of a 5 kb genomic sequence from *Cucumis melo* subcloned in the EcoRI restriction endonuclease recognition site of pCambia1301. Hygromycin phosphotransferase (*hpt*) was used as the selection marker (Figure 8A).

The plasmid pGPTV-Kan-CmGAS1p-CsRFS was constructed by a former researcher in Brian Ayre's laboratory to express *Raffinose Synthase* in the minor veins of transgenic Arabidopsis. In brief, a phloem specific *Raffinose Synthase* (*RFS*; NCBI accession no. AF073744) cDNA was generated from *Cucumis sativum* mRNA by RT-PCR, digested with KpnI and SacI restriction enzymes and inserted into the same sites of pUC-GUT-CO (Ayre et al., 2004), and then the *CmGAS1* promoter – *RFS* cDNA cassette was inserted into pGPTV-Kan as a SbfI – SacI cassette (Becker et al., 1992). Neomycin phosphotransferase (*nptII*) was used as the selection marker (Figure 8B).

The plasmid pGPTV-bar-MMVE1p-AmSTS was similarly constructed by a prior researcher to express *Stachyose Synthase* in the minor veins of transgenic plants in Ayre's lab. The sequence for *Cucumis Stachyose Synthase* (*STS1*) is not available. However, the sequence for *STS1* from *Alonsoa meridionalis* (NCBI Genbank accession no. AJ487030) is available. *Alonsoa meridionalis* also loads from the symplasm and translocates stachyose predominantly. *STS1* cDNA was generated from mRNA by RT-PCR and then digested with restriction enzymes and inserted into pGPTV-bar. The *MMVE1* promoter element (McGarry et al., 2008) was used to

express *AmSTS1* specifically in minor-veins of transgenic Arabidopsis and phosphinothricin acetyl transferase (*bar*) resistance marker was used to facilitate selection (Figure 8C).

2.2 Plant Materials

Arabidopsis, which possesses endogenous RFO metabolism in seeds and during stress, but not as a transport sugar, was chosen as a model for this research. Numerous lines of transgenic plants potentially transformed with the three biosynthetic genes to produce stachyose in the phloem were generated previously by Dr. Ayre and colleagues. Transformed Arabidopsis seeds were selected on Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) with 1% Suc and appropriate antibiotics. To make 1000 ml of MS medium, 10 g of sucrose and 4.44 g MS modified Basal Medium with Gamborg Vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS) were dissolved in 800 ml distilled H_2O. The pH was adjusted to 5.8 by using 1 M KOH and the final volume was adjusted to 1000 ml with distilled H_2O. 2.8 g/L Gel-rite gellan gum (Fisher Scientific, Fair Lawn, New Jersey) was added to solidify. The medium was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle. The medium was cooled to about 60 °C after autoclaving. 100 mg/L Kanamyicn (PhytoTechnology Laboratories), 10 mg/L Glufosinate ammonium (PhytoTechnology Laboratories), and 40 mg/L Hygromycin B (PhytoTechnology Laboratories) were used for selecting transformed Arabidopsis seedlings, and 200 mg/L Timentin (Ticarcillin Disodium Salt/Potassium Clavulanate mixture 15:1) (PhytoTechnology Laboratories,) was used to suppress bacteria growth.

Approximately 50 Arabidopsis seeds for each line in a 2 mL microtube were surface-sterilized with chlorine bleach (30 mL bleach and 1 mL concentrated HCl) for 6 hours in a vacuum sealed jar (Martinez-Zapater and Salinas, 1998). The sterilized seeds were distributed evenly on MS medium with 1% sucrose and selection. In order to identify transformants for

resistance to hygromycin, kanamycin and glufosinate ammonium, seeds were stratified for 2 days in the dark at 4°C and were then illuminated for 4-8 hours to stimulate germination. Seeds were germinated in the darkness at room temperature for four days to promote hypocotyl elongation, and scored for antibiotic resistance after two days growth with 14 / 10 light / dark cycles (Harrison et al., 2006). Seedlings which resisted all three antibiotics were transferred to soil and grown in a growth chamber under appropriate conditions for different experiments.

Seeds from mature plants were harvested manually and separated from the chaff by passing through a sieve. Cleaned seeds were stored in microfuge tubes with small holes in the lids in a sealed dessicator with desiccant for seven days for thorough drying, moved to -80 °C for 3 days to kill any insect eggs, returned to the dessicator for 1 day, and then placed in a sealed, seed-storage chamber with desiccant for long term storage.

2.3 Plant Transformation and Selection

Plasmid vectors harboring appropriate DNA constructs were introduced to *Agrobacterium tumefaciens* by electroporation (Sambrook et al., 2001). *Agrobacterium tumefaciens* containing constructs were transformed into wild type Arabidopsis (Col-0) by using the Floral Dip transformation procedure (Clough and Bent, 1998). This work was carried out by previous students in Dr. Brian Ayre's lab. In order to select transformants for all three genes, seeds were germinated on MS medium with 1% sucrose containing hygromycin, kanamycin and glufosinate ammonium. After 2 days cold treatment at 4°C, 4-8 hours light treatment and 4 days dark treatment, seedlings which resisted all three antibiotics had green, open, expanded cotyledons with long hypocotyls and were transferred to soil and grown under 12 hours light/12 hours dark cycle at 21°C. The lines with all seedlings resistant to all three antibiotics, and that did not show segregation in subsequent generations, were considered homozygous

(*CmGAS1*/*CmGAS1; CsRFS*/*CsRFS; AmSTS1*/*AmSTS1*). The homozygous lines are GRS63 and GRS47.

2.4 Sugar Analysis

GRS63 and GRS47 were grown for 21 days under 14 / 10 light / dark cycles (The light intensity is 110-150 μmol photons m^2s^{-1}). The samples were collected at two different times: 8:00 am after 10 hours of dark and 4:00 pm after 8 hours of light. Sugar extraction was performed on either fresh tissues or tissues frozen and ground in liquid N_2 to establish which method is superior. Leaves of plants were bisected and fresh weight of each half was established. One half was immediately immersed in 1 mL of ice-cold MCW extraction solution (methanol: chloroform: water, 12:5:3) containing 10 μM lactose as an internal standard and extracted for 15 minutes in a 50°C water bath (Srivastava et al., 2008). The other half was crushed in liquid N_2 in a 2 mL microtube with a glass rod, followed by adding 1 mL ice-cold MCW extraction solution with 10 μM lactose, and extracted for 15 minutes in a 50°C water bath. The volume of MCW extraction solution was at least five volumes per unit fresh weight for each extraction. Tissues were extracted twice, and extracts combined. The residue of tissues after sugar extractions were measured for starch levels. Water was added to the combined extract (three parts water per five parts extract) to separate the aqueous and organic phases. After centrifugation, the aqueous phase was collected and dried down to approximately 200 μL in a centrifugal concentrator. 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 polyvinylpolypyrolidone (Sigma, St. Louis, MO), and 250 μL of AG50-X8 cation-exchange resin (Bio-Rad, Hercules, CA; Hydrogen Form) and the columns were washed with 1 mL H_2O. Sugars were separated and quantified

using a CarboPac PA-20 column using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale CA). Six plants of each line were used to establish variation in this experiment.

2.5 Starch Analysis

2.5.1 Starch Staining with Iodine and Microscopy

GRS63 and GRS47 were grown for 21 days under 14 / 10 light / dark cycles (The light intensity is 110-150 μmol photons m^2s^{-1}). The samples were collected at two different times: 8:00 am after 10 hours of dark and at 4:00 pm after 8 hours of light. After 10 hours of dark, the 4th leaf from individual plants were excised and incubated in 70% ethanol at 37°C for 24 h to clear chlorophyll. The tissues were rehydrated in a graded ethanol series until they were in water (60% ethanol for 2 minutes, 30% ethanol for 2 minutes, and water for 2 minutes twice). The tissues were immersed in 10% I-KI staining solution and staining proceeded for 30 minutes. The tissues were washed twice with water for 5 minutes to remove excess stain. Stained leaves were recorded with a Nikon SMZ1500 stereo microscope equipped with a Nikon Digital camera DXM1200F (Melville, NY). For digital photography, exposure time and settings were equivalent for all samples (exposure time was 1/25 sec.; magnification was 1.5X). After eight hours light, the 5th leaf from the same plants was collected and the same procedures were used for starch staining with iodine. These experiments were repeated twice and there were 9 replicates in the first experiment and 6 replicates in the second experiment.

2.5.2 Starch Assay

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;

myloglucosidase/α-amylase method). 1 mL of 80% ethanol was added to remove chloroform from MCW treated leaves in 2 mL microtubes. After centrifugation, the supernatant was discarded and 70 μL of 80% ethanol was added and tissues dispersed by vortexing. 1 mL of α-amylase solution (prepared as described by the kit manufacturer) was added and the tubes were incubated in a boiling water bath for 6 minutes. 50 μL of amyloglucosidase was added and the tubes were incubated at 50°C for 30 minutes after mixing. After centrifuging at 13,000 rpm for 10 minutes, 1000 μL supernatant was transferred to a clean tube. 200 μL of supernatant was transferred to 13x100 mm glass test tubes, 2000 μL 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 2000 μL of GOPOD Reagent. Reagent Blank solutions consist of 0.1 mL of water and 2000 μL of GOPOD Reagent. The absorbance for each sample was read at 510 nm against the reagent blank.

2.6 EDTA Exudates Analysis

An EDTA-exudation method was used to collect phloem sap from cut leaves (King et al., 1974). EDTA is used to enhance phloem exudation by chelating Ca^{2+} that is required for sieve-tube plugging. GRS63 and GRS47 were grown for 30 days under the conditions described above. After 7 hours light treatment, the 6th through11th leaves from individual plants were excised at the stem and their fresh weights measured. Leaves were cut again under 10 mM EDTA and arranged into a small chamber (Coulter Counter, Pittsburgh, U.S.A.) containing 2 ml of 10 mM EDTA, 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. Phloem exudates from the first twenty minutes were discarded because there

may be some contamination from the contents of cut cells. Subsequently, exudates from each of two two-hour periods were collected. The neutral fraction of exudates was obtained and sugars analyzed by HPAEC-PAD and described above. Twelve samples for each line were tested to establish variation in this experiment.

2.7 Growth Rate Analysis

Seeds of the two independent transformed lines homozygous for each transgene, GRS63 and GRS47, were put out on soil and after two days cold treatment at 4 °C, were grown under 14 hours light/10 hours dark cycle at 21°C. Plants were well spaced with one plant in each cell of a 36-cell growth tray so as not to impact each other's growth. The seeds from wild type Arabidopsis plants were put out as growth controls and grown under same conditions. After 18 days germination, rosettes of both transgenic and wild type Arabidopsis plants were photographed and the total rosette area was measured by using ImageJ software (Rasband, 2007). Twelve plants of each line were used to establish variation in this experiment.

2.8 Flowering Time Analysis

Seeds from wild type Arabidopsis plants and two independent transformed lines homozygous for each transgene, GRS47 and GRS63, were put out on soil and after two days cold treatment at 4°C, were grown under 14 hours light/10 hours dark cycle at 21°C. 36 plants of each line were used to measure the flowering time with one plant in each cell of a 36-cell growth tray. The position of the three flats and orientation of each flat were exchanged daily to compensate for any potential microclimates in the growth chamber. Plants were 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 recorded. Thirty six plants of each line were used to establish variation in this experiment.

2.9 Aphid Feeding Experiments

2.9.1 No-choice Aphid Feeding Test for Fecundity

GRS63, GRS47 and wild type Arabidopsis were grown under standard conditions. Plants were 30-days old when used. A "no-choice" experiment was used to test aphid fecundity on test plants, and measured reproductive rates (Pegadaraju et al., 2005). 20 adult aphids were put on the rosette of each plant from both wild type and transgenic lines GRS63 and GRS47. After two days, the plants were harvested and the number of aphids residing on each plant was counted (Figure 9A). 12 plants of each line were used to test variation in this experiment.

2.9.2 Choice Aphid Test

In "choice" experiments, aphids were placed equidistant between control and experimental plants, and the distribution of aphids determined 24 hours later to establish if a feeding preference existed (Pegadaraju et al., 2007). For choice aphid experiments, 20 adult aphids were placed on the soil between WT and transgenic plants growing in the same 15 cm diameter pot. The number of aphids on each plant was monitored after 24 hours (Figure 9B). Nine plants of each line were used to test variation in this experiment.

2.9.3 Artificial Aphid Diet Test

An artificial aphid diet (Mittler and Dadd 1965) was used to test aphid fecundity and its components are listed in Table 1. The concentration of sucrose in this diet is 500 mM. This diet was mixed with 50 mM of additional sugar, including sucrose, glucose, fructose, galactinol, raffinose or stachyose, to test if the exotic sugars affect aphid fecundity directly. Three adult aphids were transferred with a fine paintbrush into a feeding chamber (a 3.5 cm Petridish; Falcon, Primaria, NJ, U.S.A.). A layer of stretched parafilm (Parafilm "M"; American National

Can, Greenwich, CT, U.S.A.) was covered over the chamber to capture the three aphids. 500 μL of artificial diet or diet with 50 mM test sugar was added on the first layer of parafilm and another layer of stretched parafilm was placed on the first layer to spread diet and form a feeding sachet from which the aphids could feed on the diet (Figure 9C) (Louis et al., 2010). Aphid numbers including adults and nymphs were counted after four days later. Three replicates were included for treatment.

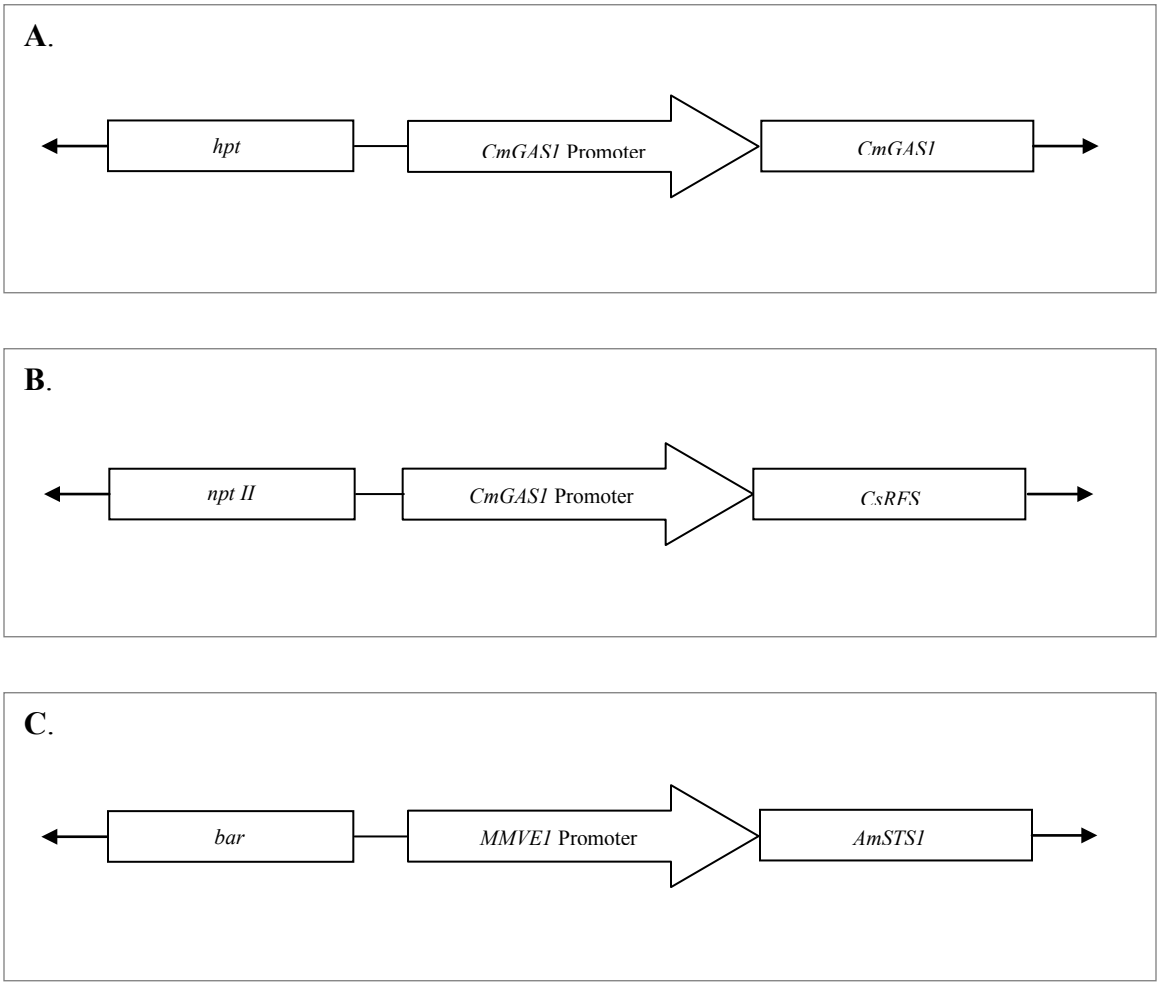


Figure 8. Schematic diagram of **A.** The *Galactinol Synthase* construct and **B.** the *Raffinose Synthase* construct and **C.** the *Stachyose Synthase* construct. (Not drawn to scale).

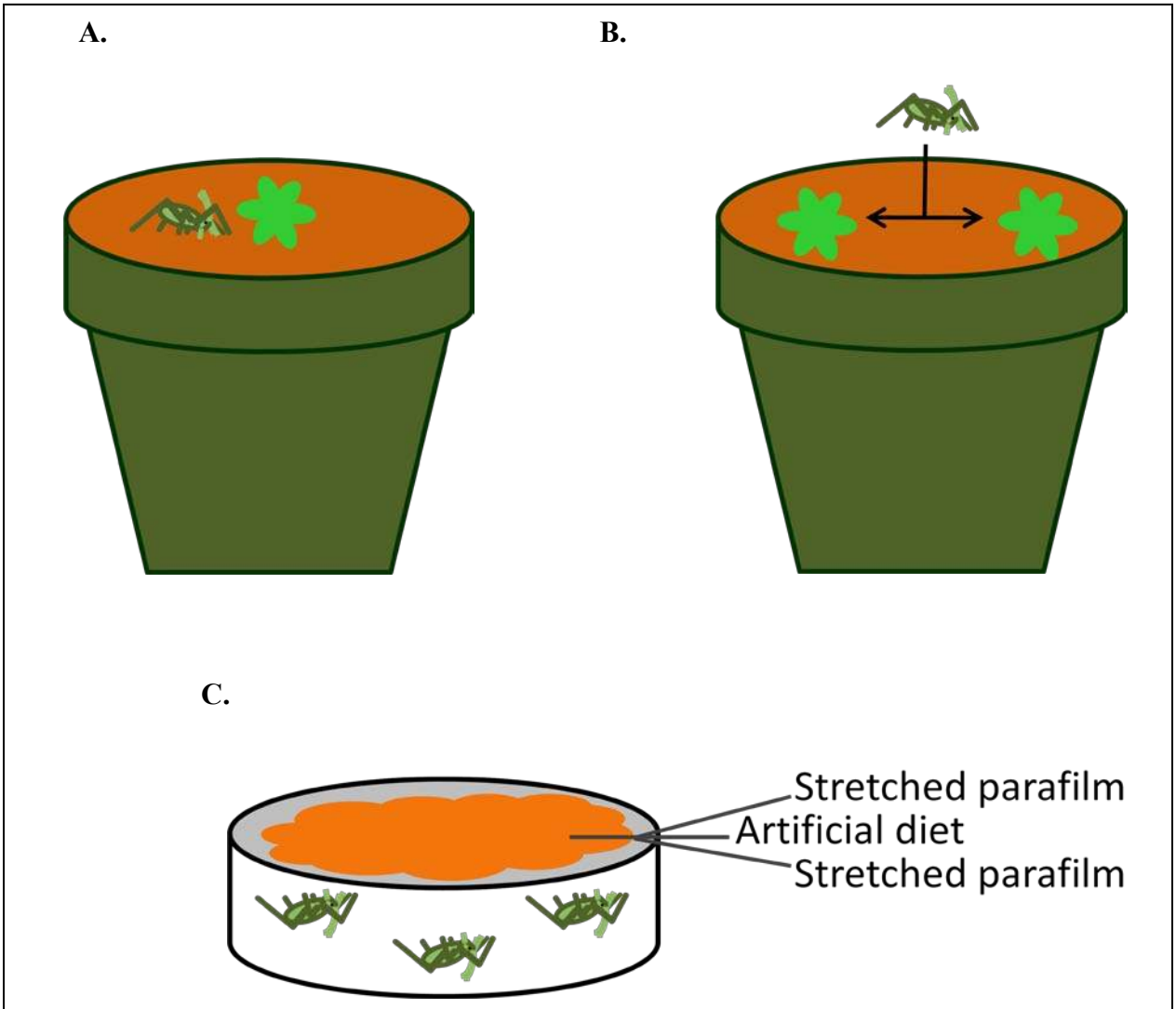


Figure 9. Schematic diagram of **A.** No-choice aphid test. **B.** Choice aphid test. **C.** Artificial aphid diet test.

Table I. *Composition of the artificial aphid diet. pH adjusted to 7.0 using 1M KOH (Mittler TE and Dadd RH, 1965)*

Component Type	Component Name	Amount (mg)
A = Amino acid	L - Alanine	100
A	L - Arginine	270
A	L - Asparagine	550
A	L - Aspartic acid	140
A	L - Cysteine HCL	40
A	L - Glutamic acid	140
A	L - Glutamine	150
A	L - Glycine	80
A	L - Histidine	80
A	L - Isoleucine	80
A	L - Leucine	80
A	L - Lysine mono HCL	120
A	L - Methionine	40
A	L - Phenylalanine	40
A	L - Proline	80
A	L - Serine	80
A	L - Threonine	140
A	L - Tryptophan	80
A	L - Tyrosine	40
A	L - Valine	80
V = Vitamin	L - Ascorbic acid	100
V	D - Biotin	0.1
V	D - Pantothenic acid	5
V	Choline chloride salt	50
V	Folic acid	0.5
V	Myo - Inositol	50
V	Nicotinic acid	10
V	L - Pyridoxine	2.5
V	Riboflavin	0.5
V	L - Thiamine HCL	2.5
O = Other	Potass. Dihydrogen-orthophosphate	500
O	$MgCl_2$ hexahydrate	200
O	Cupric-Sodium EDTA salt	0.4
O	Ferric-Sodium EDTA salt	1.5
O	$MnCl_2$ tetrahydrate	0.4
O	Zinc EDTA	0.8
O	Sucrose	17.5g
O	Water sterilised	100ml
O	Cholesterol	2.5

CHAPTER 3

RESULTS

3.1 Identification of Homozygous (*CmGAS1*/*CmGAS1; CsRFS*/*CsRFS; AmSTS1*/*AmSTS1*) Plants

The plasmid pGPTV-Hyg-CmGAS1p-CmGAS1, pGPTV-Kan-CmGAS1p-CsRFS and pGPTV-bar-MMVE1p-AmSTS were transformed into *Agrobacterium tumefaciens* competent cells by electroporation (Sambrook et al., 2001). Wild type *Arabidopsis thaliana* Col-0 plants were used for transformation using the floral dip method (Clough et al., 1998). Agrobacterium cultures harboring the plasmid pGPTV-Hyg-CmGAS1p-CmGAS1 and the plasmid pGPTV-Kan-CmGAS1p-CsRFS were grown separately and subsequently combined for the floral dip procedure. Transgenic plants were selected on sterile MS media with kanamycin and hygromycin, and were subsequently transformed by floral dip with the T-DNA of pGPTV-Bar-MMVE1p-AmSTS. Seedlings transformed with all three constructs were selected on sterile MS media with 1% sucrose and Hygromycin, Kanamycin and Glufosinate ammonium (phosphinothricin) at appropriate concentrations. Seedlings which resisted all three antibiotics were transferred to soil and seeds harvested for segregation analysis in the next generation.

Hygromycin was used as the selection marker for the *Galactinol Synthase* construct using a selection technique of germinating the seedlings in darkness. Transformed seedlings grown in medium containing hygromycin (40 mg/L) had elongated hypocotyls of approximately 0.8-1.0 cm (typical of dark grown seedlings), whereas non-resistant seedlings had short hypocotyls (0.2-0.4 cm) (Figure 10, A and B). In Figure 10, A and B, seedlings designated ‘a’, ‘b’ and ‘c’ have long hypocotyls (about 0.8-1.0 cm), indicating these three are hygromycin-resistant plants. Seedlings ‘d’, ‘e’ and ‘f’ have short hypocotyls (0.2-0.5 cm) and are hygromycin susceptible.

Kanamycin was used as the selection marker for the *Raffinose Synthase* construct. Kanamycin-resistant transformants have green, open, expanded cotyledons and kanamycin-sensitive seedlings have yellow cotyledons. Seedlings from 'b' and 'e' in Figure 10, A and B show yellow cotyledons, which means seedling 'b' and seedling 'e' are kanamycin-sensitive seedlings. Finally, a glufosinate ammonium resistance marker was used to facilitate selection for the *Stachyose Synthase* transformants. In the presence of 10 mg/L glufosinate ammonium, resistant transformants have green, open, expanded cotyledons, whereas susceptible seedlings have pale unexpanded cotyledons. In Figure 10, A and B, seedlings 'c' and 'f' show pale unexpanded cotyledons, indicating these two are glufosinate ammonium susceptible. The seedling 'a' in Figure 10, A and B shows long hypocotyls (about 1.0 cm) and green, open and expanded cotyledons, indicating this seedling is transgenic with all three selectable marker genes. Characteristics of different antibiotic-resistant seedlings are summarized in Table 2. All offsprings from homozygous plants (*CmGAS1*/*CmGAS1; CsRFS*/*CsRFS; AmSTS1*/*AmSTS1*) should resist all three antibiotics. Ultimately, transgenic homozygous lines GRS63 and GRS47 from T7 generation were selected for further analysis.

3.2 Carbohydrate Steady State Analysis in Mature Leaves

Wild type, GRS47 and GRS63 lines were analyzed for sugar content in leaves by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which can detect picomole quantities of sugar. Sugars in leaves are removed in MCW extracts (methanol: chloroform: water solvent in the ratio 12:5:3 [v/v]). This solvent extracts most low molecular weight compounds including sugars, amino, nucleic and organic acids, and lipids. Chloroform is an organic solvent for lipids and only able to solubilize relatively small amounts of water and remain in a single phase in MCW solvents. Aqueous and organic phases

are separated after water is added to the extract (0.6 volumes). Sugars were analyzed after extraction from fresh tissues and after freezing and grinding in liquid nitrogen. No significant difference was observed between extractions from fresh and frozen tissues (Figure 11). Representative chromatograms are shown in Figure 12. A Dionex CarboPac PA-20 column with 50 mM NaOH as HPAEC eluent was used and under these conditions, Glc and Gal co-elute (Figure 12). Glc and Gal can be readily separated by using a different eluent or by using a CarboPac MA-1 column. Standards containing sugars of known concentration were run with the samples (Figure 12A). Prominent peaks for galactinol (1.4 minutes), raffinose (10.7 minutes) and stachyose (12.0 minutes) are present in extracts from GRS47 and GRS63, but not in WT (Figure 12B, C and D). In Figure 13, sugars are expressed as pmol/mg fresh weight and it is apparent that transgenic homozygous lines GRS63 and GRS47 accumulate substantial levels of galactinol, raffinose and stachyose in leaf tissues (more than 50% of total soluble sugar relative to wild type plants). It is also evident that this accumulation is not at the expense of Glc, Fru, and Suc, but in addition to them. To further analyze carbon metabolism, tissues from wild type and transgenic plants were collected at two different times: after 10 hours of dark treatment and 8 hours of light treatment. Figure 14 shows comparison of sugar components at these two times among WT, GRS47 and GRS63. For wild type plants, Suc, Glc and Fru were increased at least two fold after 8 hours of photosynthesis. However, for transgenic GRS47 and GRS63 lines, Suc, Gol, Raf and Sta levels remained nearly constant after 8 hours photosynthesis and Glc and Fru increased about two-fold after 8 hours of light treatment. Starch analysis by qualitative starch staining with iodine and a quantitative starch assay was also conducted to investigate starch levels between wild type and transgenic plants. Iodine staining showed that there was no difference in starch accumulation between wild type and transgenic plants at two different times: after 10 hours of

dark and 8 hours of light (Figure 15A). In addition, there was more starch accumulation in the leaves after 8 hours of light compared to 10 hours of dark treatment (Figure 15 compare A+B). Quantitative analysis of starch using an enzymatic procedure confirmed the qualitative starch staining experiments: there was no significant difference in starch levels between wild type and transgenic plants after 10 hours of darkness and 8 hours of photosynthesis ($P > 0.1$) (Figure 15B). As expected, starch reserves were nearly depleted after 10 hours of darkness, and there was substantial accumulation after 8 hours of photosynthesis (Figure 15A+B).

3.3 Analysis of Soluble Sugars in Phloem Exudates

In order to investigate RFOs transport efficiency, phloem exudates from cut leaves were collected and analyzed. Exudates were collected for two, two-hour intervals and analyzed by HPAEC-PAD (Figure 16). Carbohydrates in exudates from wild type and transgenic plants were compared as exudation rate (pmol/mg fwt/hour). Suc is the major transport sugar in both wild type and transgenic Arabidopsis plants (Figure 16A). RFOs were identified in phloem exudates from excised leaves of transgenic plants, whereas levels were negligible in exudates from wild type leaves (Figure 16B). Therefore, RFOs are phloem mobile in transgenic Arabidopsis plants. In addition, total rates of Glc, Fru and Suc exudates from transgenic plants are greater than that of wild type plants, especially for Suc which is present in exudates of GRS47 and GRS63 at nearly twice the level of exduates from wild type plants. In order to test if RFOs are degraded to Suc in phloem, Gal in phloem exudates was separated from Glc by using 10 mM NaOH eluent instead of 50 mM eluent. Table 3 shows there is no difference for Gal in phloem exudates between wild type and transgenic lines, limiting the possibility of degradation of RFOs in phloem. It is possible that transgenic plants producing RFOs transport more sugars through the

phloem than wild type plants because production of RFOs in source tissue increases the hydrostatic pressure.

3.4 Growth Rate Analysis

The total rosette area of the two independent homozygous lines GRS63 and GRS47 and wild type plants was measured using ImageJ software (Rasband, 2007). Transgenic Arabidopsis plants producing RFOs did not show significant differences in vegetative growth (Figure 17).

3.5 Flowering Time Analysis

In addition to growth rate analysis, flowering time was monitored to analyze transition to reproductive growth between wild type and transgenic plants. Transgenic lines, GRS47 and GRS63, were observed to flower earlier than wild type plants (Figure 18). Under long-day conditions, 50% of 36 wild type plants started to grow inflorescence from rosettes 23 days after germination. In contrast, under identical conditions transgenic GRS47 and GRS63 lines started to grow inflorescence from rosettes 21 days after germination (Figure 18A): this difference is significant (T-test $P<0.01$, $n=36$). Furthermore, the number of rosette leaves produced at flowering in transgenic GRS47 and GRS63 lines is less than wild type plants and shows significant difference ($P< 0.01$, $n=36$) (Figure 18B). GRS63 and GRS47 lines 36 days after germination had longer primary inflorescence stems than WT because of earlier flowering (Figure 19).

3.6 Aphid Experiments

Recently, RFOs were shown to play an important role in stress tolerance, including drought-stress tolerance, cold-stress tolerance and oxidative-stress tolerance (Bailly et al., 2001; Taji et al., 2002; Peters et al., 2007; Nishizawa et al., 2008). Galactinol has been suggested to be

a signaling component of the induced systemic resistance caused by pathogens (Kim et al., 2008). In order to test whether the RFOs also impact resistance against aphid damage, "no-choice" aphid experiments and "choice" aphid experiments were performed by using wild type Arabidopsis and transgenic Arabidopsis plants producing RFOs. In order to determine if transgenic plants producing RFOs can influence aphid growth, we compared aphid growth on wild type and transgenic plants in "no-choice" aphid bioassays. 20 adult aphids were put on each wild type and transgenic plant (n = 12). After 48 hours, the plants were harvested and the number of aphids residing on each plant was counted. Aphids on plants producing RFOs had a lower reproduction rate than WT ($P<0.05$) (Figure 20).

To investigate further the role of RFOs in aphid feeding, choice aphid experiments were also performed. Adult aphids were given the choice of feeding on WT or transgenic plants by releasing 20 aphids equidistant from WT and transgenic plants grown in the same pot. The number of aphids that had settled on each plant was determined after 8 h and 24 h. The results presented in Figure 21 A and B show that aphids have a significant preference for WT plants ($P<0.001$) over RFO producing plants after 24 h aphid release. However, there is no significant preference after only 8 h aphid release (Figure 21C). This indicates that aphids did not show preference until they were feeding on different plants (i.e., there appears to be no difference in the initial attraction to either plant). In addition, as a control for the choice aphid bioassays, aphids were given a choice between two plants of the same kind (i.e., WT#1 and WT#2 or GRS63#1 and GRS63#2) in the same pot, and no feeding preference was observed ($P > 0.2$) (Figure 22). To summarize, the results from no-choice aphid experiments and choice aphid experiments indicate that RFO-producing plants negatively impact aphid feeding and fecundity.

To test if this resistance to aphid feeding is a direct or indirect effect of the exotic sugars, aphid fecundity on artificial media with and without RFOs was performed. The artificial diet used consisted of amino-acids, mineral salts, vitamins and 500 mM sucrose (Table I). To this basic diet, 50 mM of one of sucrose, fructose, glucose, galactinol, raffinose or stachyose was added. After 4 days, no difference in aphid populations were observed among treatments ($P > 0.1$, $n=3$) (Figure 23). These results indicate that the resistance to aphid feeding is an indirect effect of the exotic sugars. The synthesis of RFOs may contribute other defense responses, such as secondary metabolism, which provides the resistance to aphid feeding.

A. **B.**

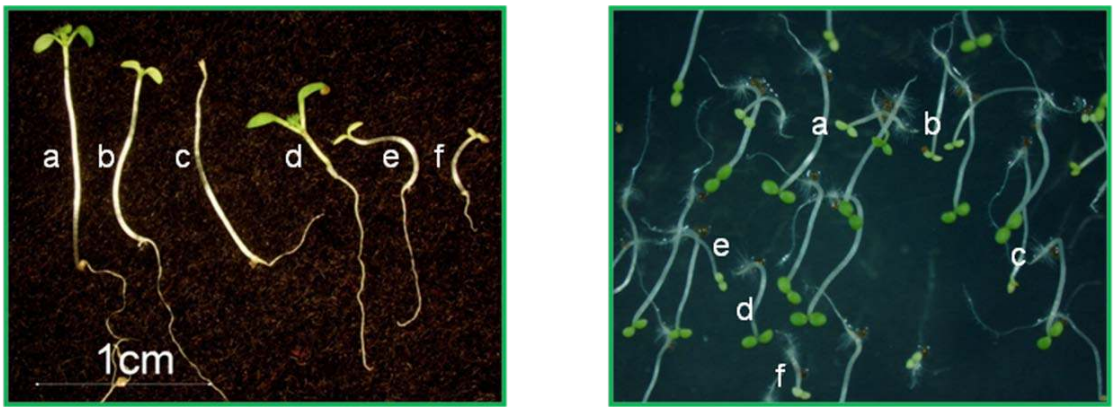


Figure 10. Selection of seedlings based on antibiotic/herbicide resistance. **A.** Phenotype of seedlings based on antibiotic resistance. **B.** Phenotype of segregating population of seedlings based on their antibiotic resistance. "a" seedling is transformed with all three selectable markers; "b" seedling is resistant to phosphinothricin and hygromycin, but not to kanamycin; "c" seedling is resistant to hygromycin only; "d" seedling is resistant to both kanamycin and glufosinate ammonium, but is susceptible to hygromycin; "e" seedling is resistant to only phosphosphinothricin, and "f" seedling is susceptible to all three antibiotic.

Table II. *Summary for different antibiotic-resistant seedlings*

	Hygromycin		Kanamycin	Phosphosphinothricin
	Resistant	long hypocotyls	green, open, expanded cotyledon	green, open, expanded cotyledons
	Susceptible	short hypocotyls	yellow cotyledons	pale unexpanded
a seedling	Resistant		Resistant	Resistant
b seedling	Resistant		Susceptible	Resistant
c seedling	Resistant		Susceptible	Susceptible
d seedling	Susceptible		Resistant	Resistant
e seedling	Susceptible		Susceptible	Resistant
f seedling	Susceptible		Susceptible	Susceptible

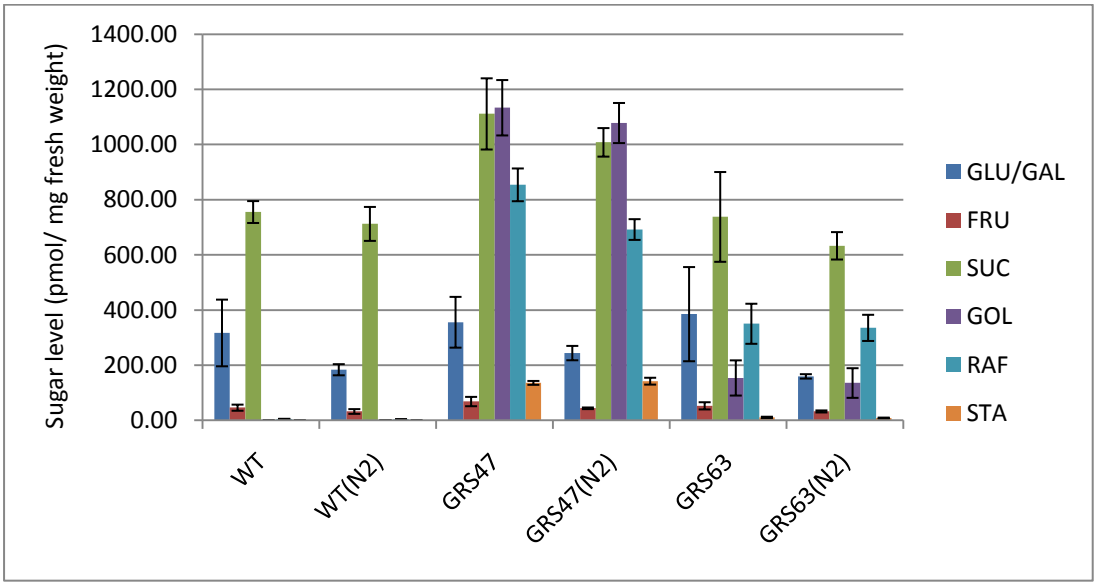
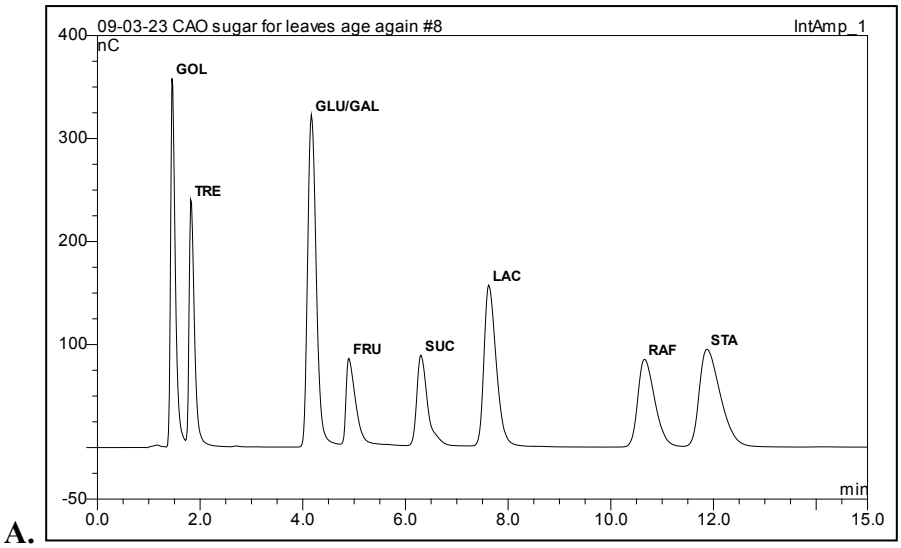
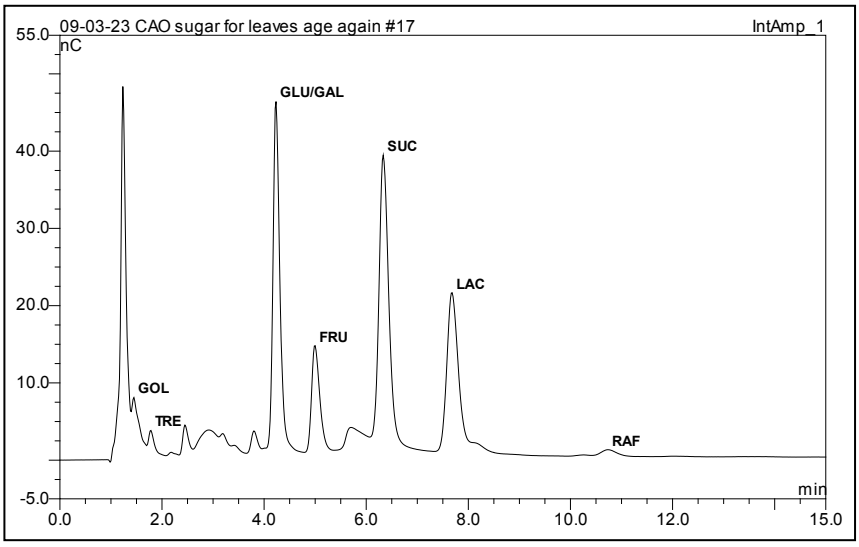


Figure 11. Comparison of sugar concentrations extracted from fresh tissue (labeled as plant line names) and from tissue frozen and ground in liquid nitrogen (labeled as plant line names with N2 in parentheses) (pmol/mg fresh weight). Variation is expressed as SE; n=6 sibling plants.



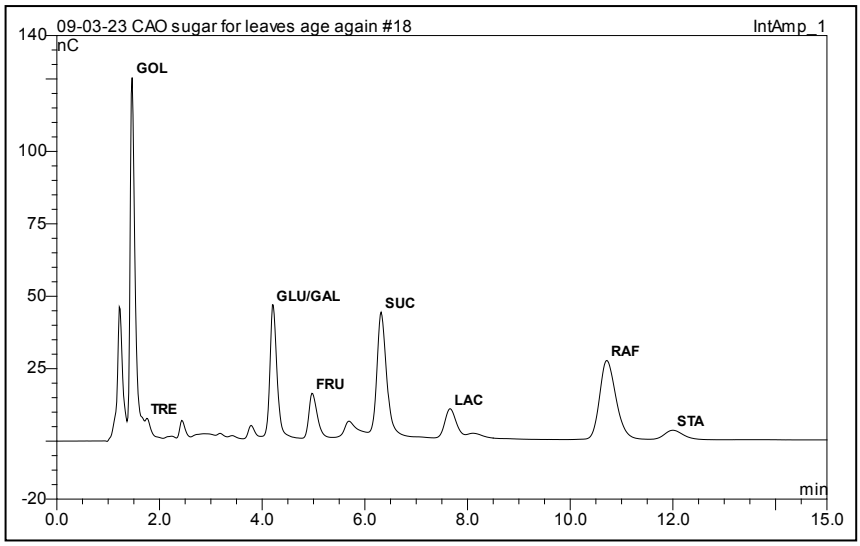
A.



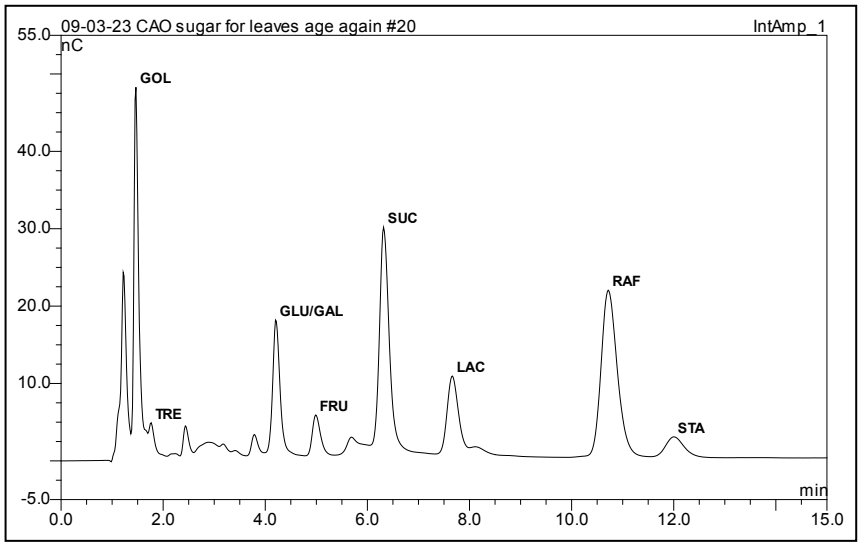
B.

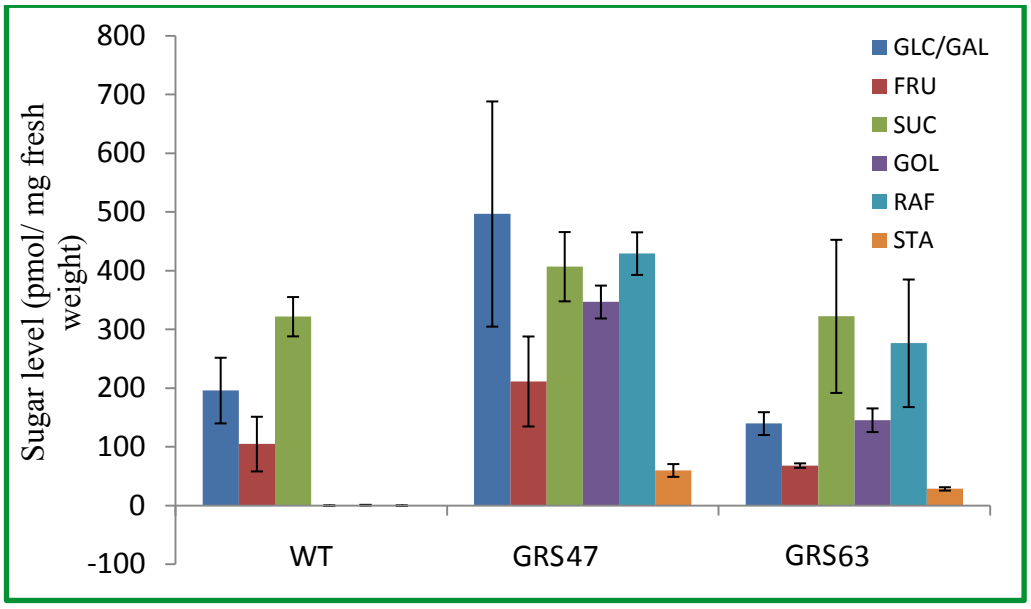
Figure 12. Representative chromatograms of sugar extracts from high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). X axis, elution time; Y axis, nano coulombs. **A.** Sugar standard. **B.** Wild type Arabidopsis plants. **C.** Transgenic line GRS47. **D.** Transgenic line GRS63. A Dionex CarboPac PA20 analytical column was used and the eluent was 50 mM NaOH.

C.

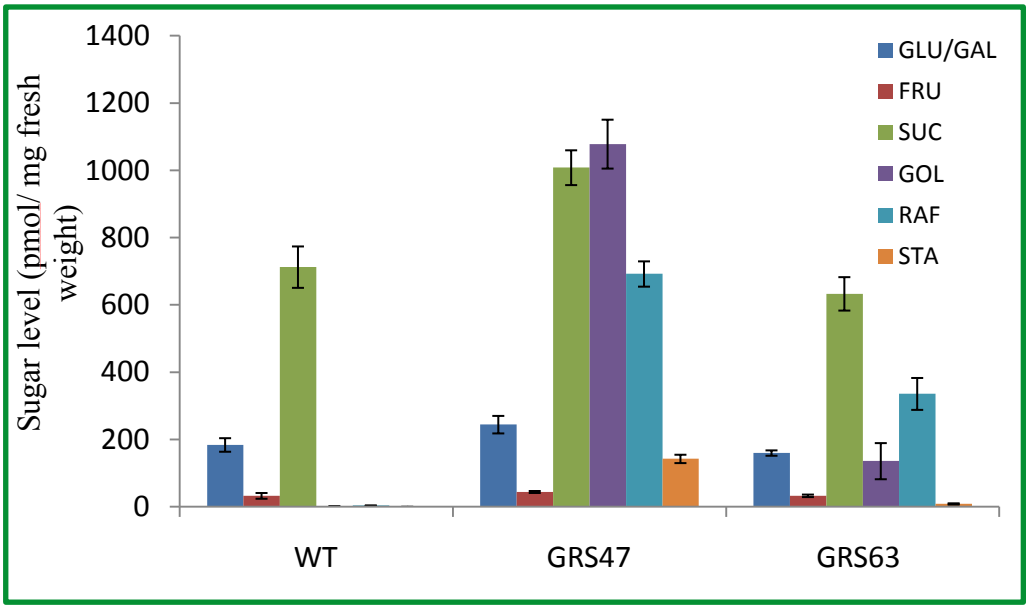

09-03-23 CAO sugar for leaves age again #18
IntAmp_1
nC
140
100
75
50
25
-20
GOL
TRE
GLU/GAL
FRU
SUC
LAC
RAF
STA
min
0.0
2.0
4.0
6.0
8.0
10.0
12.0
15.0

D.


09-03-23 CAO sugar for leaves age again #20
IntAmp_1
nC
55.0
40.0
30.0
20.0
10.0
-5.0
GOL
TRE
GLU/GAL
FRU
SUC
LAC
RAF
STA
min
0.0
2.0
4.0
6.0
8.0
10.0
12.0
15.0

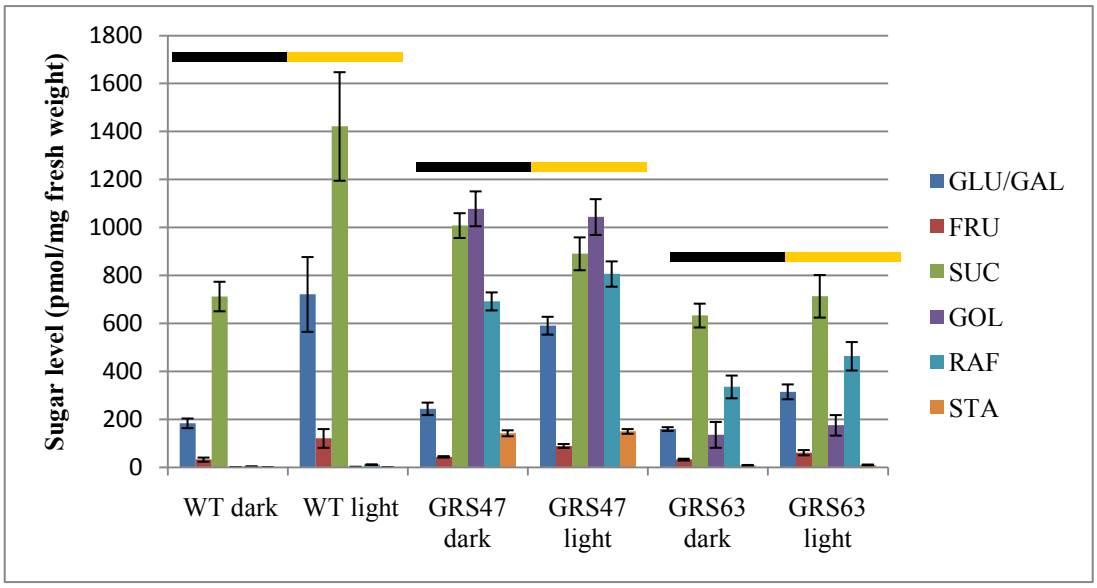


A.



B.

Figure 13. Sugar analysis in leaves of WT and transgenic Arabidopsis plants (pmol/mg fresh weight). Results from two experiments are represented. **A.** The results from experiment on 05/17/2008. Variation is expressed as SE n=3 sibling plants. **B.** The results from experiment on 11/14/2009. Variations is expressed as SE n=6 sibling plants.



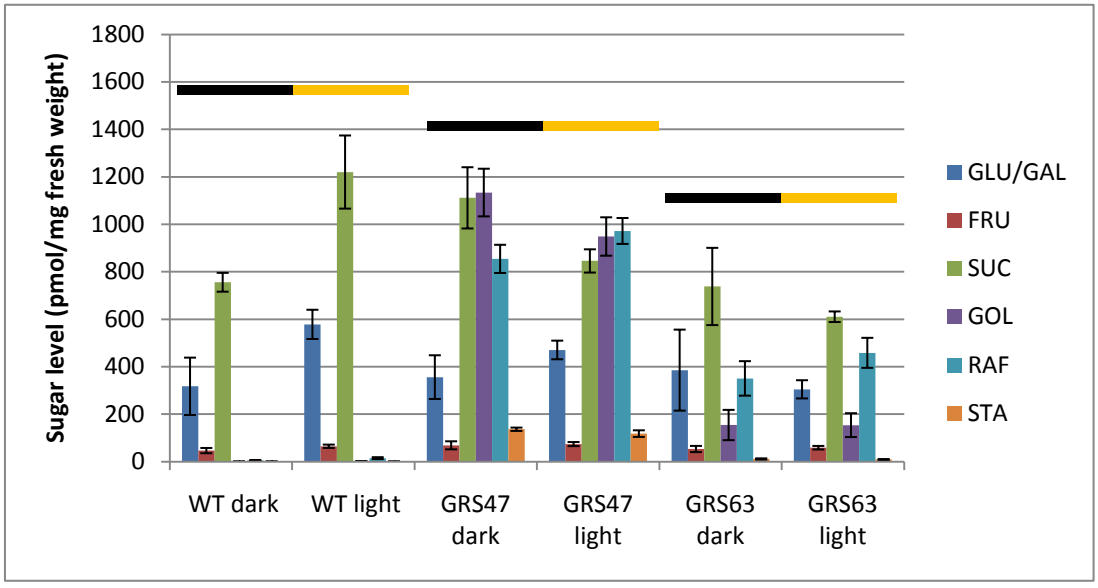


Figure 13. Comparison of sugar components between 10 hours dark treatment and 8 hours light treatment (pmol/mg fresh weight). Variation is expressed as SE; n=6 sibling plants. **A.** Results from tissues frozen and ground in liquid N_2. **B.** Results from fresh tissues.

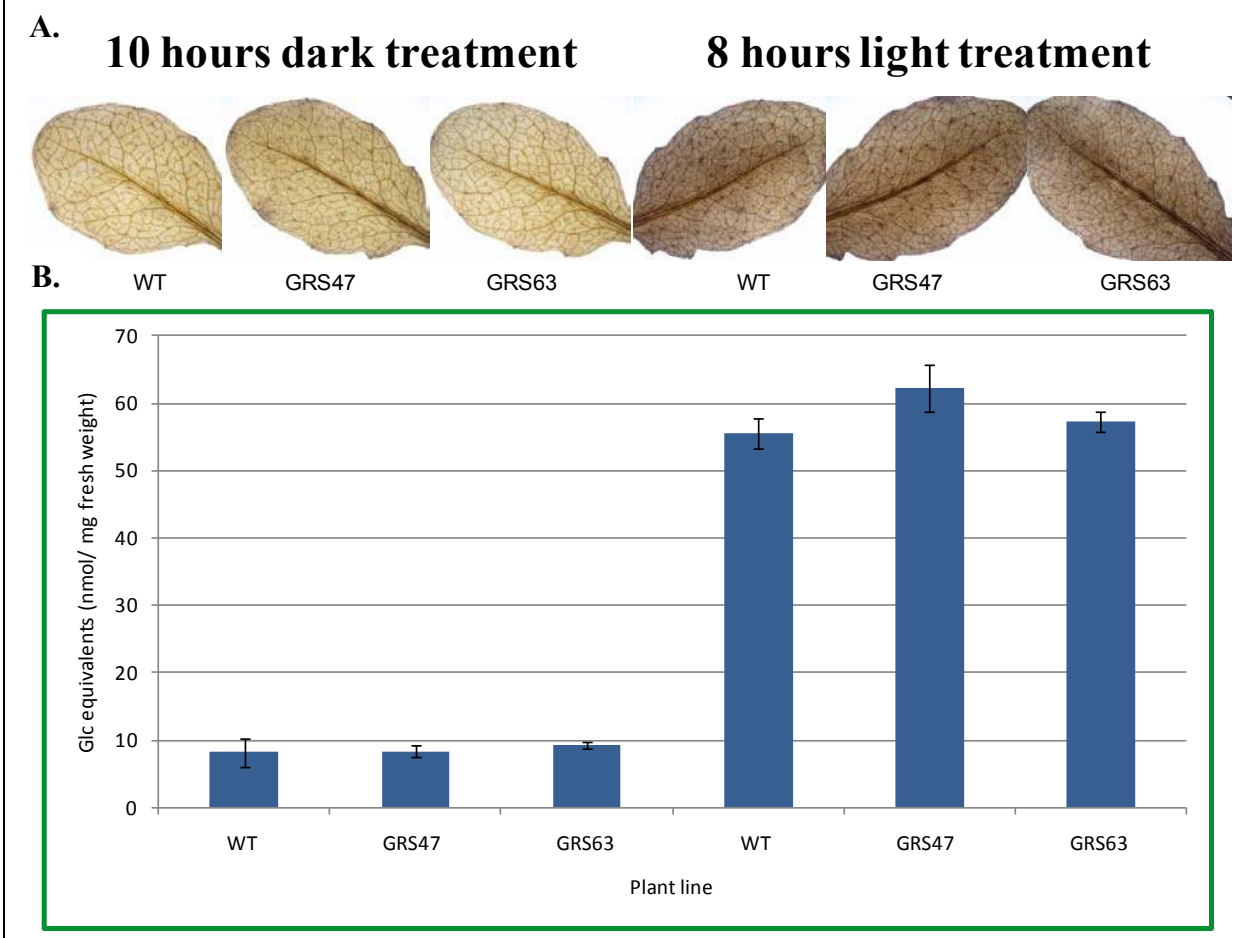
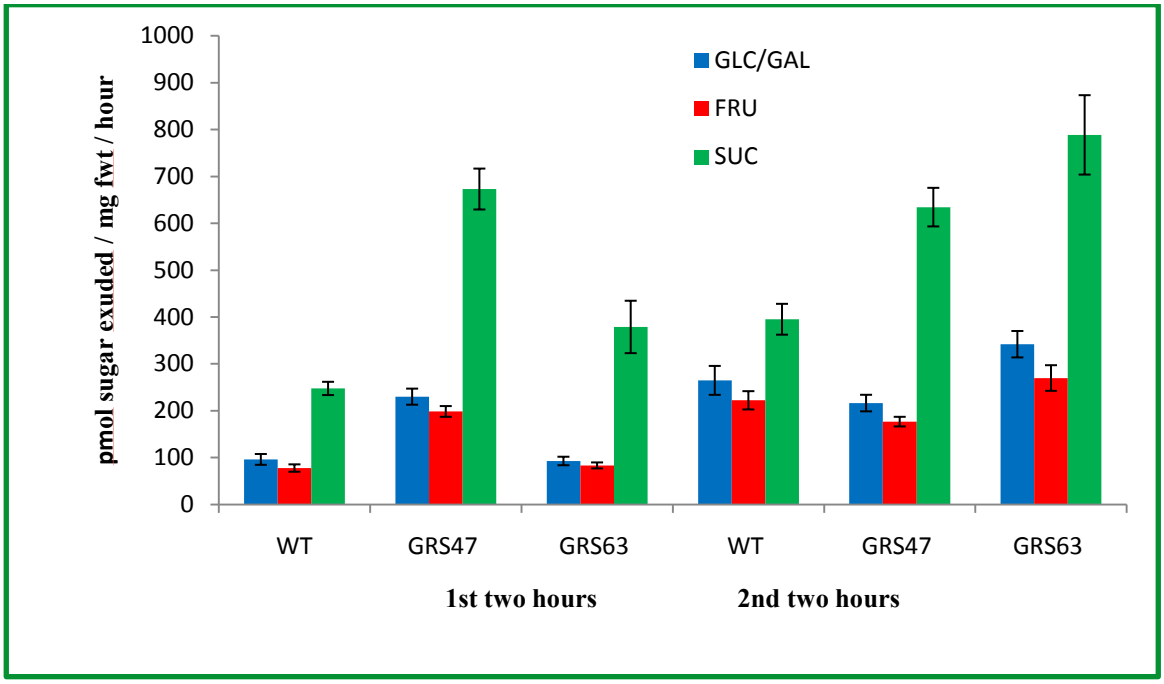


Figure 15. Starch staining and starch assay in wild type (WT) and transgenic plants (GRS63 and GRS47) after 10 hours of dark treatment and 8 hours of light treatment. **A.** Pictures from starch staining experiment. **B.** Chart from starch assay. Exposure time and settings were equivalent for all samples.

A.



B.

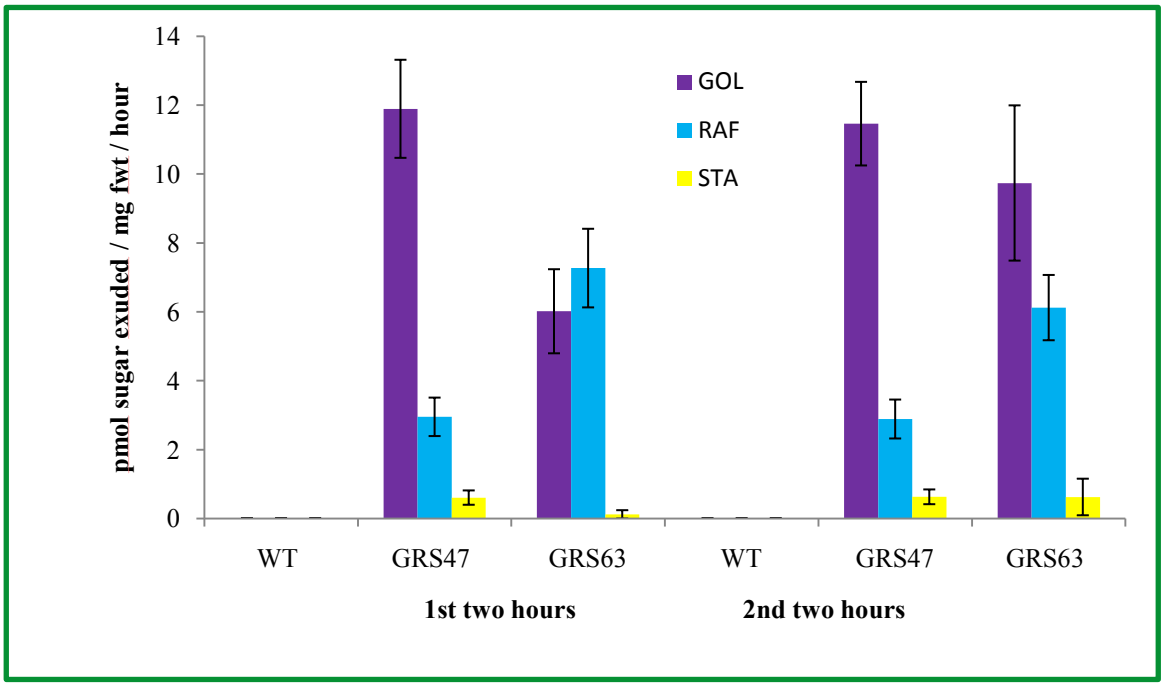


Figure 16. Transport carbohydrates in phloem exudates from wild type and transgenic plants. **A.** Comparison for glucose, fructose and sucrose. **B.** Comparison for galactinol, raffinose and stachyose. Variation is expressed as SE; n=12 sibling plants. Note the difference scales of the Y access between A and B.

Table III. *Galactose in phloem exudates from wild type and transgenic plants with exudation rate (pmoles sugar exuded / leaf mg fwt / hour) by using 10 mM or 50 mM NaOH eluent. Variation is expressed as SE; n = 12 sibling plants*

	1st 2 hours			2nd 2 hours		
	WT	GRS47	GRS63	WT	GRS47	GRS63
Gal (10 mM)	2.1±0.6	2.7±1.0	2.0±1.0	1.4±0.6	3.0±0.7	4.6±0.8
Glc (10 mM)	130.1±10.2	206.9±24.9	68.8±18.0	194.5±31.7	196.8±20.6	282.2±27.2
Glc/Gal (10 mM)	132.2±10.8	209.6±25.8	70.8±19.0	195.9±32.3	199.7±21.3	286.9±28.0
Glc/Gal (50 mM)	96.2±11.5	230.0±17.2	92.8±9.1	264.8±30.8	216.4±17.6	341.9±28.2

A.

B.

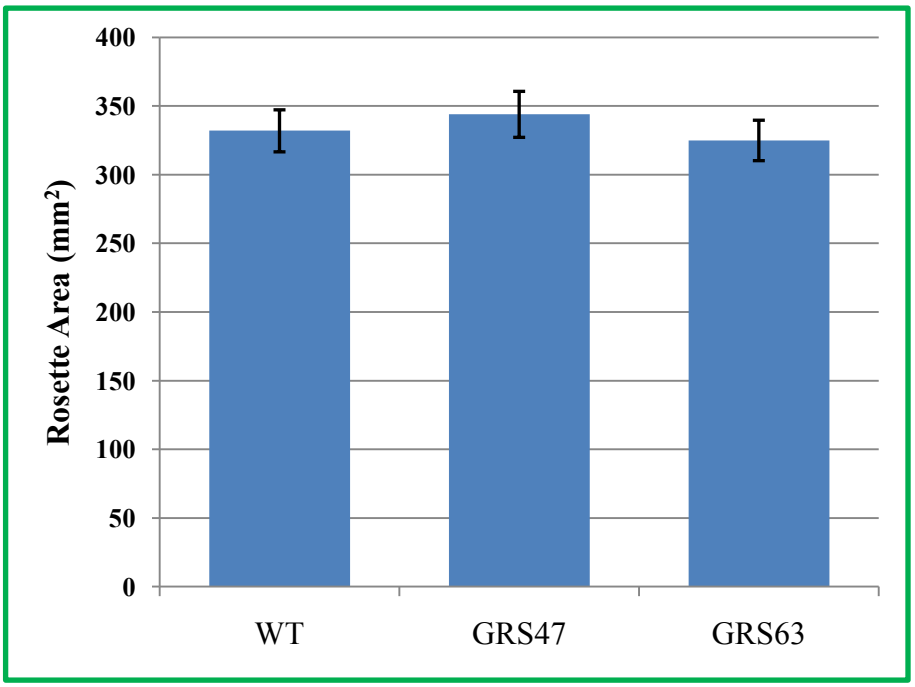
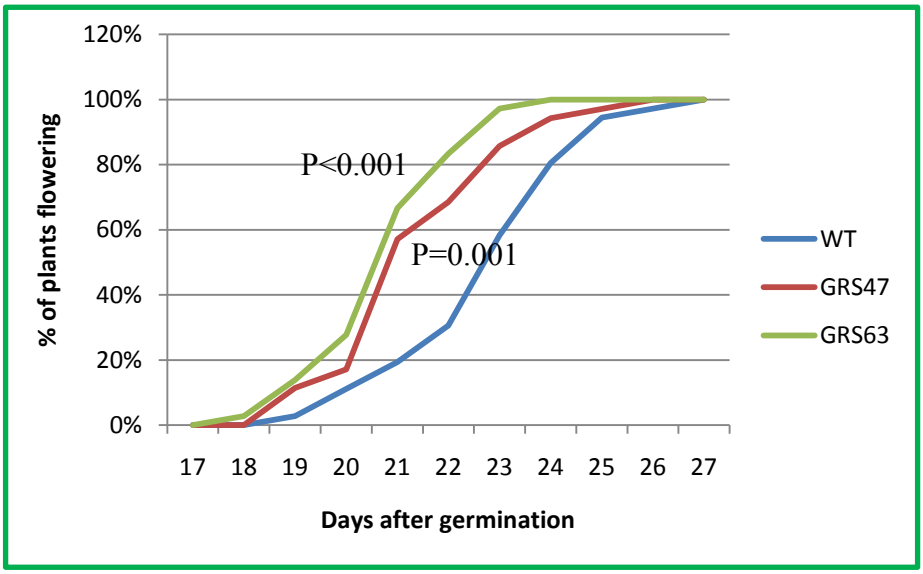


Figure 17. Rosette growth analysis. **A.** Qualitative analysis (Phenotypic comparison between transgenic and wild type plants); Scale bar = 1 cm. **B.** Quantitative analysis and comparison of total rosette area of wild type and transgenic plants. Average and SE values from 12 replicates are represented.

A.



B.

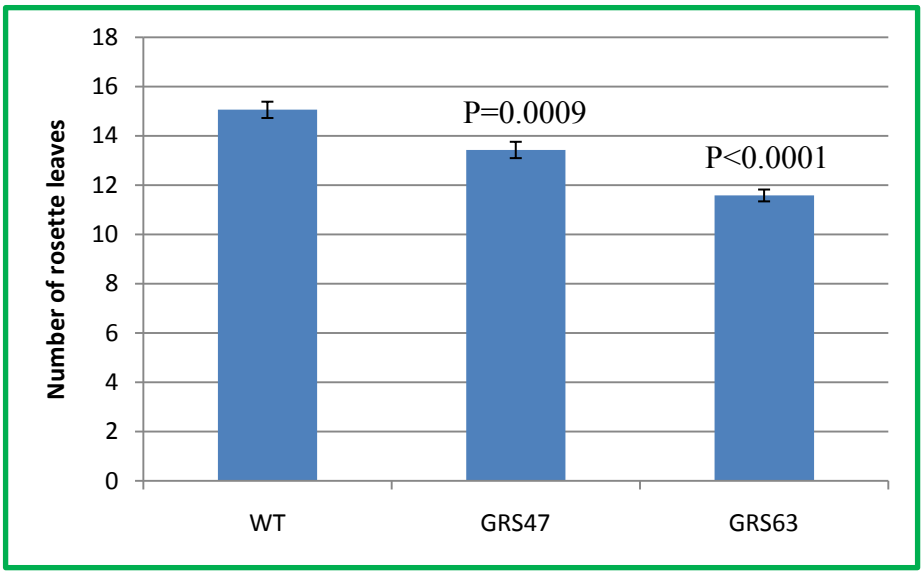
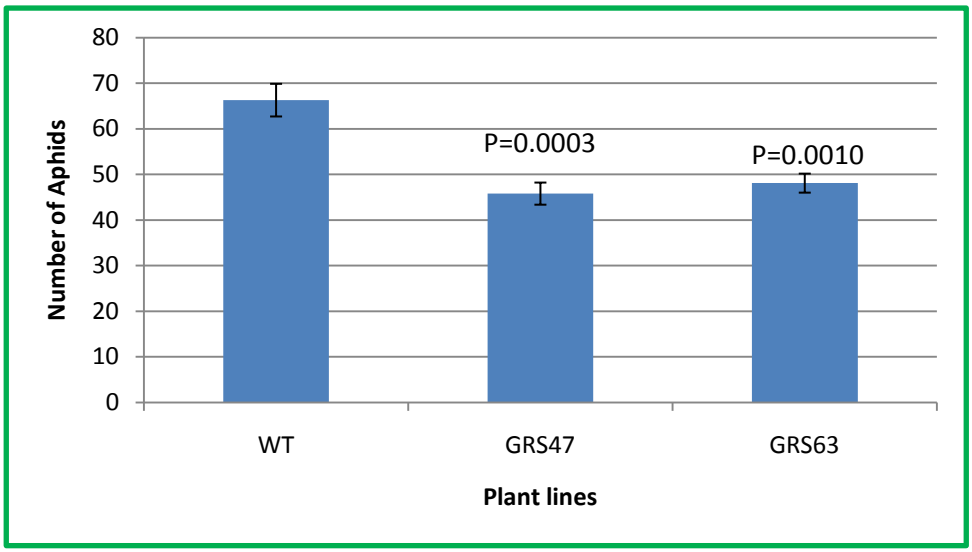


Figure 18. Flowering time analysis. **A.** Percentage of plants flowering relative to days post germination. **B.** Number of rosette leaves at the time of flowering. Average and SE values from 36 replicates are represented. The same experiment was repeated twice. P is from Student's t-test.

Figure 19. GRS63, WT and GRS47 lines 36 days after germination.

A.



B.

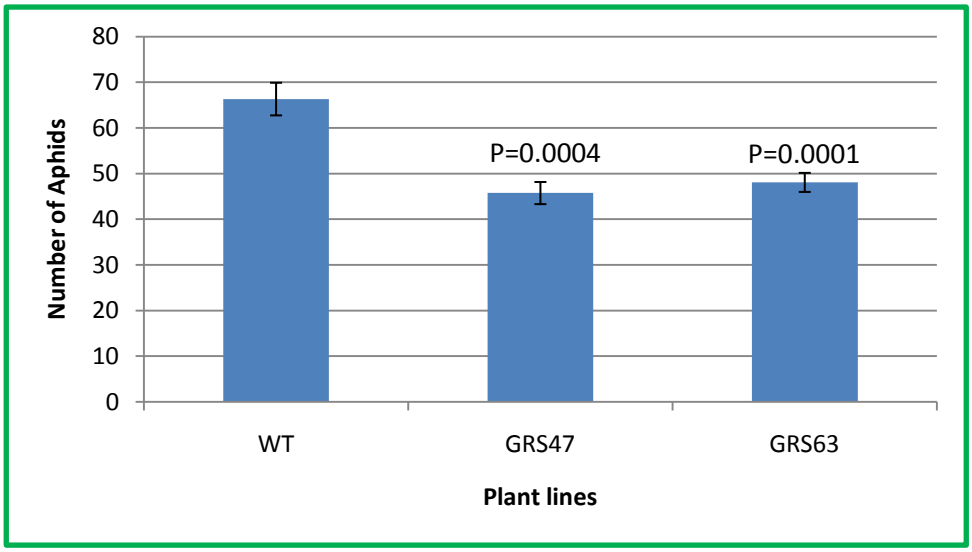
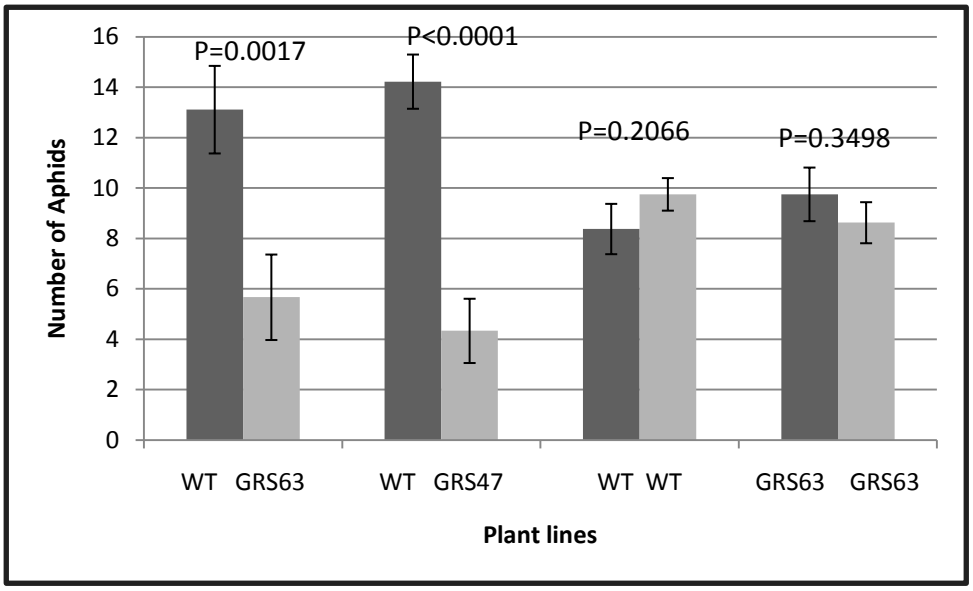


Figure 20. No-choice test comparison of aphid growth for wild type (WT) and homozygous lines GRS63 and GRS47. Average and SE values from 12 replicates are represented. The same experiment was repeated twice. **A.** The results from the first experiment. **B.** The results from the second experiment. P is from Student's t-test.

A.



B.

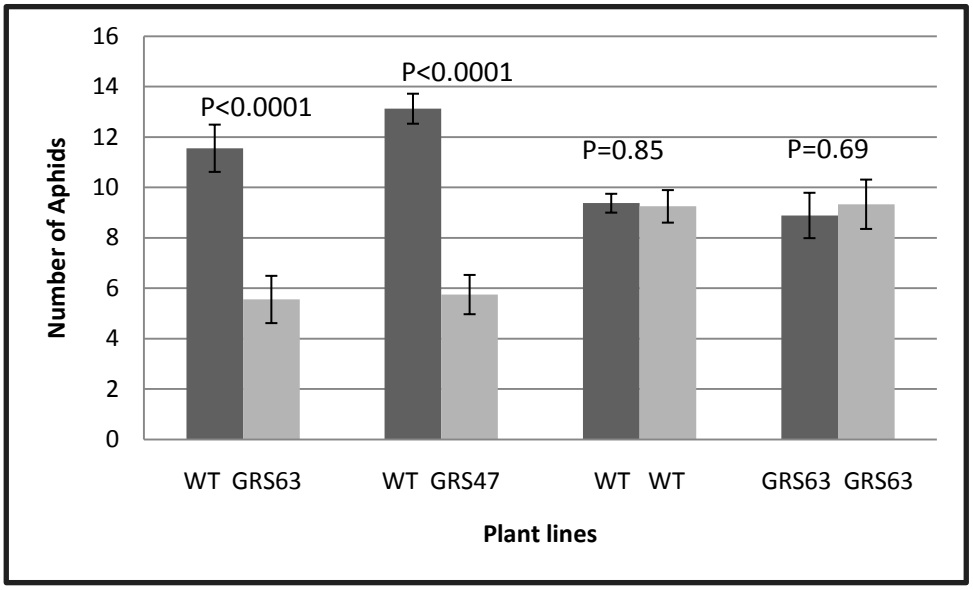
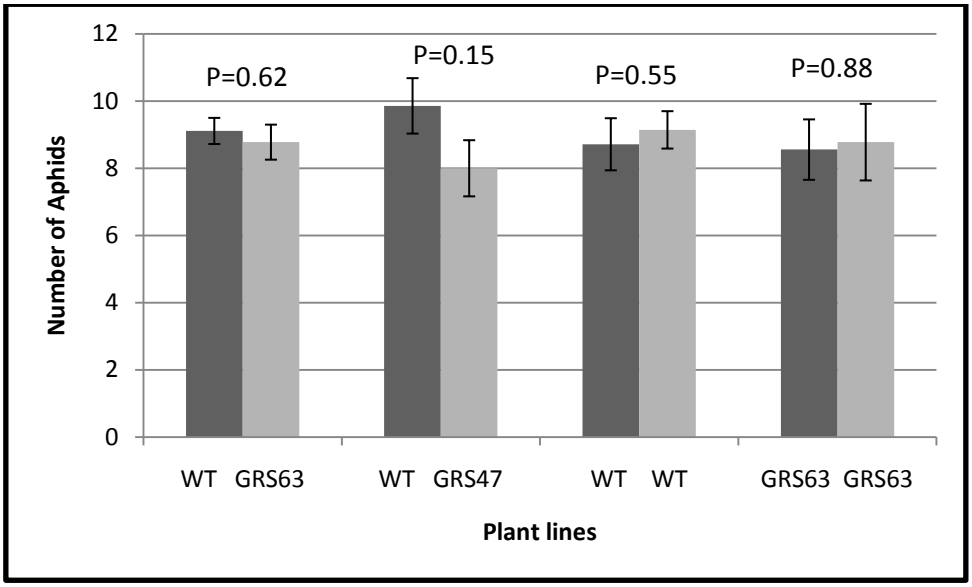
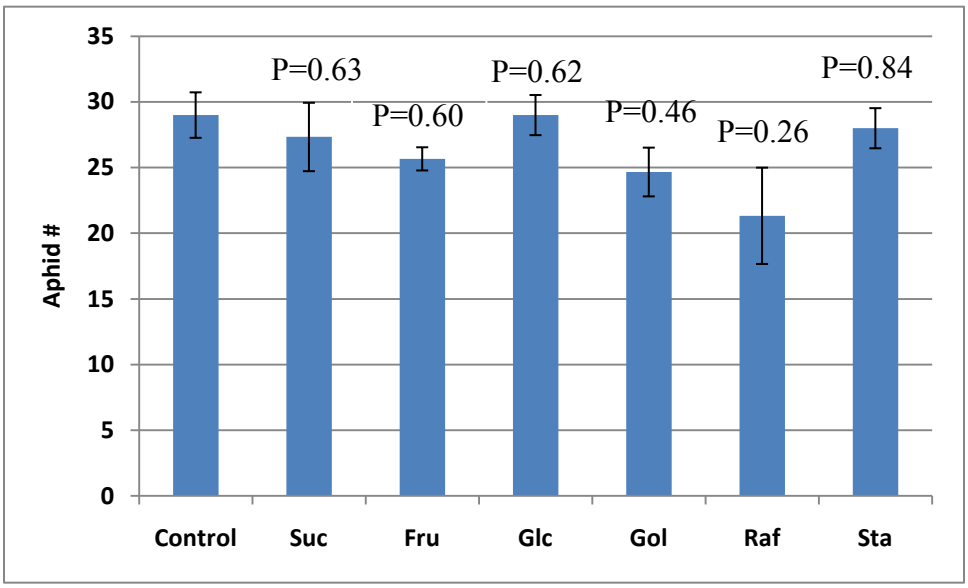


Figure 21. Choice test comparison of aphid preference for wild type (WT) and homozygous lines GRS63 and GRS47. **A.** The results after 24 hours aphid release from first experiment. **B.** The results after 24 hours aphid release from second experiment. **C.** The results after 8 hours aphid release from second experiments. Average and SE values from 9 replicates are represented. P is from Student's t-test.

C.


12
10
8
6
4
2
0
Number of Aphids
P=0.62
P=0.15
P=0.55
P=0.88
WT GRS63
WT GRS47
WT WT
GRS63 GRS63
Plant lines

A.



B.

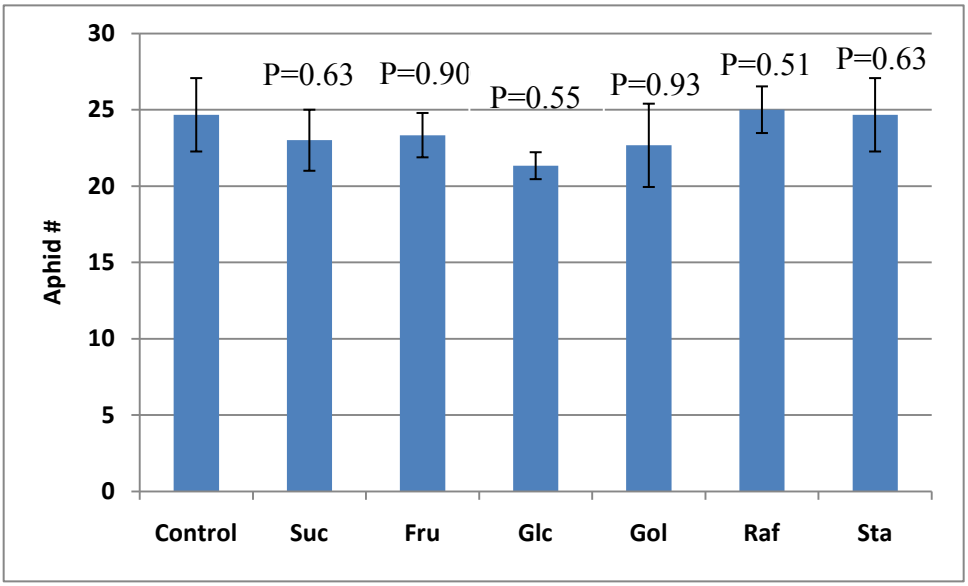


Figure 22. Artificial aphid diet test. Average and SE values from 3 replicates are represented. Experiment was conducted twice with similar results each time. **A.** Results from first experiment. **B.** Results from second experiment. P is from Student's t-test.

CHAPTER 4

DISCUSSION

4.1 The Effect of RFOs Synthesis in Transgenic Arabidopsis

One of the purposes of this study was to generate novel sugars specifically in the minor veins of source leaves and analyze carbon catabolism. Introducing genes encoding GolS, RafS, and StaS driven by phloem-specific promoters had a dramatic impact on the levels of RFOs. Sugars in two lines are expressed as pmol/mg fwt in Figure 13 and are compared with wild type Arabidopsis. It is apparent that RFOs accumulated to as much as 50% of the soluble carbohydrate without a compensating decreasing in Suc, Glc, or Fru. In these studies, suc produced in mesophyll cells through photosynthesis is used to produce RFOs in the phloem. However, the levels of RFOs observed suggest they are not confined to the SE-CCC since the phloem constitutes only a minor portion of the leaf. RFOs are likely distributed throughout the leaf and stored in vacuoles or accumulated in the apoplast. *Coleus blumei* is an archetype for RFOs translocation (Turgeon et al., 1990). As a positive control in this study, Coleus was detected to produce about 1835 pmol/mg fwt Glc/Gal, 802 pmol/mg fwt Fru, 1044 pmol/mg fwt Suc, 195 pmol/mg fwt Gol, 988 pmol/mg fwt Raf and 250 pmol/mg fwt Sta in green leaves (data not shown). In previous study, Haritatos *et al* detected radiolabel distribution in source leaves of Coleus after exposure to $^{14}CO_2$ and radiolabel was found in Suc (100), Sta (118), Raf (37) and Gol (40) (Haritatos et al., 2000).

Plants generally do not store carbohydrate in the form of soluble sugars, but rather as starch, and starch levels can fluctuate significantly as levels of soluble sugar remain relatively constant. In response to salinity stress, coleus has been shown to build temporary carbon reserves

as the synthesis of high-degree of polymerization RFOs to replace the normal starch storage reserves, which are greatly reduced during the initial phases of salinity stress (Gilbert et al., 1997). Comparing starch levels between WT and transgenic lines revealed no significant difference either dawn or dusk (Figure 15). The production of 50% the soluble carbohydrate in transgenic plants was not from degradation of starch in source leaves.

Another purpose of this study was to analyze the impact of increasing RFOs on plant vegetative growth and reproductive growth. How do plants respond to increasing levels of novel sugars, mainly RFOs? In order to answer this question, growth rates were analyzed. From the results of rosette area measurements, homozygous lines GRS63 and GRS47 did not show alterations in rosette area at eighteen days post germination (Figure 17). This result shows that RFOs synthesis does not affect vegetative growth rate. However, GRS47 and GRS63 lines flowered earlier than wild type (Figure 18 and Figure 19). It is possible that increasing export of sugars in transgenic plants caused earlier flowering, since it is well known that elevated sugar-levels either endogenous to the plant or add exogenously can accelerate the transition to flowering (Corbesier et al., 1998). Sugars are important in the accomplishment of sexual reproduction in the grapevine because they are the main source of energy and also signal molecules involved in the regulation of reproductive development (Lebon et al., 2008). In addition, *StSUT4*-RNAi (sucrose transporter from potato) led to early flowering at least 6 days before wild-type flowering and had significantly fewer leaves at flowering time than wild-type plants because Suc export from phloem exudates in *StSUT4*-RNAi plants was twice as much as in wild type plants at the end of the light period (Chincinska et al., 2008). StSUT4 has been shown to express prominently in sink tissues and play an important role in flowering by interacting with StSUT1 to regulate carbon availability. *StSUT4* was an inhibitor of *StSUT1*

highly expressed in source leaves, the most important Suc transporter for Suc efflux from mature leaves. Suc efflux from leaves of *StSUT4*-RNAi plants was increased by the missing *StSUT4*-mediated *StSUT1* inhibition.

4.2 Hexoses Transport in Transgenic Arabidopsis

EDTA exudation was used to assess sugar transport out of leaves. The HPAEC-PAD system was applied to quantify which sugars are being transported as a result of metabolic engineering. Sugars in EDTA exudations show Suc, Glc and Fru, with Suc predominating (Table IV). Glc and Fru were present at almost equal concentration (Table IV). It has previously been concluded that transport sugars in the phloem are non-reducing sugars, like Suc, alcohol sugars (sorbitol, mannitol) and RFO, because reducing sugars are too reactive. The presence of Glc and Fru in phloem exudates, although commonly observed, was thought to be due to Suc degradation (Corbesier et al., 1998). More recently, however, hexose was proposed to be legitimate phloem transport sugars (van Bel et al., 2008). van Bel and colleagues measured sugars from EDTA phloem exudates from a large number of plant families, and found that the Ranunculaceae and Papaveraceae translocated more than 80% of carbohydrates in the form of hexoses. They proposed that hexose translocation is a normal mode of carbohydrate transfer by phloem in these lineages because the rate of Suc cleavage is too low to explain the large proportions of hexoses. By contrast, Asteraceae and Fabaceae, two families used as controls, showed low hexose: sucrose ratios in the exudates with high sucrose concentrations in phloem sap. For example, *Dahlia* hybrid from Asteraceae family has been shown that the hexose: sucrose ratio in exudates is 0.13, which means 11.5% hexose and 88.5% sucrose in phloem. For another example, *Galinsoga parviflora* also from Asteraceae family had 0.71 as hexose: sucrose ratio in exudates, which means 41.5% hexose and 58.5% sucrose in phloem exudates. The authors suggested that

higher hexose: sucrose ratios in exudates compared to leaf extracts would lend credibility to the view that hexoses act as a prominent phloem translocate. Comparing Table V and Table VI, it is obvious that hexoses are transport sugars because of higher hexoses: sucrose ratios in exudates than in leaves. van Bel and colleagues did not study *Arabidopsis thaliana*. However, Haritatos and colleagues reported most of the translocated sugar in *Arabidopsis thaliana* is sucrose without mentioning hexose translocation (Haritatos et al., 2000). Hexose translocation in *Arabidopsis thaliana* needs further analysis in the future.

4.3 Galactinol Transport in Transgenic Arabidopsis

Wild type Arabidopsis normally transports sucrose, but not galactinol nor RFOs. However, an endogenous RFOs metabolism exists in Arabidopsis and is often induced during stress. During the biosynthesis of RFOs, galactinol serves as the donor of the galactosyl moiety. At 3 hours after oxidative stress by treatment with 50 μM methylviologen (an enhancer of the production of O_2^-), the levels of galactinol and raffinose in leaves of Arabidopsis were 32.2 ± 3.6 and 44.5 ± 9.8 nmol/ g fresh weight, respectively, and 130.7 ± 10.5 and 177.2 ± 46.5 nmol/ g fresh weight after 6 hours treatment, respectively, whereas galactinol and raffinose were hardly detectable in the untreated plants (Nishizawa et al., 2008). Therefore, galactinol is found to accumulate to substantial amounts in leaves of Arabidopsis in response to oxidative stress and gradually converted to RFOs. From Figure13B, 1078 pmol/mg fwt of galactinol for GRS47 and 136 pmol/mg fwt for GRS63 were accumulated in leaf tissues. Despite 33.6% of total sugar being galactinol for GRS47 and 10.4% of total sugar being galactinol for GRS63 (Table V), the relative amount of galactinol in phloem exudates from excised leaves of transgenic plants was negligible: 1.1% for GRS47 and 0.7% for GRS63 (Table VI). A similar result was also reported by Ayre et al (2003). Tobacco plants with companion cell specific expression of galactinol

synthase were shown to accumulate galactinol in the leaf, but only limited levels of galactinol were transported in phloem (Ayre et al., 2003). In a subsequent study, Hannah *et al* produced raffinose in potato companion cells by using either the *rolC* promoter or the CaMV 35S promoter and found that galactinol accumulated to similar high levels in the leaves from both rolC: GS and 35S: GS plants due to the majority of GS being expressed in the companion cell, with less translocation of galactinol (about 1%) into phloem (Hannah et al., 2006). In this paper, the *rolC* promoter was used to drive direct companion cell specific expression for *Galactinol Synthase* (GS) from *C. sativus* to produce transgenic line "rolC: GS", while 35S promoter was used as control for constitutive non-specific overexpression to produce "35S: GS". Similarly, coleus, which synthesizes and translocates RFOs naturally, also produces substantial galactinol in leaves, which cannot be detected in phloem exudates (Gilbert et al., 1997).

4.4 RFOs Transport in Transgenic Arabidopsis

High levels of RFOs (about 50% of total soluble sugars) accumulated in leaf tissues in transgenic Arabidopsis (Figure13). This accumulation is not at the expense of Glc, Fru, and Suc, but in addition to them. In Figure 16B, RFOs were identified in phloem exudates from leaves of transgenic plants, whereas levels of RFOs were negligible in exudates from wild type leaves. Therefore, RFOs are phloem mobile in transgenic Arabidopsis plants. This observation was also reported in previous papers. Labeled sucrose, raffinose, and stachyose were transported from green to nonchlorophyllous regions of detached *Coleus blumei* leaves after exposure to $^{14}CO_2$ (Turgeon et al., 1990). In addition, labeled stachyose, sucrose and raffinose were detected in phloem exudates after labeling treatment in leaves of *Coleus blumei* with $^{14}CO_2$ (Gilbert et al., 1997). Transgenic potato, which produced raffinose in potato companion cells by using the *rolC* promoter, had significant amounts of raffinose in phloem exudates (Hannah et al., 2006). In this

paper, the *rolC* promoter was used to drive direct companion cell specific expression for *Galactinol Synthase* (GS) from *C. sativus* to produce transgenic line "rolC: GS", while 35S promoter was used as control for constitutive non-specific overexpression to produce "35S: GS". rolC: GS 25 and 31, and 35S: GS 41expressing *Galactinol Synthase*, were selected to retransform with *Raffinose Synthase* from *C. sativus* by using the same promoter (*rolC* promoter or control promoter 35S) to produce "rolC: GS/RS" and "35S: GS/RS" lines. Raffinose in "rolC: GS/RS" line accumulated to only 20-30% of that in "35S: GS/RS" lines presumably because the 35S promoter expresses in most plant organs and not only in company cells. Raffinose was shown to translocate from source tissues to sink tissues through phloem by using grafting experiments. Higher raffinose in sink tissue (tubers) was detected in rolC:GS/RS self grafts than rolC:GS/RS stocks grafted to WT scions indicating a contribution of shoot supplied raffinose (Hannah et al., 2006). However, in our studies, the proportion of RFOs identified in phloem exudates from excised leaves of transgenic plants was much lower than the proportions observed in whole-leaf samples (Figure 16). This implies that RFOs are not confined to the companion cells. In previous study, Arabidopsis has been shown to be Suc-transporting "type 1-2a" plants and small amounts raffinose are also transported (Haritatos et al., 2000). Through labeling experiment with $^{14}CO_2$ to source leaves of Arabidopsis, radiolabel was found in sucrose, raffinose and galactinol in source leaves, which were translocated to sink leaves. Coleus was used as positive control and was found to synthesize and translocate substantial raffinose and stachyose, but little galactinol. "Type 1-2a" plants have relatively abundant plasmodesmata between minor-vein phloem and surrounding cells, such as vascular parenchyma cells and bundle sheath cells. It is possible that RFOs produced in transgenic lines are transported into the vacuole, which functions as storage, waste disposal, protection, and growth. If so, how are RFOs

translocated from companion cells to vacuole? Are there RFOs transporters in plants membranes? Sugar transporters for Suc and hexoses have been identified and characterized for a variety of plants. However, RFOs transporters have received little attention. RFOs were proposed to be transported through plasmodesmata as part of the polymer-trap mechanism for symplasmic phloem loading (Turgeon, 1996). Furthermore, RFOs may leak from phloem and return to the leaf in the xylem stream. ^{14}C-Sucrose labeling experiment, with distribution analysis of labeled sugars in phloem exudates and sink tissues, can be used to resolve this question. If labeled RFOs exist in phloem exudates, but not detected in sink tissues, RFOs may leak from phloem and return to the leaf in the xylem stream.

4.5 RFOs Metabolism in Transgenic Arabidopsis

RFOs appear to be stable in transgenic plants because of quantitative analysis for galactose, one product from RFOs degradation by α-galactosidases, between WT and transgenic plants. Sugar analysis in leaves showed that for total hexoses, including Glc, Gal and Fru, there are 217 pmol/ mg fwt from WT, 288 pmol/ mg fwt from GRS47 and 193 pmol/ mg fwt from GRS63 (Figure 13). In addition, the total amount of Glc, Fru and Suc in phloem exudates of transgenic plants (1101 pmol/ mg fwt/ hour for GRS47 and 555 pmol/ mg fwt/ hour for GRS63 in first two-hour phloem exudates) are more than the total amount of Glc, Fru and Suc in phloem exudates of wild type plants (421 pmol/ mg fwt/ hour) (Figure 16), raising the possibility that RFOs are degraded to Glc, Fru and Suc in phloem. Measurement of galactose in phloem exudates can answer whether RFOs are degraded. In table 3, there is no significant difference for galactose in phloem exudates between wild type and transgenic lines arguing against degradation of RFOs in phloem. There are four acidic α-galactosidases in Arabidopsis, three of which are cell wall enzymes and one is a vacuolar enzyme. In addition, two alkaline cytoplasmic α-

galactosidases were elucidated to play a potential role in degrading RFOs during germination. The acidic α-galactosidases are most likely to degrade RFOs in the cell wall compartments and the vacuole. Because RFOs accumulate to high levels in the existing transgenic plants, it is not clear if any of these enzymes are acting on RFOs. RFOs are produced as compatible solutes during stress and seed maturation, and are degraded during germination (Taji et al., 2002; Peters et al., 2007; Nishizawa et al., 2008; Downie et al., 2000; Blochl et al., 2007). However, the fate of RFO after recovery from stress is not addressed.

4.6 New Function of RFOs to Protect Plants from Aphids

The occurrence of RFOs in plants is nearly ubiquitous. In some species, it is for carbon transport and is thought to function in carbon storage in many seeds (Keller and Pharr, 1996; Turgeon, 1996; Ayre et al., 2003). *GALACTINOL SYNTHASE* in numerous species is stress-inducible to function in the accumulation of galactinol and raffinose during drought, high salinity and cold stress. *AtGOLS2*-overexpressing transgenic Arabidopsis plants showed drought-stress tolerance due to increasing galactinol and raffinose as osmoprotectants (Taji et al., 2002). Sucrose and RFOs, particularly raffinose, function as compatible solutes during drought stress in the leaves of the resurrection plant *Xerophyta viscosa* (Peters et al., 2007). Furthermore, galactinol and raffinose have been shown as novel intracellular antioxidants in plants by scavenging hydroxyl radicals as under oxidative stress (Nishizawa et al., 2008). In addition, Galactinol has been suggested to be a signaling component of the induced systemic resistance caused by pathogens, supporting a role in defense against biotic stresses (Kim et al., 2008). Aphids are phloem-feeding insects that constitute a biotic stress since they extract nutrients and reduce plant vigor. To test for an impact of RFO production on aphid feeding, “no choice” fecundity tests and “choice” feeding preferences were conducted. In this study, transgenic

Arabidopsis producing RFOs were a less preferred host for aphids, and aphid reproduction was reduced on transgenic plants (Figure 20 and 21). There were no significant preferences between WT and transgenic plants 8 h after aphid release, suggesting that the resistance to aphids is unlikely to result from chemicals released at the surface of plants (Figure 21C). The preferences after 24 h can result from RFOs increasing in transgenic plants or due to presence of a metabolite that adversely impacts insect reproduction. We are unaware of RFOs levels increasing in direct response to aphid feeding. However, since the carbohydrate content of the phloem stream is altered in the transgenic plants, we rationalized that aphid feeding preferences may also be altered. It is worth noting that many animals cannot digest the αGal1-6 linkage of RFOs, and RFOs can cause bloating particularly in mammals. Aphid feeding on synthetic media was used to test if RFOs directly impacted aphid behavior. Aphids did not show differences in reproduction four days after they were fed on synthetic media with or without RFOs (Figure 22). This result indicates that the resistance to aphid feeding is an indirect effect of the exotic sugars. The aphid resistance can result from higher hydrostatic pressure produced in our engineered source tissue which could make aphids lose body water to the gut as they feed. RFOs may be as signaling components of the systemic resistance for protecting plants against aphids. Another possibility is that RFO accumulation in transgenic Arabidopsis promotes a general stress response which in turn results in compounds that resist aphids. The synthesis of RFOs may contribute the secondary metabolism which provides the resistance to aphid feeding. For example, glucosinolates, as a secondary metabolite, were reported to accumulate in the phloem sap to serve as reservoirs for the release of toxic cyanates and nitriles to defend against herbivores and pathogens (Halkier et al., 2006). Toxic breakdown products of glucosinolates can also deter aphid feeding (Kim et al., 2008).

Table IV. *Sugars in phloem exudates from wild type and transgenic plants with exudation rate (pmoles sugar exuded / leaf mg fwt / hour). Variation is expressed as SE; n = 12 sibling plants*

	GLC/GAL	FRU	SUC	GOL	RAF	STA
WT 1st	96±40	78±27	248±49	0±0	0±0	0±0
GRS47 1st	230±59	198±40	673±151	12±5	3±2	1±1
GRS63 1st	93±29	83±20	379±177	6±4	7±4	0±0
WT 2nd	265±107	222±68	395±114	0±0	0±0	0±0
GRS47 2nd	216±61	177±36	634±143	11±4	3±2	1±1
GRS63 2nd	342±89	270±86	789±268	10±7	6±3	1±2

Table V. *Percentage of sugars in leaves from wild type and transgenic plants*

	GLU/GAL	FRU	SUC	GOL	RAF	STA	Hexoses
WT	19.7%	3.5%	76.3%	0.1%	0.4%	0.0%	23.2%
GRS47	7.6%	1.4%	31.4%	33.6%	21.6%	4.4%	9.0%
GRS63	12.2%	2.5%	48.5%	10.4%	25.7%	0.6%	14.8%

Table VI. *Percentage of sugars in phloem exudates from wild type and transgenic plants*

	GLC/GAL	FRU	SUC	GOL	RAF	STA	Hexoses
WT 1st	22.8%	18.4%	58.7%	0.0%	0.0%	0.0%	41.3%
GRS47 1st	20.6%	17.8%	60.3%	1.1%	0.3%	0.1%	38.4%
GRS63 1st	16.3%	14.7%	66.6%	1.1%	1.3%	0.0%	31.0%
WT 2nd	30.0%	25.2%	44.8%	0.0%	0.0%	0.0%	55.2%
GRS47 2nd	20.8%	17.0%	60.9%	1.1%	0.3%	0.1%	37.7%
GRS63 2nd	24.1%	19.0%	55.7%	0.7%	0.4%	0.0%	43.2%

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