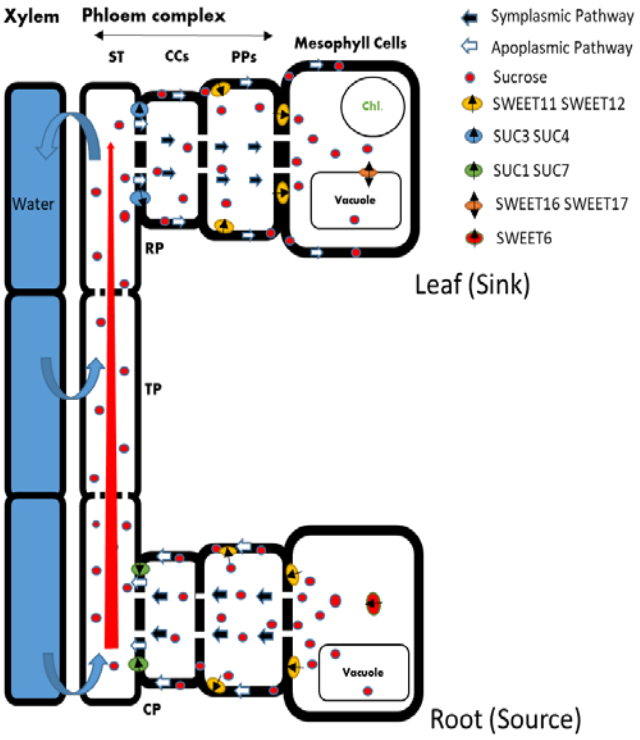
SWEET genes mediate sugar translocation and allocation as a key step in roots of *in vitro* grown *Arabidopsis*



M.Sc. Thesis Report 2018

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Abstract

The aim of this research is to provide a basic understanding of how exogenous carbon is being translocated from roots in culture and identify key genes involved in sucrose translocation of plants grown *in vitro*. Understanding and optimizing the sucrose translocation system of plants grown *in vitro* could help to increase the growth rate in culture but also might overcome tissue culture barriers such as recalcitrance. Currently, the complete pathway by which exogenous sucrose is being translocated from the source roots to the sink leaves, it is not known. In *Arabidopsis,* 17 *SWEET* genes and 9 SUC genes have been identified up until now, with the former family to be associated with cell-to-cell transportation and the latter with phloem loading. Gene expression analysis using *SWEET* and *SUC* primer pairs was performed for both roots and leaves of *Arabidopsis ecotype* Columbia (Col-0) plants grown *in vitro* and *ex vitro*. We are the first to present a potential representation of sucrose translocation system *in vitro, as well as* confirming the already known data of sucrose translocation in *ex vitro* grown *Arabidopsis* and drawn them down in two representative model schemes. *SWEET11* and *SWEET12* were found to be important proteins for sucrose translocation both in the source (roots) and the sink (leaves) of *in vitro* grown *Arabidopsis* plants. In addition, *SWEET11 SWEET12* and *SWEET15* can mediate sugar translocation and allocation in roots of *in vitro* grown *Arabidopsis*. Mutation of *SWEET12* may enhanced activity of its homolog *SWEET11* but the vice versa scenario maybe not, according to physiological and biochemical analysis. Photosynthetic activity was also tested in the mutant lines as an alternative method of plants to gain their sugar molecules (beside the sucrose uptake from the medium) to obtain a spherical opinion regarding the physiology of the mutant lines, using photo marker genes that each represents a major component of the photosynthetic pathway. After gene expression analysis, the expression levels suggested a light-induced reduction in the photosynthetic capacity of the mutant plants compared to the *Arabidopsis* wild-type. The photosynthetic apparatus of the mutant lines probably saturated due to the inability of exporting sugars from the leaves of the mutant lines.

Introduction

Background

Sucrose is an important metabolite and signalling compound in plants (Smeekens, 2000**;** Solfanelli *et al*., 2006) that is produced in mesophyll cells by conversion of photosynthetically fixed CO_2 into organic carbon. As the main form of sugars in long-distance translocation, sucrose is being transferred through the plant's veins and especially via the phloem (Chen *et al.*, 2015). Sucrose long-distance translocation starts from the source leaves and is exported to the growth and storage organs (called sinks) such as roots, stems, flowers, fruits and seeds (Williams *et al*., 2000; Ludewig and Flügge, 2013). In cultivated crops, these sink tissues will eventually be harvested and utilized for food, fibre, feed, fuel, and more, depending on the applicability of the harvested crop (Bihmidine *et al*., 2013). Following that, sucrose can be defined as a key instrument for determining total yields in crop production. Numerous studies have indicated the significant role of sucrose in plant development, including germination, senescence, flowering (Gibson, 2004; Pourtau *et al.*, 2004) and stress tolerance (Ruan *et al.* 2010).

Besides their vital role as a carbon source and energy molecules (Ruan, 2014), sugars are a key factor for various interactions between plants and external organisms. Plants evolutionary developed synergies with other organisms by exchanging carbon for their beneficial activities. For example, flowers secrete sugars for nectar production to attract pollinators or either to house and feed the predators of their herbivores (Fürstenberg *et al*., 2013). In other cases, plants secrete sugars from their roots to feed nitrogen-fixing bacteria like rhizobia as an exchange for nitrogen compounds (Júnior, *et al*., 2017). On the other side of the food chain, biotrophic pathogens have also developed strategies to gain access to the plant's energy resources. Chen *et al.,* (2010) has found that pathogens and symbionts may target and highjack their host sugar translocation system by retooling physiological functions for their benefits.

Phloem loading

The phloem system of plants is one of the two prominent conducting tissues of plants vascular system and can be subdivided into three functional regions: collection, transport and release phloem (Taiz and Zeiger 2010). Briefly, sucrose enters the phloem vascular system from the collection phloem (CP) and released into the sink tissues from the release phloem (RP). Transport phloem (TP) is the intermediate piece of the CP and RP, and proportionally the largest within the phloem network in plants (van Bel, 2003). Phloem is primarily composed of sieve-tubes elements (SE/ST), companion cells (CCs) and phloem parenchymal cells (PPs). Sucrose is actively transported from source leaves into the collection phloem (first entering CCs and then STs) and increases the solute concentration there. As a result, the water potential is being reduced, leading to water inflow from the xylem to the phloem through osmosis (Braun *et al*., 2014). Transpiration causes the upward transfer of water to the leaves through the xylem vessels. The resulting hydrostatic pressure forces the sucrose-water mixture down towards to sink tissues where sucrose is unloaded (from STs to RP, Lalonde *et al*., 2003; Wippel and Sauer, 2012; Patrick, 2013).

Plants can be divided into predominant apoplasmic or symplasmic phloem loader species (Braun *et al*., 2014)). Symplasmic loading can also be referred to as passive loading due to the lack of energy required (Slewinski and Braun, 2010). Whereas, in apoplasmic loading species such as *Arabidopsis thaliana*, sucrose is imported into the ST in CP by the sucrose-H^+ cotransporter *AtSUC2* (Wippel and Sauer, 2012; Chen *et al*., 2012).

Sucrose Transporters

The sugar translocation system is regarded to be highly dependent on the function and activity of sucrose transporters genes (called *SUCs* or *SUTs*). Those genes are the main gears for the whole sugar translocation system, as they orchestrate sucrose movement from higher plant source leaves to heterotrophic sink tissues (Gong *et al*., 2014). Supporting their name, sucrose transporters are the link between the collection of photoassimilates (CP) and their relocation to sink areas (RP) for utilization. For this to occur, first the sucrose needs to be loaded into the phloem and then from there its long journey begins. For example, the *AtSUC2* gene encodes a phloem loader in *Arabidopsis* (Srivastava *et al*., 2008). Silencing of this gene leads to reduce carbon partitioning, due to defective sucrose export from source leaves. Phenotypic changes such as anthocyanin accumulation and stunted growth with low reproduction rate were also mentioned (Srivastava *et al*., 2009). In *Arabidopsis*, nine *SUCs* (*AtSUCs*) genes have been identified of which their

expression and function are well studied (Gong *et al*., 2014). After phylogenetic analysis, those nine *SUCs* genes were divided into three distinct subfamilies called clade I, II, and III (Aoki *et al*. 2003).

In plants, three main pathways of sucrose transportation have been identified for cell-to-cell transport of sugars. First, it is the symplasmic pathway by which sucrose diffuses via plasmodesmal connections. The second is the apoplasmic pathway via the plasma membrane and cell wall (apoplast, Patrick, 1997; Chen *et al*., 2012). The last mechanism involves the efflux of sucrose and again influx during sucrose long-distance movement within cells to support axial sinks (Bihmidine *et al*., 2013). However, the molecular nature of cellular sugar efflux systems in plants is still unclear (Chen *et al*., 2010).

SWEETs

SWEET sugar transporters are a recently discovered subfamily of predominantly sucrose efflux transporters that started gaining attention due to their involvement in phloem loading (Chen *et al.,* 2010; 2012; Xu *et al*.; 2014). *SWEETs* are small proteins predicted to have seven transmembrane domains and are divided into four distinctive clades. In *Arabidopsis,* 17 *SWEET* genes have been identified up until now and their classification in clades can be seen in Figure 1. *SWEET* gene members that belong to clade III are typically involved in cellular efflux processes (Chen *et al.,* 2010; 2012; Xu *et al*.; 2014). For example, *SWEET11* and *SWEET12* localize in the plasma membrane of the phloem parenchyma cells and are important for phloem loading. *SWEET11* and *SWEET12* were found to be expressed in leaves and to participate in the export of photoassimilates from the mesophyll cells to the phloem apoplast (Eom *et al*., 2015). Induced mutations in those genes lead to accumulation of sucrose in leaves as the partitioning of exported photoassimilates is blocked (Chen *et al*.; 2012). In brief, they are mediated sucrose transporters that efflux sucrose from the cytoplasm of mesophyll cells to the apoplast of CCs and PPs. In addition, it has been discovered that all members of clade III *AtSWEET* family (AtSWEET9 to 15) have the potential to transport sucrose across the plasma membrane and participate in the phloem loading process (Chen *et al*., 2010; 2012).

In *Arabidopsis*, many *SWEET* genes were studied as they were identified to participate in several important physiological functions of the plant. For instance, *AtSWEET13* and *AtSWEET8* are essential for pollen development (Han *et al*., 2017) and pollen fertility (Guan *et al*., 2008), respectively. Besides their role in phloem loading, *AtSWEET11* and *AtSWEET12* have also an autonomous role in seed filling, as well as their homolog *AtSWEET15* (Chen *et al*.; 2015). Furthermore, *AtSWEET16* and *AtSWEET17* are vacuolar hexose transporters localized to the tonoplast with the latter being a key candidate of determining fructose levels in the leaves (Klemens *et al*., 2013; Guo *et al*., 2014;). Lastly, *SWEET9* is expressed in nectaries and it is essential for nectar production and secretion (Lin *et al*., 2014; Eom *et al*., 2015). A list including the functions and further information about AtSWEET1-17 can be found in Supplementary data 5.

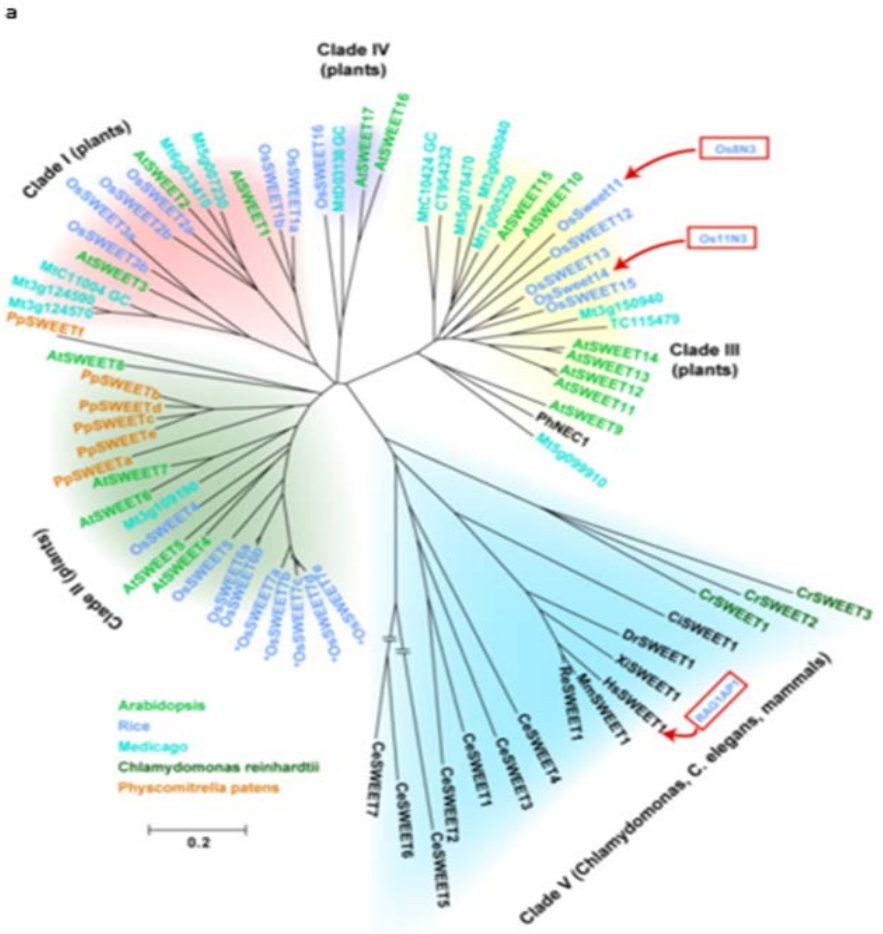

Figure 1: Phylogenetic tree of the four distinctive clades of the SWEET family. The Arabidopsis SWEET genes are represented with a light green colour. SWEETs 1–3 in clade I, SWEETs 4–8 in clade II, SWEETs 9–15 in Clade III, and SWEETs 16–17 in clade IV (Chen et al. 2010)

Plant tissue Culture

Plant tissue culture relies on the concept of cell totipotency and has become an important tool-set for plant biotechnology. Shortly, tissue culture involves the growth of plant material on an artificial medium, most commonly Murashige and Skoog (MS) medium, supplemented with sugar amongst others under sterile conditions and in a sealed environment. Exogenous sucrose is provided as a carbon and energy source for the plants inside the container. Tissue culture (TC) exploits the fundamental ability of plant parts to reinitiate the formation of novel meristems or to activate dormant ones and in this way to replicate themselves for many and diverse applications. For instance, TC is applied for rapid multiplication of elite cultivars on a large-scale in a comparatively short time (Rao, 1993; Jain, 1997). For breeding, tissue culture is considered a strong tool, as it can overcome specific breeding barriers as well as maintain existing germplasm stocks (Wawrosch *et al*., 2001; Jain and Ochatt, 2010). Initially, the technique was employed for ornamental crops only (Chu *et al*., 1990), but presently, *in vitro* multiplication or micropropagation is being used for many different plant species. Currently, plant tissue culture represents a multibillion-dollar industry throughout the world, that has created new opportunities in global trading (Prakash, 2006). Compared with conventional methods of multiplication, micropropagation has some very significant advantages, such as the generation of genetically homogeneous and virus free plant material with a high propagation efficiency (Hussain *et al*., 2012).

Despite these advantages, micropropagation as a technique also has certain drawbacks, e.g. high costs and recalcitrance for still many plant species that limits their industrial use and exploitation. The explanation for the observed recalcitrance might be the insufficient uptake of nutrients from the medium by the explants. In *ex vitro* conditions, nutrients (minerals) are being taken up by the roots and transported to sink tissues through the xylem, while as mentioned above, photoassimilates-derived nutrients such as sucrose, are transported through the phloem. On the other hand, photosynthesis and transpiration (transport driver) are low *in vitro* due to the suboptimal conditions in the controlled environment (Kozai, 2010). Those two important functions in plant development and growth are low due to photosynthetic barriers (e.g. low CO_2 supply, low light intensity) and high relative humidity in the vessels. This all results in a relatively lower growth rate of tissue culture grown plants compared to the plants growing in a greenhouse or field. Understanding and optimizing the sucrose translocation system of plants grown *in vitro* could help to increase their growth rate in culture but also might overcome tissue culture problems such as recalcitrance.

Currently, the complete pathway by which exogenous sucrose is being translocated from the source roots to sink leaves, it is not known. Sugar migration occurs at the interface of medium and submersed plant organ, and transport may be driven by an osmotic gradient or by diffusion (Williams, 1995). Our working hypothesis is that sucrose is being translocated through the vascular system and still within the phloem tissue, but this time with a different direction (from downwards to upwards). Knowing the activity and the intracellular expression of sucrose translocators under *in vitro* conditions may shed light on the current mystery. Moreover, identifying key genes that participate in sucrose transportation in *in vitro* growing plants will provide clearer information regarding plant physiology with respect to nutrient flow and availability for sustaining growth in a contained environment. This might enhance the micropropagation efficiency and *in vitro* growth of crops in general and could overcome recalcitrance and thereby broaden the application of *in vitro* propagation. However, information regarding sucrose uptake and specifically its transport during *in vitro* growth of plants is quite limited. This study intends on shedding light on this issue and contribute to the understanding of sucrose translocation on cellular and plant level.

Research Aim

Plants grown *in vitro* get their carbohydrates exogenously from the medium, mostly as sucrose due to low photosynthetic conditions within the vessels (Kozai, 2010). However, it is still unknown how exogenous sugar translocates and how sugar transporters facilitate carbon transport across membranes *in vitro*. The aim of this research is to provide a basic understanding of how exogenous carbon is being translocated from roots in culture and identify key genes involved in sucrose long-distance translocation in *in vitro* growing plants. With sucrose as the main nutrient in mind, this research focused on the relative expression levels of *SWEET* and *SUC* genes in roots and leaves of *in vitro* growing *Arabidopsis thaliana* plants using *ex vitro* grown plants for comparison. Applying a gene expression analysis using qPCR, a model was designed using *SWEET* and *SUC* primer pairs as well as literature research. Another aim of this research was to confirm the involvement and role of *SWEET11* and *SWEET12* in sucrose translocation under *in vitro* growth conditions. Photosynthetic activity was also tested in the mutant lines as an alternative method of plants to gain their sugar molecules (beside the sucrose uptake from the medium) to obtain a perimetric vision regarding the physiology of the mutant lines. For this experiment, six photo marker pairs were used with each one to represent a major component of the photosynthetic pathway

Hypothesis:

Nutrient uptake and sugar flow are greatly impaired under *in vitro* conditions in comparison with *ex vitro* conditions due to abnormal source-sink nutrient migration. This switch within the system may lead to different sugar translocators activity between the two growth conditions. The working hypothesis for this thesis is that *SWEET11* and *SWEET12*, regulate sucrose long-distance transportation (from downwards to upwards) in *in vitro* growing plants. In addition, probably photosynthesis may be enhanced in the mutant lines due to deficient sucrose translocation from roots to leaves. In this way, the photosynthesis may be more active in the leaves of the mutant plants compared to the control plants to cover the sugar needs.

Results

Gene Expression Analysis

Quantitative polymerase chain reaction (qPCR) was performed to quantify the relative gene expression levels of sugar transporter genes from the *SWEET* and *SUC* family, using 17 primer pairs for the former and 9 primer pairs for the latter family. The primer pairs were used to determine the expression of members from the two sugar transporting families in roots and leaves of *Arabidopsis* (Col-0) plants, growing both *in vitro* and *ex vitro*. The relative changes in gene expression were quantified using real-time qPCR and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The raw data were normalized against the reference gene *UBQ10* and their quantitative analysis expressed relative to leaf *SWEET1* activity (calibrated as 1) for each environment.

In vitro Col-0

A higher number of *SWEET* genes were recorded to be expressed in the leaves compared to roots, although the expression levels in the roots are much higher. Four genes from the *SWEET* family were expressed in roots, *SWEET4*, *SWEET6*, *SWEET11* and *SWEET12.* The last three genes were all higher expressed than the first one, with a similar expression pattern. Ten genes from the *SWEET* family were expressed in leaves, with different relative expression levels. *SWEET16* and *SWEET17* had the highest expression levels from the *SWEET* family in leaves, followed by *SWEET11*. Similar results regarding the number of expressed genes as well as the level of expression in both plant tissues were recorded for the *SUC* family. Seven genes were shown to be expressed in leaves with *SUC3* and *SUC4* to be the genes that had the highest expressions, while in roots only two genes were expressed, *SUC1* and *SUC7.* These results are displayed in Figure 2. Overall, for both gene family's higher number of expressed individual genes was observed within the leaf tissue but in contrast, the relative expression levels of the fewer active individuals in roots were much higher in fold changes. Possibly indicating a higher activity of sucrose transporters genes in roots, which can be supported as plants grown *in vitro* get their carbohydrates exogenously from the medium.

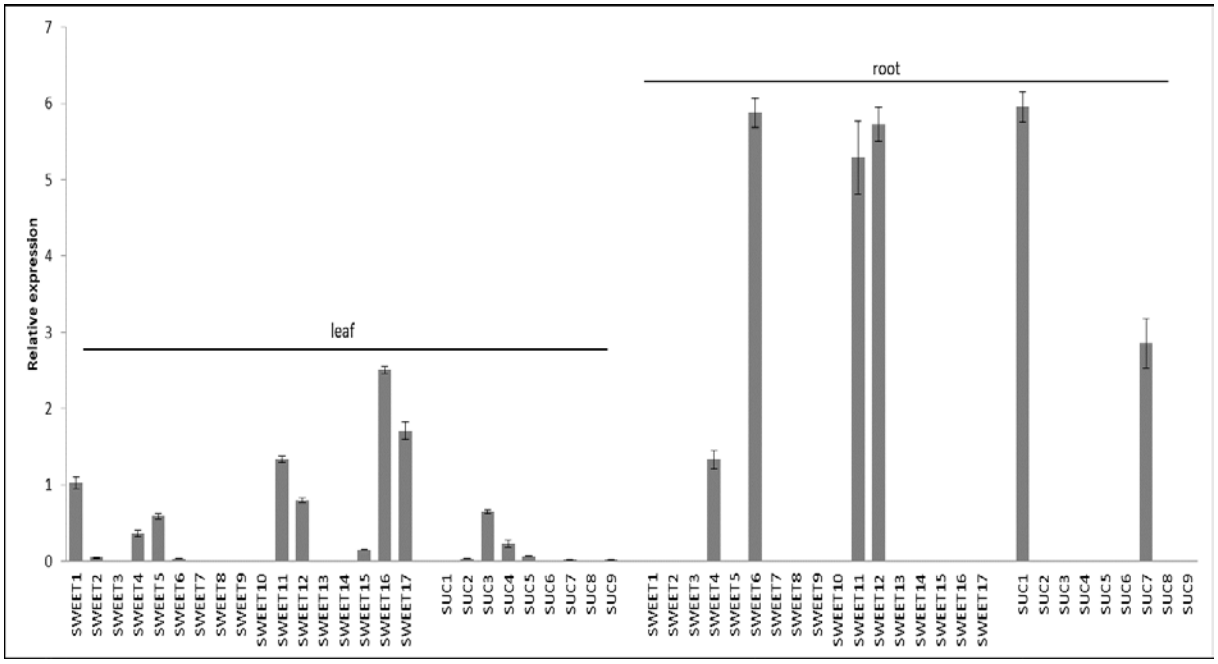

Figure 2: Transcriptional levels of SWEETs and SUCs genes in leaves and roots of Col-0 growing in vitro conditions. The relative changes in gene expression were quantified using real-time qPCR and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The raw data were normalized against the reference gene UBQ10 and their quantitative analysis expressed relative to leaf SWEET1 activity (calibrated as 1)

Ex vitro Col-0

Under ex vitro conditions, similar numbers of expressed genes were quantified from both families in the two tested plant tissues. In addition, many genes were expressed both in roots and leaves, with their relative expression levels being fluctuated among the plant tissue. For example, *SWEET11* had the highest relative expression level in the leaves with an 8.3-fold change related to leaf *SWEET1,* while in the root much lower expression levels for *SWEET11* were recorded. It is Interesting to mention that for both plant tissues *SWEET11* was the highest expressed gene from the *SWEET* family. Expression levels of the rest *SWEET* genes for both plant tissues were similar or even smaller relative to leaf *SWEET1*. For the *SUC* family, there is a switch on which gene had the highest expression among the tested plant tissue, as in leaves *SUC2* and *SUC3* were the highest expressed genes while in roots were *SUC3* and *SUC4*. Additionally, *SUC3* and *SUC4* were also the highest expressed sucrose transporter genes in the roots. Higher number of *SWEET* genes were expressed in both plant tissues compared to the *SUC* family, though the level of expression was found to be higher in the expressed *SUC* genes (besides the leaf *SWEET11*). Those results are displayed in Figure 3.

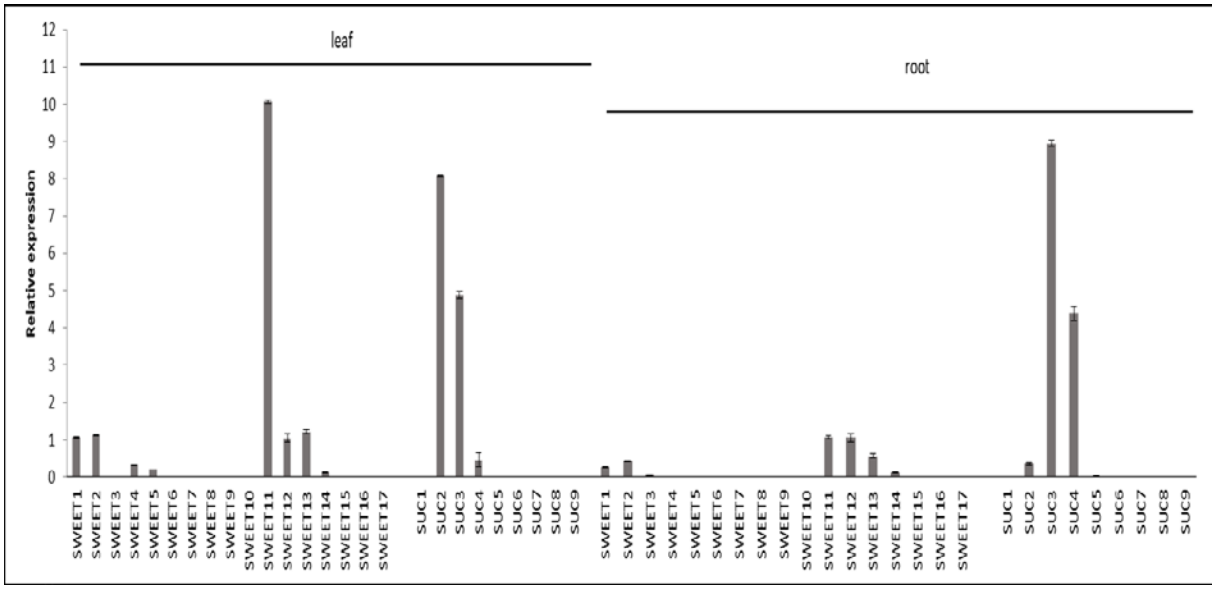

Figure 3 Transcriptional levels of SWEETs and SUCs genes in leaves and roots of Col-0 grown ex vitro. The relative changes in gene expression were quantified using real-time qPCR and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The raw data were normalized against the reference gene UBQ10 and their quantitative analysis expressed relative to leaf SWEET1 activity (calibrated as 1)

Identification of inserted Mutations

The tested *Arabidopsis* mutant lines were provided by Huayi Li (Table 2) while gene expression analysis to measure the expression of the genes in the mutant lines was performed using the above primer pairs. *Arabidopsis SWEET* mutant lines were tested using RT-qPCR, and their relative expression levels were calibrated using *UBQ10* as a reference gene. Quantitative analysis of each target gene in the mutant lines was analysed compared to the same genes from *in vitro* grown Col-0 plants as controls. The mutation(s) for the six tested *Arabidopsis* mutant lines were confirmed as their activity compared to Col-0 *SWEET* genes showed a dramatic decrease. Specifically, gene activity of *SWEET11* and *SWEET12* was knocked down in the mutant lines. For example, lines *sweet11* and *sweet12* had a decrease of more than 70% on *SWEET11* and *SWEET12* gene expression levels compared to Col-0 plants as controls (Supplementary Data1). Relative expression levels of mutated gene *SWEET15* in both doubles (*sweet12;15 and sweet11;15)* and triple mutant (*sweet11;12;15*) lines were zero, probably indicating a knock-out of this gene.

Physiological Analysis

Phenotyping

Seeds from the homozygous mutant *Arabidopsis* lines as well from the wild-type Col-0 were germinated in 2% sucrose (w/v) MS medium for 3 weeks after sterilization. The mutant lines used had single mutations (*sweet11* and *sweet12*), double mutations (*sweet11;12*, *sweet11;15* and *sweet12;15*) and triple mutations (*sweet11;12;15*). In addition, seeds from transgenic Col-0 plants overexpressing *SWEET11* and *SWEET12* genes by a 35S promoter were also grown in the same conditions. The phenotype of those lines after 3 weeks can be seen in Figure 4. According to this figure, Col-0 plantlets looked normal and had a higher growth compared to all mutant lines. Overall, the inserted mutation(s) seemed to influence the plant growth, as all the mutant lines seems to have smaller plants compared to the control Col-0 plantlets. Similar growth was observed between the two overexpression lines and the WT Col-0. Unexpected results on growth between the mutant lines were observed. The insertion of an extra mutation was expected to affect the plant growth in a negative way that can be distinctive (linear). However, all mutants; single, double and triple had a similar growth pattern

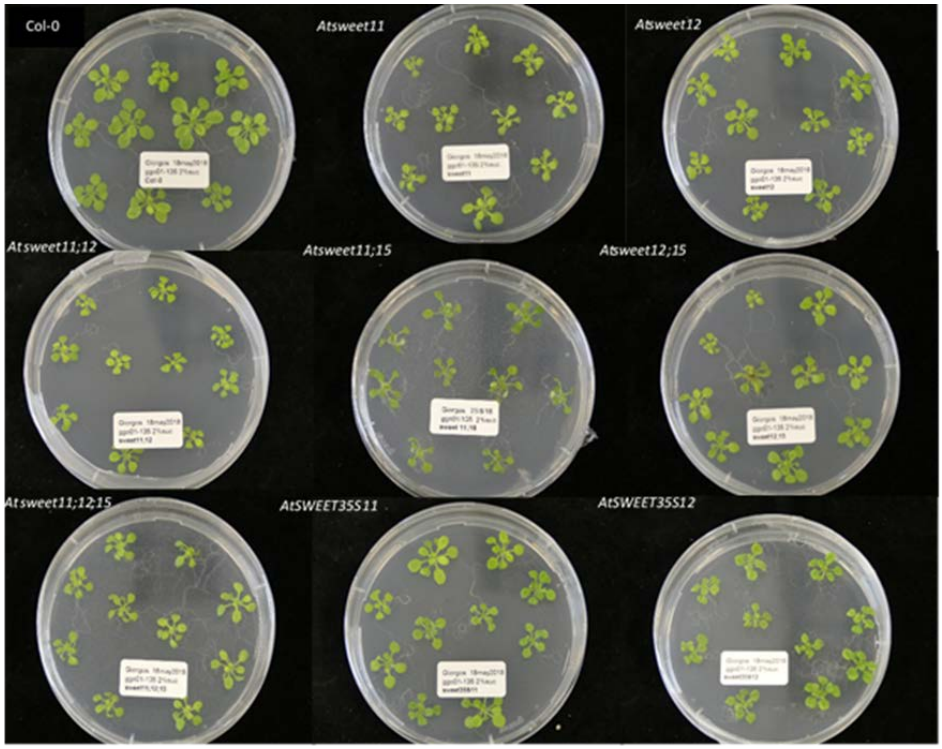

Figure 4: Arabidopsis plantlets growth phenotype after 3 weeks in medium containing 2% (w/v) sucrose in MS. Top left is the wild type Col-0 and the rest are the mutant lines. The last two from right to left are transgenic overexpressing SWEET lines 35S-SWEET11 and 35S-SWEET12

Further experiments were done to understand the role of *SWEET* genes by growing the *Arabidopsis* mutant lines and the WT Col-0 on media containing different sucrose concentrations. This experiment was performed to check the effect of sucrose (low to high levels) on the plant growth of *Arabidopsis* mutants and wild-type. Figure 5 represents the growth of the tested lines within 0%, 2% and 4% sucrose after 3 weeks. A linear increase in plant growth in all tested lines was observed when sucrose concentration inside the growing medium was increased. First, all tested lines had similar growth in the 0% sucrose medium, being relatively weak compared to the other sucrose mediums. Results from the 2% sucrose medium were mention above. Finally, when sucrose concentration was doubled in the medium (4% sucrose), all plant lines were bigger with a dark greener colour on leaves compared to the same lines growing in 2% sucrose.

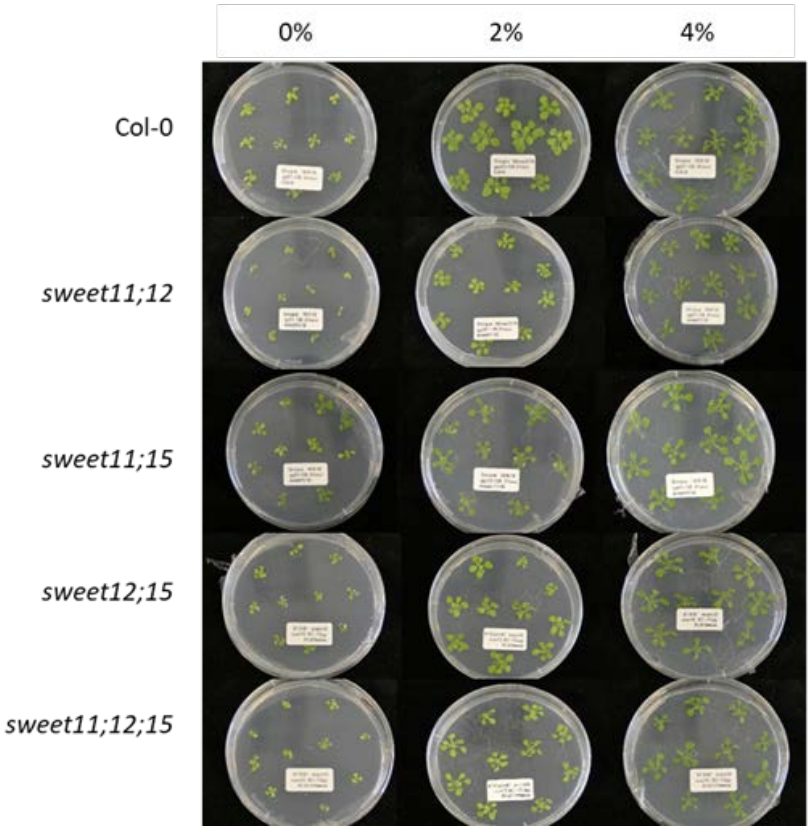

Figure 5: Arabidopsis plantlets growth phenotype after 3 weeks in MS medium containing 0%, 2% and 4% sucrose concentration respectively. Col-0 and the rest are the transgenic lines

Arabidopsis plants (Figure 4) were harvested five weeks after sowing for fresh weight (FW) and dry weight (DW) measurements (n~10). Two formulas were designed to calculate the average mass and water percentages. The means of those parameters from each line can be seen in Table 1. *Arabidopsis* mutant lines *sweet11;15* and *sweet12;15* were excluded from this analysis due to the limitation of individuals caused by a contamination. Healthy plants from those two lines were harvested and used for sugar extraction and determination analysis.

Single mutant lines *sweet11* and *sweet12* had the highest FW with an average of 175.88mg and 172.83 mg per mutant plant, respectively. In addition, *sweet11* and *sweet12* had the highest DW averages with 9.60mg and 10.24mg per plant respectively. The average mass percentage was formulated as the DW percentage of the FW measurements (DW/FW*100). According to this formula, Col-0 had the highest biomass production with 8.03%, while single mutants *sweet11* and *sweet12* had the lowest with 5.96% and 5.97% respectively. Water percentage was also formulated as the delta of FW to DW percentage of the FW measurements ((FW-DW)/FW*100).

Table 1: Fresh weight (FW) and Dry weight (DW) of tested Arabidopsis lines growing on 2% sucrose MS medium. Also, average water and mass percentages were calculated for each tested line.

Cultivars	FW (mg)	DW (mg)	Water (%)	Mass (%)
Col-0	109.46	8.35	91.97	8.03
sweet11	175.88	9.60	94.04	5.96
sweet12	172.83	10.24	94.03	5.97
sweet35S11	69.92	4.38	93.60	6.40
sweet35S12	111.56	7.00	93.68	6.32
sweet11;12	67.29	5.04	92.69	7.31
sweet11;12;15	133.46	9.23	92.95	7.05

An analysis of variance (ANOVA) using GenStat (19th Edition) with a threshold value of $p<0.05$ to indicate significant differences between the different lines, was carried out for all the measured para meters. After the analysis, significant differences were observed only within the average mass and water percentages of the mutant lines *sweet11*, *sweet12, SWEET35S11* and *SWEET35S12* compared to Col-0 data (Figure 6).

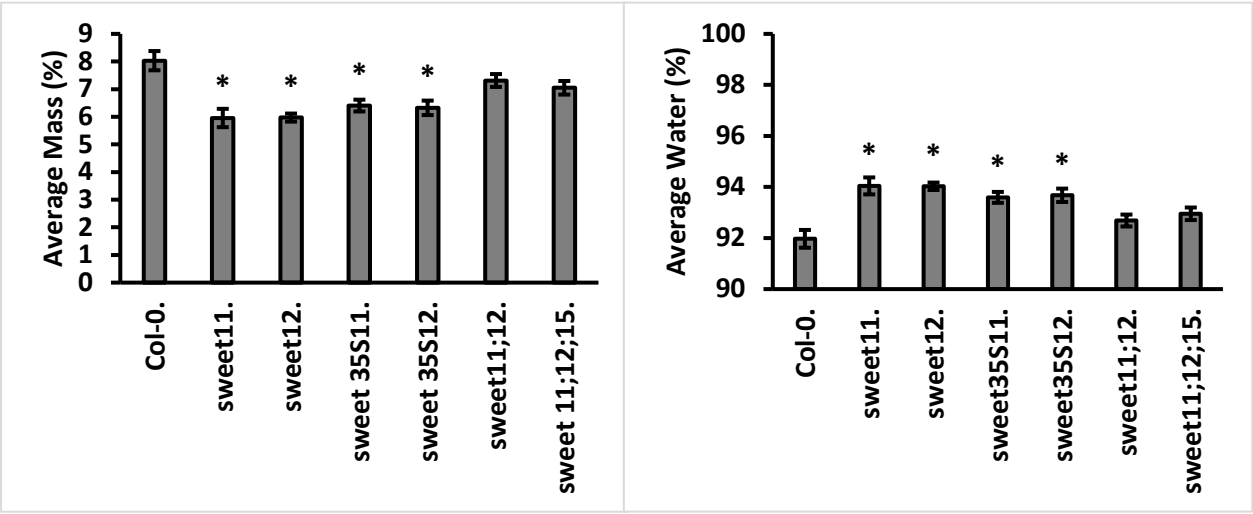

*Figure 6: Graph on the left represents the average Mass percentage of the tested Arabidopsis lines (n~10) on the x-axis. Graph on the right represents the average Water percentage of the same tested Arabidopsis lines (n~10) on the x-axis. * indicating the significant difference (p < 0.05) of a line to Col-0.*

The effect of sucrose on plant growth and overall biomass production was confirmed both in *Arabidopsis* WT and the mutant lines. The increase of sucrose concentration within the medium caused a linear increase of biomass production for Col-0 plants (Figure 7). Significant differences were also found between Col-0 plants grown on 2% and 4% of sucrose compared to sugar-free medium regarding average FW and DW, but not with each other (Figure 7). In addition, tested transgenic lines grown on 4% sucrose had significantly increased Mass percentage compared to the same lines grown on 2% sucrose (Supplementary data 2). Significant differences between the tested genotypes grown on 4% sucrose were not found among any parameter (data not presented).

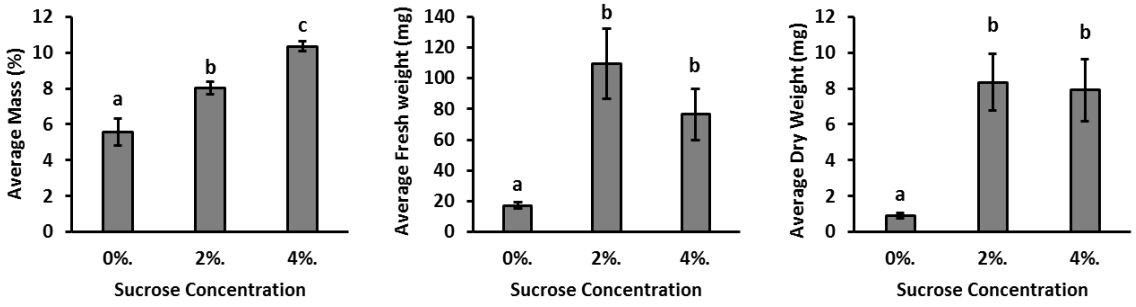

Figure 7: Arabidopsis Columbia plants growing in 0% 2% and 4% sucrose concentrations. From left to right graphs are represented the average (n~10) Mass%, Fresh weight and Dry weight respectively of the Columbia plants in the different sucrose concentrations. The letters are indicating significant differences (p<0.05).

Biochemical Analysis

Sugar Components

Sugars from *Arabidopsis* tested lines (Figure 4) were extracted using 80% ethanol, determined and quantified using High-performance liquid chromatography (HPLC). Root and leaf tissues of each tested line (n~3) were used for this analysis. Eight different sugar components were used to construct standard curves within the HPLC analysis on different concentrations. The standard sugar data from the HPLC analysis were then used to calculate the content of each neutral sugar (mg/FW) in the tested samples. Besides that, only glucose and sucrose data were used for further analysis to simplify the spectrum of this research. The rest of the sugars concentrations of each line were also analysed, but due to unclear results and time limitation were not included in this thesis. Data concerning the analyses of the rest of the sugars can be collected from Huayi Li. Figure 8 displays the average amount of sucrose and glucose (mg/FW) both in leaves and roots of the tested lines grown on 2% (w/v) sucrose. Multiple comparison analysis was performed between all the tested *Arabidopsis* lines for each plant tissue and any significant differences were indicated with letters.

All the transgenic lines were found to contain higher glucose and sucrose amounts in their leaves compared to the control plants (Figure 8A). However, only the mutant lines *sweet12*, *sweet11;12* and *sweet11;15* as wells as the overexpressing line *SWEET35S11* were found to have significantly higher glucose amounts in the leaves compared to Col-0, with the last one containing a significantly higher amount than the former lines. The sucrose amounts in the leaves of the Arabidopsis tested lines was similar to glucose results, although few differences were observed between some lines. The double mutant line *sweet11;12* had significantly higher glucose in the leaves but not sucrose compared to Col-0, and *vice versa* for *SWEET35S12*.

On the other hand, results from the glucose and sucrose amounts in the roots of the Arabidopsis mutant lines and the WT were more diverse (Figure 8B). Significantly higher amounts of glucose in the roots of *sweet12*, *SWEET35S11*, *sweet12;15* and *sweet11;12;15* were observed, while sucrose results fluctuated among the tested lines. First, significantly higher amounts of sucrose compared to the WT were found in the lines *sweet12*, *SWEET35S11* and *sweet11;12;15*. Secondly, the double mutant line *sweet11;15* was the only line that showed the significantly lower amount of sucrose compared to Col-0 roots. Surprisingly, sucrose levels within the roots of the double mutant line *sweet11;12* were not identified, but possibly due to experimental errors. The other lines did not show any statistical differences compared to the wild-type.

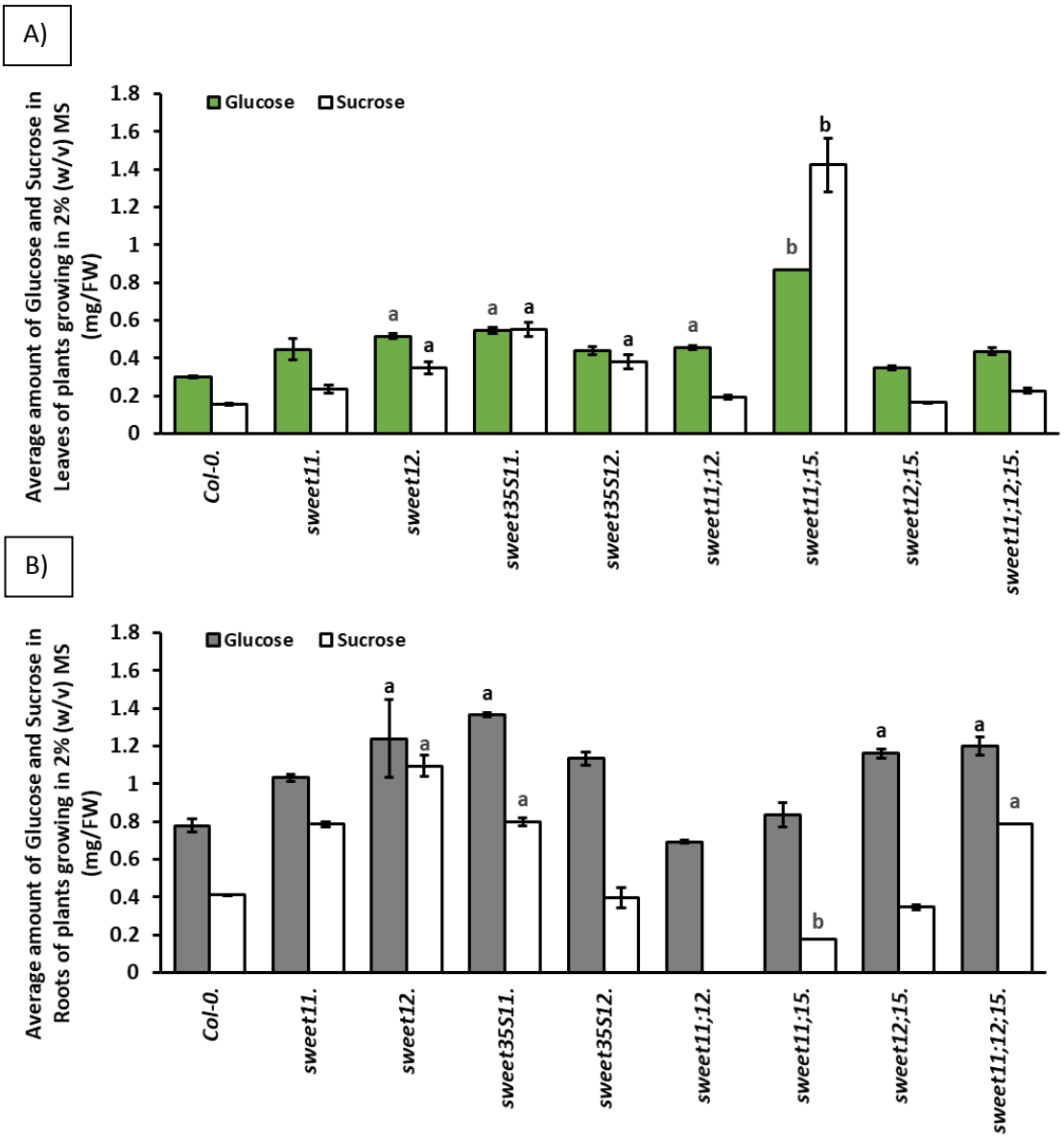

Figure 8 Average amount of Sucrose and Glucose both in A) leaves (n~3) and B) roots (n~3) of Col-0 and mutant lines. The letters represent significant differences between the tested line compared to the Col-0 control plant tissue ($p < 0.05$).

Photosynthetic Assessment

Photosynthesis and SWEET functionality

Expression of six photosynthetic marker genes that each represents a major component of the photosynthetic pathway (Figure 12) were quantified within 4 mutant lines. The relative changes in gene expression were quantified using real-time qPCR and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The raw data were normalized against the reference gene *PP2AAC* and quantitative analysis was expressed relative to Col-0 plants expression levels for each marker gene (calibrated as 1). The same process as above was also performed for the expression quantification of two light stress marker genes within the tested lines. Multiple comparison analysis was performed between all the tested *Arabidopsis* lines and any significant differences were indicated with letters. All those results are displayed in Figure 9.

The expression level of beta-carbonic anhydrase 3 (*ATBCA3*) within all the mutant lines was reduced by half related to control Col-0 plants. Different expression levels of photosystem I P700 chlorophyll A apoprotein A1 (*psaA*) were found for each tested line. Triple mutant *sweet11;12;15* had the highest relative expression level of *psaA* gene with a 7-fold difference from Col-0, followed by the double mutants *sweet12;15*, *sweet11;12* and *sweet11;15* respectively. Higher expression levels of photosystem II reaction centre protein A (*psbA*) compared to Col-0 were observed only in *sweet11;12;15* and *sweet11;12* lines, with the former being even significantly higher than the later. Photosynthetic electron transfer A (*PetA*) expression levels were higher in the mutant lines compared to the control plants. Still, only *sweet12;15* and *sweet11;12;15* lines were statistically higher expressed compared to Col-0 with a 5-fold change for both lines. Last but not least, *ATFD2* (ferredoxin 2) and *LHCB1.1* (light harvesting chlorophyll A/B-binding protein 1.1) were expressed in higher levels related to Col-0 only in *sweet12;15* and *sweet11;12;15*, respectively.

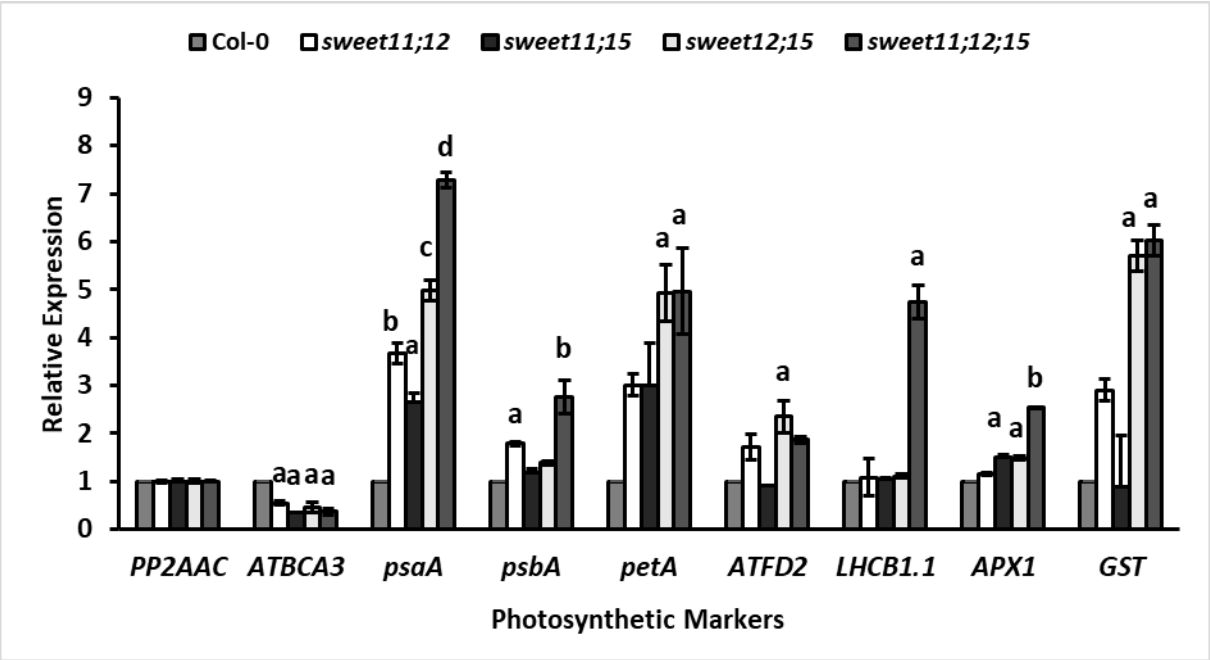

Figure 9: Expression of marker genes implicated in photosynthesis and light stress. Overall six photosynthetic marker genes and two light stress marker genes relative expression level quantified in 4 SWEET mutant lines. The relative changes in gene expression were quantified using real-time qPCR and calculated using the ΔΔCt method. The raw data were normalized against the reference gene PP2AAC and quantitative analysis expressed relative to Col-0 expression levels for each marker gene

The mutant lines *sweet12;15* and *sweet11;12;15* were identified to have greater light stress expression than Col-0, as both light stress marker genes, ascorbate peroxidase 1 (*APX1*) and glutathione s-transferase (*GST*) were highly expressed. Additionally, *sweet11;15* also showed higher expression in *APX1* compared to the control gene but not with *GST*.

Discussion

Molecular Analysis

Ex vitro and *In vitro*

Gene expression analysis for *SWEET* and *SUC* genes was performed for both roots and leaves of *Arabidopsis ecotype* Columbia (Col-0) plants grown *in vitro* and *ex vitro*. There is a distinctive difference on which genes from both families were activated at each growing environment as well as their expression levels within the same tissue. For example, *SWEET16* and *SWEET17* were highly expressed within *in vitro* leaves while in *ex vitro* leaves expression levels were so low that they couldn't be quantified. Same results were recorded on the activity of specific *SUC* genes in the roots between *in vitro* and *ex vitro* conditions. *SUC1* and *SUC7* were the only activated genes in roots of *in vitro* grown Col-0, as for the *ex vitro* Col-0 roots only *SUC2*, *SUC3* and *SUC4* were expressed. Those results can be explained as the pathway and direction of sugar movement is different between the two environments.

Further evidence for this hypothesis, is the high expression level from genes that belong both in the *SWEET* and *SUC* families that were quantified with the source tissue of each environment. Specifically, the level of expression of both *SWEET* and *SUC* genes was higher in the roots of *in vitro* grown Col-0, and *vice versa* for *ex vitro* grown Col-0. Those results suggest a switch of plant tissues functioning on the source-sink transport, more likely a reverse of the system under *in vitro* conditions. This phenomenon of sink transition to source tissue has been described before as a strategy to unfavourable conditions in *Cassava* plants (Fernie and Sonnewald, 2018) meaning that this system is also present in nature. *Cassava* plants sacrifice their leaves while carbon is mobilized from the roots to enable the generation of new leaves (Fernie and Sonnewald, 2018). According to that, plants may have established a plastic source-sink system that can alter its activity and flow movement of sugar transportation, thus sugar transporter functionality.

This research tried to provide a better understanding of how exogenous carbon is being translocated from roots in culture and identify key genes involved in sucrose long-distance translocation in *in vitro* growing plants. After gene expression analysis using primer pairs of genes involved in sucrose translocation from source tissues to sinks, a model was designed to visualise this pathway both *in vitro* and *ex vitro* conditions. The models were designed with the qPCR expression levels of *SWEET* and *SUC* genes both in roots and leaves, as well as data from literature research.

Ex vitro Model

Plants that are grown *ex vitro*, must convert photosynthetically fixed CO_2 to sucrose in mesophyll cells of sources leaves. Then its long-distance translocation via phloem to the sink tissues comes on a downwards direction. First, the produced photoassimilates must be relocated from sites of synthesis via cell-to-cell movement and then loaded into the phloem vessels (ST). *Arabidopsis* is a predominant apoplasmic phloem loader species (Braun *et al*., 2014) meaning that sucrose must cross into the plasma membrane (apoplast) as a prerequisite before being loaded to the phloem (ST). As mentioned in the literature *SWEETs* are responsible for this function as efflux sucrose to the phloem apoplast (Chen *et al*., 2012) with *SWEET11* and *SWEET12* being the key genes for this function (Chen *et al*., 2012; Eom *et al*., 2015). Then a second protein called *SUC2* (H^+ cotransporter) will take the imported sucrose and load it into phloem ST (Wippel and Sauer, 2012; Chen *et al*., 2012). Those two statements are confirmed by the findings of this study as *AtSWEET11* and *AtSUC2* were highly expressed in *ex vitro* leaves (Figure 3). Afterwards, sucrose unloaded to sink tissues and potential key candidates for this action can be *SUC3* and *SUC4* as they were highly expressed in *ex vitro* roots (Figure 3). *SUC3* protein is localized in the sieve-elements of *Arabidopsis* phloem cells and expression levels were also identified in several sink tissues such as trichomes and root tips by Meyer *et al.*, (2004). They also imply a role in delivering sucrose to sink cells from that protein. Gong *et al*., (2014) have also mentioned the importance of *AtSUC4* in sucrose distribution. *SWEE11* and *SWEET12* may also have an impact on phloem unloading as they were also expressed in roots. A model of the above source-sink pathway as well as the identified genes involving in the sucrose translocation system on *ex vitro* grown *Arabidopsis* plants can be seen in Figure 10

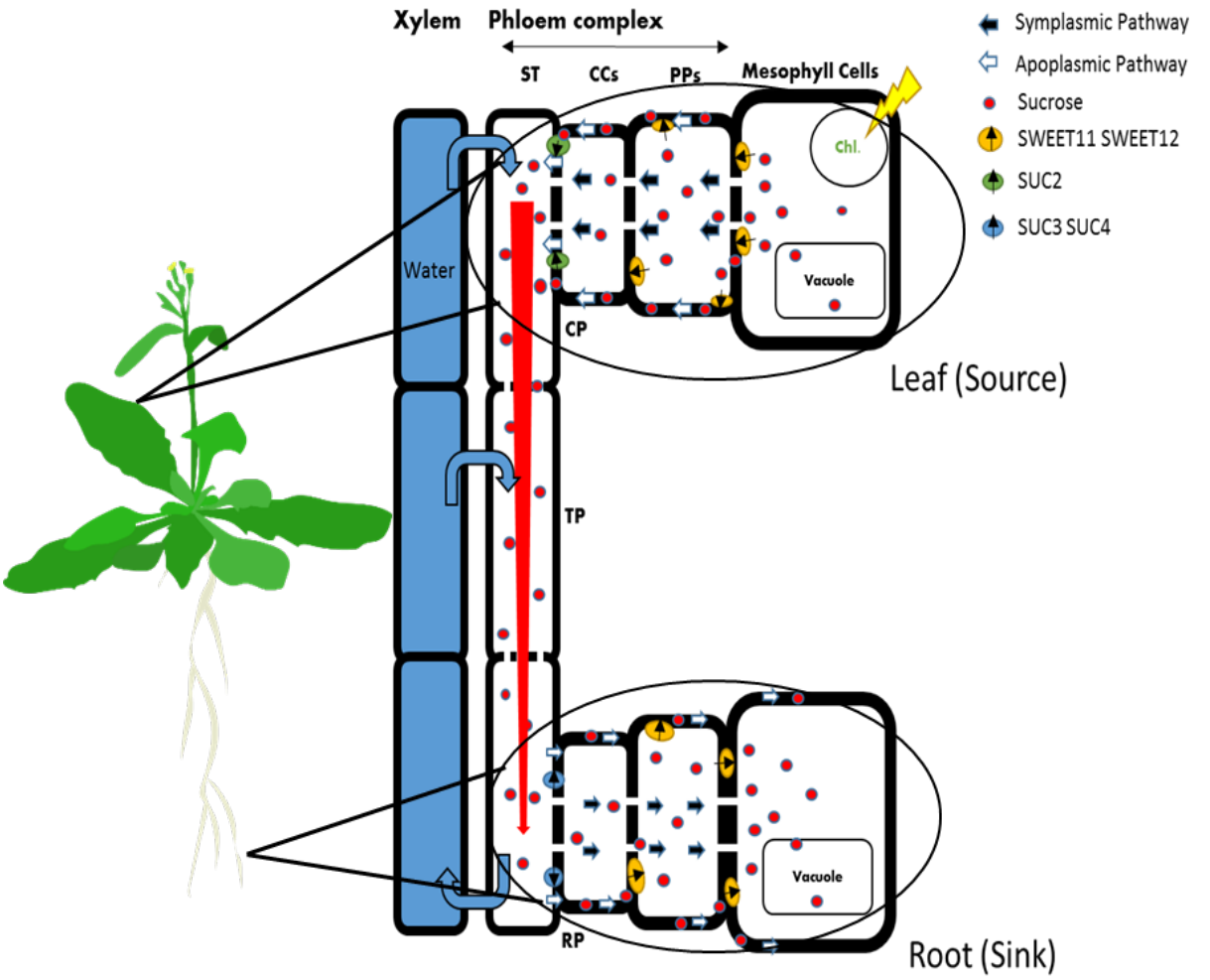

Figure 10: A model representation of the source-sink pathway of sucrose translocation on ex vitro grown Arabidopsis plants as well as the genes involving for this sucrose translocation (short-distance and long-distance transport). The model was designed according to the qPCR data from the gene expressions levels of SWEETs and SUCs both in leaves and roots using 17 primer pairs for the former family and 9 primer pairs for the latter. In addition, literature research was also applied regarding the cellular localization of the expressed genes as well for their function. Sucrose is being translocated from the source leaves where it gets synthesized and travel to the sink roots via phloem, from an upwards to a downwards direction.

In vitro Model

On the other hand, *in vitro* growing plants take their exogenous carbon substance from the growing medium meaning that roots act as a source. Sugar accumulates into the roots and needs to be dispersed by transporting upward to sink tissues such as leaves. This possibly leads to an increased expression and activity of sugar transporting genes within the roots as well as different expressions of individual genes compared to *ex vitro* system. Indeed, the expression levels of genes from both *SWEET* and *SUC* family were much higher in roots (Figure 2). Different genes also seem to play a role for phloem loading from roots and unloading to leaves areas. As mentioned and confirmed above, *AtSUC2* is responsible for phloem loading under *ex vitro* conditions but its expression could not be quantified under *in vitro* conditions. However, *AtSUC1* which belongs to the same phylogenetic group as *SUC2* (Sauer, 2007) was highly expressed in roots (Sivitz *et al*., 2007). Studies have shown that *SUC1* can also load the phloem successfully when *SUC2* is mutated (Wippel and Sauer, 2012) meaning that *AtSUC1* could be responsible for phloem loading *in vitro*. The pseudogene transporter *AtSUC7* (Sauer *et al*., 2004) and *SWEET6* were also highly expressed in roots, implying that they may also have a role on sucrose transportation system but not many information were reported for those two genes in literature. *SWEET11* and *SWEET12* were again expressed within both tissues and specifically highly expressed in roots. As previously mentioned, those two genes are responsible for sucrose transport to the phloem apoplast where a second protein will take on for phloem loading, possible *AtSUC1* under *in vitro*. Carpaneto *et al*., (2005) have discovered that the direction of sucrose transport by a transporter can be reversed depending on the direction of sucrose gradient, which can support the involvement of *SWEET11* and *SWEET12*.

Then with a downward to upward direction sucrose must then be unloaded in the sinks and specifically in the leaves. Here is getting slightly more complicated as many genes were similarly expressed, including also the sudden highly expression of the two vacuolar transporters *SWEET16* and *SWEET17*. Those two genes play a critical role in the vacuolar storage of sugars which is important for plant growth and development (Klemens *et al*., 2013; Chardon *et al*., 2013; Guo *et al.,* 2014;). Knowing that photosynthesis is limited under *in vitro* conditions the high expression of those genes could be implied as the supply of sugars to the vacuole. Expression of *SWEET11* and *SWEET12* in leaves suggests a role on sucrose secretion, maybe a revered mechanism (Carpaneto *et* al., 2005) compared to *ex vitro*. Interesting expressions were found on the *SUC* family as the genes *SUC3* and *SUC4* that were proposed before to participate in the phloem unloading in *ex vitro* were also expressed in the leaves of in vitro grown Col-0 plants. This association can indicate a role of *SUC3* and *SUC4* on phloem unloading. A model of the above source-sink

pathway as well as the identified genes involving in the sucrose translocation system on in *vitro* (2% sucrose MS) grown *Arabidopsis* plants can be seen in Figure 11.

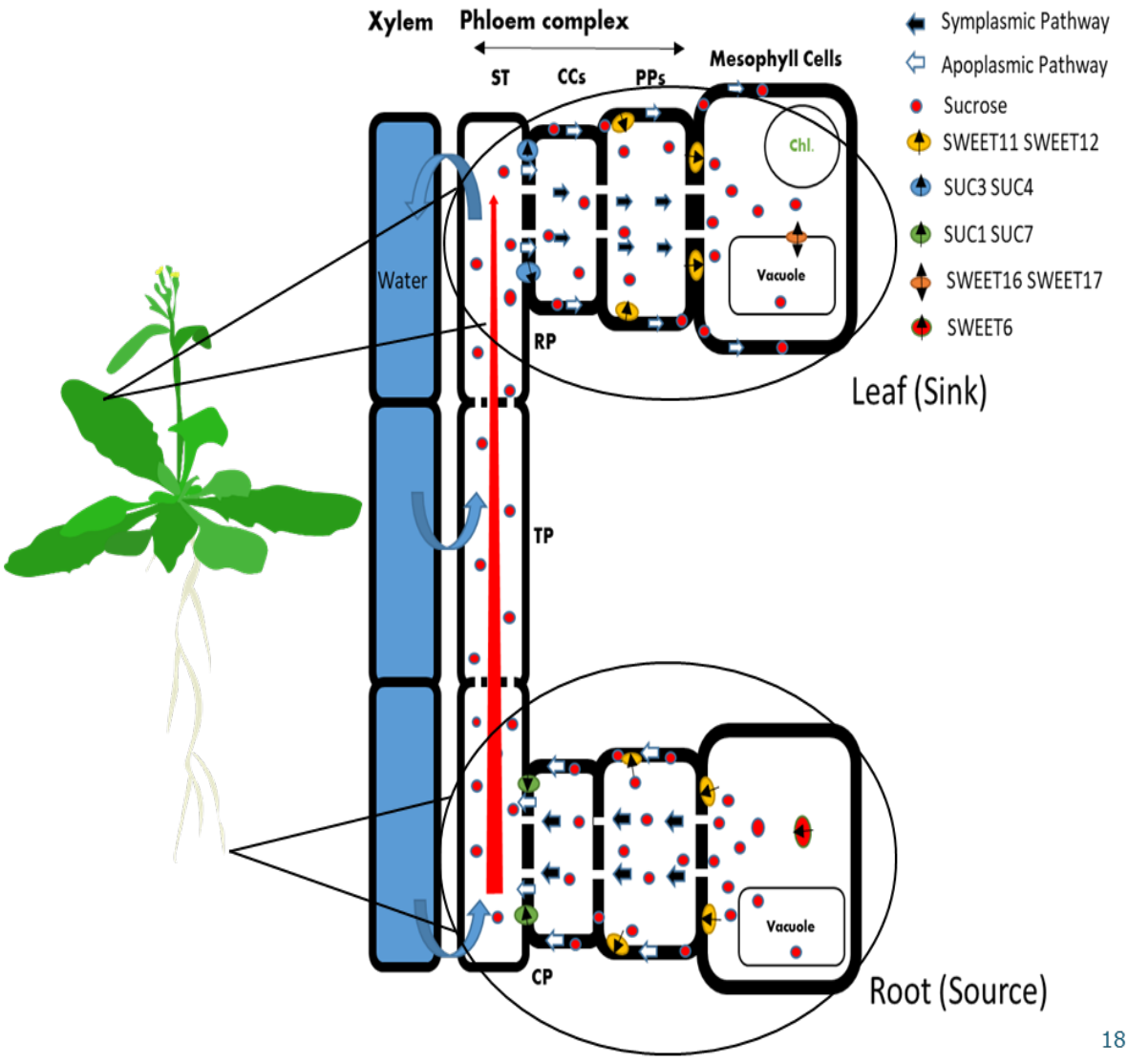

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Figure 11: A model representation of the source-sink pathway of sucrose translocation on in vitro (2% sucrose MS) grown Arabidopsis plants as well as the genes involving for this sucrose translocation (short-distance and long-distance transport). The model was designed according to the qPCR data from the gene expressions levels of SWEETs and SUCs both in leaves and roots using 17 primer pairs for the former family and 9 primer pairs for the latter. In addition, literature research was also applied regarding the cellular localization of the expressed genes as well for their function. Sucrose is being translocated from the source roots and travel to the sink leaves via phloem, from a downwards to an upwards direction. Sucrose is being uptake in the roots from the growth medium in culture.

Besides the clear differences between the two system, some similarities were also observed. *SWEET* genes had a higher number of active individuals compared to the *SUC* family for both environments and plant tissues. This may indicate a simpler role for the *SUC* genes like the phloem loading as mentioned in this literature. On the other side, *SWEET* genes may have more complicated tasks to do as sucrose transporters, due to diverse functions (mentioned in the Introduction). Overall, both families are important and working together as a link forming a chain that contributes to the sucrose translocation in plants.

According to this statement, the "chain" starts with the function of *SWEET* genes as they are exporting sucrose from source tissues and *SWEET11* and *SWEET12* are the main targets. This study wanted to further explore the functionality of *SWEET11* and *SWEET12* under *in vitro* conditions and gain clearer information regarding their role. *Arabidopsis* mutant lines were selected with insertions generating single, double and triple mutations of those two genes as well as their homolog *SWEET15*. For this analysis, *AtSWEET15* was also used for the double and triple combination of mutations as it is also believed to be involved in sugar transportation (Chen *et al.,* 2012;2015). All the members of the SWEET clade III (*AtSWEET9-15*) were reported to transport sucrose across the plasma membrane (Chen *et al.,* 2012), but only *AtSWEET15* showed some expression levels under in vitro (Figure 2). The creator of each mutant line can be seen in Table 2, locating in the Material and Methods sector.

Physiological Analysis

Three weeks after sowing all mutant lines showed less growth than Col-0 plants and two weeks later fresh and dry weight analysis was performed to define growth differences. For this analysis only, the average biomass productions (DW/FW*100) was presented as non-significant differences were found with FW and DW data. This formula was created because no differences were observed in the DW measurements between the transgenic (mutants and overexpressing) lines compared to Colombia control plants. Knowing that sucrose represents the main growth building block inside the medium and that the control environment is stable under *in vitro* conditions (assumingly being the same within each tested line), then the average DW of each line divided by their FW will indicate the average percentage of produced biomass. This produced biomass can be correlated as a growth indicator. Sucrose plays a critical role in this biomass production, meaning that when a higher amount of sucrose being utilized, then a higher amount of biomass should also be presented (Figure 7). In the same direction, a smaller increase of biomass percentage can also be associated with a lesser sucrose utilization process within the plant. Supposing that the mutations affected the sucrose translocation pathway, then less sucrose should be transfer across the

mutants thus less total biomass. Col-0 had the highest biomass production (regarding its fresh weight) with 8.03% followed by the double mutant *sweet11;12* and triple mutant *sweet11;12;15* with 7.31% and 7.05% respectively. Although, significant lower differences were observed only with the single mutation of *SWEET11* and *SWEET12* as well as with their respectively overexpressed lines (Figure 6). This suggests that reducing expression of a sucrose translocator gene such as *SWEET11 and SWEET12* can negatively influence the biomass production of *in vitro* grown plants. In the same direction, the opposite effect was expected to occur when the same genes were overexpressed. The overexpression of *SWEET11* and *SWEET12* was expected to increase the biomass production in the transgenic lines but this hypothesis was rejected. Surprisingly, the biomass percentage for both overexpressing lines was significantly lower compared to the Col-0 control plants, but higher than the single mutant lines. Gene expression analysis was performed to confirm the overexpressing lines and higher expression levels were found for both lines compares to the Col-0 gene activity (Data not presented).

According to our results, a single mutation of *SWEET11* and *SWEET12* genes can cause a higher deficiency in the average biomass production than a combined mutation in both. Those results were unexpected as with the insertion of double and even triple mutation, a linear decrease of the overall biomass production was expected to occur. On the other hand, both single mutants *SWEET11* and *SWEET12* had higher fresh and dry weight than the double and triple mutants which was expected (Table 1). As reported by Chen *et al*., (2012), double mutant *sweet11;12* was able to acquire sucrose from the medium (*in vitro*) to restore root growth after carbohydrate restriction, thus sucrose uptake and utilization function were not affected. A potential explanation could be referred to as the redundancy/complementation of the *SWEET* system. The *Arabidopsis* genome encodes several *SWEET* paralogs, including closely related transporters, that under normal conditions are expressed in lower levels, but when mutations occur specific transporters can be induced (Chen *et al.,* 2012) and replace the lost function. This complementation of *SWEET* genes was described several times in the literature (Chen *et al*., 2012; Feng and Frommer, 2015; Latorraca *et al*., 2017; Jia *et al.,* 2017). Such a complementation in the *SWEET* family might appear to increase system robustness (sucrose translocation) and reported to evolved as a defence mechanism for pathogens (Chen *et al*.; 2012; 2015). Chen *et al.,* (2010) has found that pathogens can highjack their host sugar translocation system by targeting *SWEET* genes and specifically members from the clade III. The pathogen may have developed this strategy to gain higher sugar levels from the apoplast by retooling the sucrose translocation system in plants (Eom *et al*., 2015). As a result, plants might develop a counter mechanism that can mutate the highjacked gene(s) to promote resistance (Chen *et al*., 2012), while on the same time enhance other genes

to fulfil the loss function. This hypothesis could be the reason why double and triple mutant lines were not lethal, but also why non-significant differences were recorded in mass percentage compared to Col-0 plants.

Water proportion was calculated by dividing the subtraction from FW and DW, with the FW ((FW-DW)/FW). This formula can indicate the average water percentage that has been kept within each tested line. Those numbers are correlated as the Mass % subtraction to 100. Bearing that, same mutant lines that exhibited significantly lower average mass percentage than Col-0, have exhibited significantly higher average water concentration levels (Figure 6). Plants may perhaps uptake nutrients and sucrose from the medium but due to defective *SWEET* genes the sucrose was not transported upwards and accumulated more in the root area. Consequently, the reduction of the average biomass production within the plant.
The single mutant *sweet12* was found to have significantly higher sucrose and glucose amounts (Figure 8) in the roots compared to Col-0 roots. In addition, significantly lower biomass percentage and higher water patterns within this mutant line were also observed (Figure6), supporting the previous hypothesis. Further experiments are needed to confirm this assumption. Higher sucrose and glucose amount were also found in single mutant *sweet11* but not significant than Col-0, probably due to low sampling number (n~3). Same biomass and water percentages as *sweeet12* were reported for *sweet11* (Table 1). Higher FW and DW figures (Table 1), probably appeared within the single mutant lines due to higher inflow of water in the plants as a strategy to obtain more sucrose from the growing medium (pumping more water). Plants consisting with more water levels may be associated to some extent with the higher average FW levels and thus DW due to cell elongation (more cellulose), but as a total, a lower representative of the average utilized product.

Sucrose concentration within the growing medium influenced the overall Mass production. All the mutant lines that grew on 4% sucrose mediums had significantly higher amounts of average mass production compared to the same plants growing on 2% sucrose (Supplementary Data 2) but also lower water percentages respectively (Data not presented). Osmosis has also an effect on this, as by increasing the sucrose concentration within the medium then more sucrose might accumulate in the roots which can regulate the osmotic pressure on the water that inflow to the roots (Ruan, 2014), probably in a negative way. Comparing Col-0 plants growing at 2% and 4% sucrose, non-significant differences were found regarding DW and FW, but only with the average Mass percentage (Figure 7). Suggesting that more sucrose was utilized within the *Arabidopsis* plants *in vitro* when sucrose concentration was increased from 2% to

4% in the medium. This higher amount of sucrose had, as a result, the reduction of water levels in the plans growing at 4% sucrose, as the FW of those plants was lower than plants grown on 2% sucrose. Therefore, plants grown in 4% sucrose had a higher amount of utilized sugar for biomass production (Mass %) as wells as lower water levels in plant and vice versa for plants grown on 2% sucrose, acting as an equilibrium regarding DW. That could be the reason why non-significant differences were observed for DW measurement between a plant grown on 2% and 4% sucrose (Figure 7).

Sugar Determination

From all the tested Arabidopsis transgenic lines, only two lines had higher sucrose and glucose content both in leaves and root tissues compared to Col-0 sample tissues. Surprisingly, those lines were the single mutant *sweet12* and the overexpressed line *SWEET35S11*, suggesting that induced mutation on *AtSWEET12,* may enhance the activity of its homolog *AtSWEET11* to overcome the lost function. The other way around regarding *sweet11* and *SWEET35S12* was not observed. However, higher levels of sucrose and glucose amounts were found in both tissues of those lines compared to the Col-0 tissues as controlled but were not significant, probably due to low replication number This assumption could also support the similar results that were found during the Physiological analysis between the mutation and overexpressing lines of both *SWEET11* and *SWEET12* (Figure 6). Further experiments are needed to confirm this hypothesis. In addition, the two overexpressing lines *SWEET35S12* and *SWEET35S11* had higher amount of sucrose in the leaves than the wild-type, implying that they might indirect translocated more sucrose from the source roots to the sink the leaves (L.-Q. Chen *et al*., 2012; Le Hir *et al*., 2015) but the activity of the phloem loader *in vitro* couldn't be defined in this thesis report to provide better understanding.

Other interesting results from this analysis, were the significantly higher amounts of sucrose and glucose in the roots of the triple mutant line *sweet11;12;15* which were expected (Figure 8B). These results implying that *SWEET11*, *SWEET12* and *SWEET15* can mediate sugar translocation and allocation in roots of *in vitro* grown *Arabidopsis*. However, the relative amounts of glucose and sucrose in leaves were not reduced in the triple mutant line compared to the Col-0 plants. Strange results were observed in *sweet11;15* line as a lower amount of sucrose was determined in the roots but in the leaves, an enormous amount compared to the control was recorded (Figure 8A; B). Those results may indicate that photosynthesis was stimulated within all the mutant line due to deficient sucrose transportation in the leaves, and for an unknown reason at a higher level in the double mutant *sweet11;15*.

Photosynthetic Capability within Genotype and sucrose concentration

Arabidopsis plants grown *in vitro*, receive carbon molecules from the growing medium. The reason behind this concept is to provide the growing plants with sugars to overcome the decreased photosynthetic rate (Donnelly and Vidaver 1984; Kozai, 2010). The double mutant line *sweet11;15* showed a higher concentration of sucrose and glucose in leaves compared to the control, while glucose and sucrose levels in leaves of the rest mutant lines were similar to Col-0 (excluding the overexpressing SWEET genes lines). Knowing that sugar translocation is affected in the mutant lines, a decrease of sugar components in leaves was expected, but not observed. By connecting those observations, a new hypothesis was raised regarding the presumed upregulation of photosynthetic activity within the mutant lines. Photosynthesis can be regulated by the availability of light interacting with the source leaves, as well as by the demand for photoassimilates depending on the plant's sink activity (William *et al*., 2014). Reduced sucrose translocation (from downwards to upwards) in the mutant plants grown in vitro could lead to higher photosynthetic activity in the leaves, so the plants cover their energy needs (William *et al*., 2014). An experiment was conducted in order to clarify the above dilemma using six photosynthetic marker genes that each represent a main component of the photosynthetic apparatus (Figure 12).

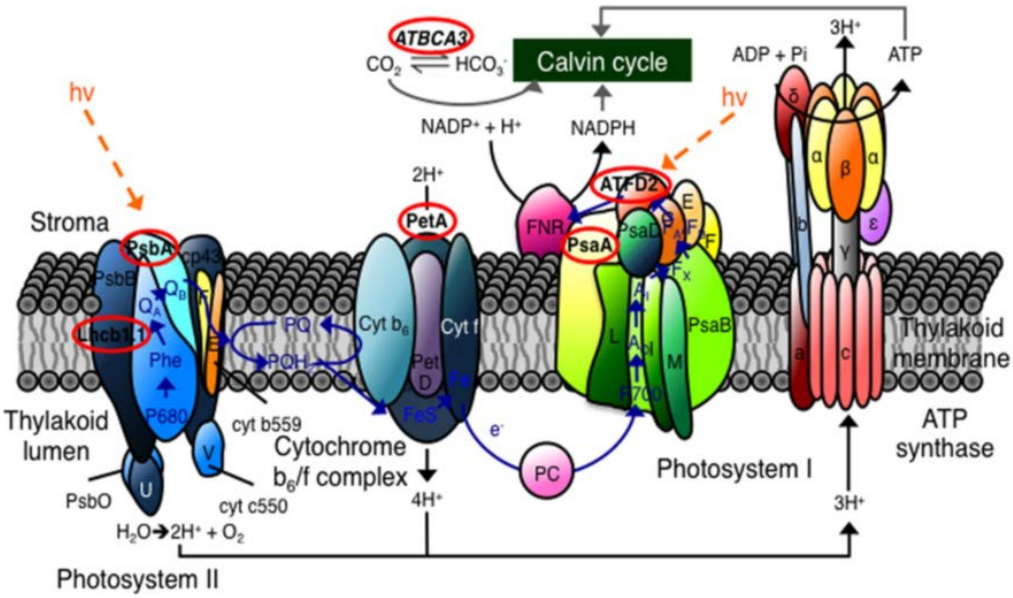


Figure 12: Diagram representing plant's photosynthetic pathway (Ooi et al., 2016)

Two genes, *psaA* and *psbA* are responsible for the primary electron donor of Photosystem I (PSI) and photosystem II (PSII) respectively (Thum *et al*., 2001; Gong *et al*., 2013). Those two genes can be associated with photo-inhibition and predominantly for *psbA,* as PSII is more susceptible to damage under high light intensity (Thum *et al*., 2001; Ooi *et al*., 2016). Combined mutations of both *SWEET11* and *SWEET12* seem to be required to induced photoinhibition as the relative expression levels of *psbA* were significantly higher in the mutant lines *sweet11;12* and *sweet11;12;15* (Figure 9). Those results can be accepted as *SWEET11* and *SWEET12* are expressed in the leaves and are responsible for sucrose migration from the synthesize areas to the apoplast (Chen *et al*., 2012). The same scenario likewise in vivo probably occurred within the mutant lines grown *in vitro*. The mutant lines *sweet11;12* and *sweet11;12;15* might have a higher photosynthetic activity than Col-0 but due to the mutation of important sucrose translocators, sucrose accumulated in the leaves. As a result, photoinhibition might get induced due to full capacity of photoassimilates in the leaves. This hypothesis might also support the high levels of sucrose that been found in all mutant lines in Figure 8A. Higher expression levels of the photosynthetic gene *psaA* were also recorded in all mutant lines compared to the Col-0 plants, suggesting induced photoinhibition in all lines (Ooi *et al*., 2016). The indications for photo-inhibition were also supported by the high transcript levels of the two light stress genes *APX1* and *GST*. Moreover, photoinhibition in the mutant lines leaves was again supported by the low transcript levels of *ATBCA3* which involved in carbon utilization during photosynthesis (Price *et al.*, 1994; Ooi *et al*., 2016).

Taken together the gene expression data of the photosynthetic markers, suggest a light-induced reduction in the photosynthetic capacity of the mutant plants compared to the *Arabidopsis* wild-type. The photosynthetic apparatus of the mutant lines probably saturated due to the inability of exporting sugars from the leaves. The rate of sugar production exceeded the rate of export thus photoassimilates accumulated in the leaves (William *et al.,* 2014) which can lead to an inhibition of photosynthesis (Rolland *et al*. 2006). Although direct conclusions cannot be drawn from these outcomes regarding the photosynthetic activity of the mutant lines as photoinhibition was induced possibly due to an already existing photosynthetic activity which could not be defined in this experiment.

Conclusion and Further studies

This thesis report tried to give a better understanding regarding the functionality of *SWEET* sucrose transporters at cellular and plant level. *SWEET11* and *SWEET12* were found to be important proteins for sucrose translocation both in the source and the sink of *in vitro* grown *Arabidopsis* plants. Moreover, these two genes were important for both phloem loading and unloading on *in vitro plants*. In addition, we are the first to present a potential schematic representation (model) of the sucrose translocation system of plants grown *in vitro.* Also, confirming the already known data of sucrose translocation in *ex vitro* grown *Arabidopsis* and drawn them down as well in a model scheme. Mutation of *SWEET12* may enhance the activity of its homolog *SWEET11* and vice versa but further experiments are needed to test this hypothesis. A candidate experiment to work on this hypothesis could be the insertion of a fluorescence carbon molecule such as esculin or a radioactive isotope of carbon ^{14}C in culture and compare the sucrose flow between the single mutant lines and the overexpressing lines (quantify light intensity). This experiment could also be applied to identify the conductive tissue that sucrose transport within *in vitro* grown plants. Another experiment to apply could be complementation assays. Moreover, complementation assays can also be used to confirm and further study the complementation system (redundant) within the *SWEET* gene family. Besides *SWEET* genes, other genes such as *SUCs* have a more direct role in long-distance sucrose translocation and especially for the phloem loading and unloading process. *AtSUC1* may play a prominent role in phloem loading *in vitro,* which will be interesting to further study. Same for the *SUC3* and *SUC4* genes as potential key proteins for phloem unloading both *in vitro* and *ex vitro*.

Finally, further studies are needed to be conducted to gain clearer answers regarding sucrose translocation *in vitro*. Sucrose transporters could be the answer on improving growth rate of plants grown *in vitro* as well as overcoming tissue culture barriers like recalcitrance. This thesis study wanted to shed light on the current mystery of how sucrose translocated in plants grown *in vitro*. Hoping that the results and the conclusions of this thesis will set the foundations for future studies to carry on by the successors.

Materials and Methods

Plant Growth Conditions

Arabidopsis wild-type (WT) Columbia (Col-0) seeds were germinated both *in vitro* and *ex vitro*. Seeds of the *Arabidopsis* cultivar Col-0 were germinated and grown in pots under glasshouse conditions with the temperature maintained at 24°C during the day, and during the night. Plantlets were exposed to a photoperiod of 16h light and 8h dark cycle. For *in vitro*, Col-0 seeds were sterilized using 2% (w/v) sodium hypochlorite (NaClO) and then germinated in Murashige and Skoog medium (MS) with 2% (w/v) sucrose at 4°C in dark for four days. After a week, ten plants from each line were transferred into new 2% (w/v) sucrose MS contained petri dishes in triplicates, under climate control conditions with the temperature maintained at 24°C during da, and night. Plantlets had the same photoperiod exposure with *ex vitro* grown plants.

Seeds from homozygous *Arabidopsis* mutant lines (Table 2) were germinated in the same way as mentioned above for *in vitro* Col-0 seeds. Plantlets from the *Arabidopsis* WT and *SWEET* mutant lines were also transferred both in 4% and 0% (w/v) sucrose 0.5 MS contained petri dishes independently, in triplicates.

Table 2: The creators and the stock names of all the tested Arabidopsis lines

Cultivars	*Stock Name*	*Creator*	Publication
Col-0			
sweet11	CS68843	Wolf Frommer	Chen *et al*., (2012)
sweet12	CS68844	Wolf Frommer	Chen *et al*., (2012)
sweet35S11		Wolf Frommer	Chen *et al*., (2012
sweet35S12		Wolf Frommer	Chen *et al*., (2012
sweet11;12	CS68845	Wolf Frommer	Chen *et al*., (2012)
sweet11;15	CS68996	Wolf Frommer	Chen *et al*., (2015)
sweet12;15	CS68997	Wolf Frommer	Chen *et al*., (2015)
sweet11;12;15	CS68998	Wolf Frommer	Chen *et al*., (2015)

Harvesting Plant Material

Arabidopsis plants of both mutant and WT were used for the measurement of fresh and dry weights, biochemical content, gene expression and histological analysis. Plants were collected 5 weeks after sowing. Tissues harvested for analysis were source leaves (youngest fully expanded leaves) and roots for RNA isolation and sugar extraction, while whole plants were harvested for the Fresh and Dry weight measurements. Plants that used for fresh and dry weight were measured using XPE105 DR analytical balance (Mettler-Toledo). Samples that were used for RNA isolation were directly frozen in liquid nitrogen immediately following the harvest.

RNA Isolation

Frozen tissue samples in liquid nitrogen were ground in Eppendorf tubes containing two steel balls, by agitating for 2 min at 30 Hz using a Retsch TissueLyser II (QIAGEN, Chadstone Centre, VIC, Australia). RNA was isolated from approximately 100 mg of ground material using the plant RNeasy® kit (QIAGEN) according to the manufacturer's instructions. The total RNA extracted was quantified and the quality was checked using NanoDrop ND-100 spectrophotometer (Isogen).

cDNA Synthesis

Complementary DNA (cDNA) was synthesized from 1 µg of the extracted RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Synthesized cDNA was first diluted before was used for the following qPCR reaction

SWEET and *SUC* Transcript quantification

Primers used for the amplification of the genes studied were designed and provided by Huayi Li (Supplementary data 3). Quantitative qPCR was carried out on CFX96 Real-Time PCR (Bio-Rad Laboratories, USA) using SYBR green (QIAGEN) and PCR cycles according to the manufacturer's instructions. Gene expression was measured relative to the housekeeping gene *Arabidopsis polyubiquitin 10* (*UBQ10; AT4G05320*).

Photosynthetic capability and light stress-related gene expression

Primers used in the amplification gene studied were designed by Ooi *et al*., (2016) and provided by Huayi Li (Supplementary data 4). The expression of photosynthetic and stress-related genes for *in vitro* growing Col-0 and *AtSWEETs* mutant plants was quantitated by CFX96 Teal-Time PCR (Bio-Rad Laboratories, USA) using SYBR green (QIAGEN) and PCR cycles according to the manufacturer's instructions. Gene expression was measured relative to the housekeeping gene *Arabidopsis* phosphatase 2A subunit A3 protein (*PP2AA3; AT1G13320*).

Sugar Extraction and mutant lines determination

Sugars were extracted from approximately 100 mg of leaves and roots materials independently, in triplicate for each tested line. Samples were added into already pre-weighted labelled 12 ml glass tubes and re-weighted using XPE105 DR analytical balance (Mettler-Toledo). Roots were carefully washed in dH_20 to remove any attached exogenous sugar from the growing medium before added into the glass tubes. Then 2 ml of 80% ethanol was added into the tubes and incubated for 40 minutes in an HLC thermoshaker (MHR 23 DITABIS), 500 rpm on 80^oC. After the incubation, the tubes were centrifuged on spin machine Multifuge 3s (Heraeus) on 4000 rpm for 10 minutes, and then the supernatant was transferred to new 12 ml glass tubes by disposable glass pasture pipettes. This procedure of the 80% Ethanol washing was repeated for 2 more times leading to a total of 6 ml of 80% ethanol for each glass tube. The supernatants were then evaporated until dried by vacuum using the RapidVap N2 (Labconco) to remove the ethanol. The evaporated dried samples were re-suspended by adding 1000 µl of dH_2O and incubated for 10 minutes in the HLC thermoshaker, 200 rpm on 25^oC and then transferred to a 1.5 ml Eppendorf tube. Tubes were centrifuge on 13000 rpm for 5 minutes and then 100 µl of the clear supernatant transferred in Dionex tubes (32x11.6 mm Thermo Scientific) contained a 0,1ml Mikroeinsatz, 31 x 6mm, Klarglas (Thermo Scientific) which were used for the HPLC measurements by Dionex ICS-500 DC (Thermo Scientific). The standard curves of the standard sugars were constructed manually from different dilutions of rhamnose, galactose, arabinose, glucose, sucrose, mannose, xylose and fructose. Each sugar component was dissolved in MQ water in the following stock concentrations: 0.005, 0.010, 0.025, 0.050 and 0.100 mg/ml. Those concentrations were used as the standards to calculate the content of each neutral sugar in the tested samples.

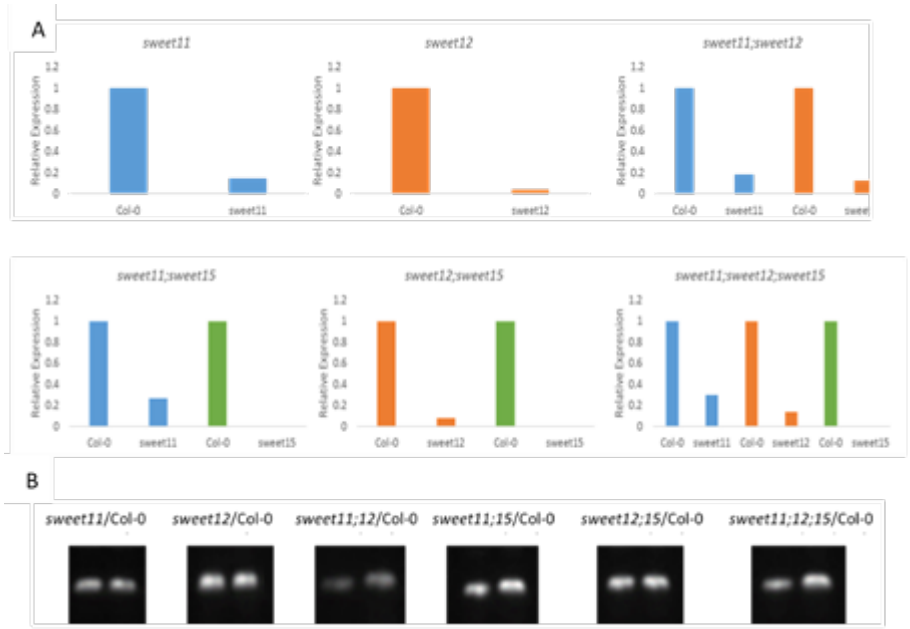
Digital photography

All photographs were taken using the Olympus Stylus SP-100 Digital Camera.

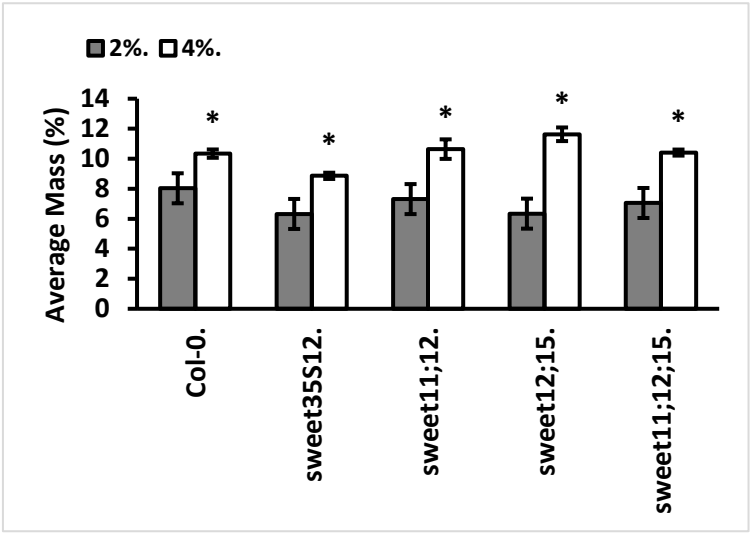
Statistical Analysis

Statistical analysis was performed using ANOVA with GenStat for Windows 19th Edition and Daniel's XL Toolbox in Microsoft Excel 2016. Significance was set to a threshold of $P < 0.05$. Raw data were also normalized and homogenized using the Shapiro-Wilk test and a Chi-Square test respectively, again with a threshold of $P < 0.05$.

Supplementary Data



SD1: A) Expression analysis of the target genes in the Arabidopsis mutant lines compared to the SWEET genes from Col-0 in vitro plants. B) Agarose gel electrophoresis revealed that the primers for the reference gene UBQ10 *within both the tested mutant line as well the WT had similar or the same amplification.*



SD2: Average Mass% in Arabidopsis wild type and mutant lines in 2% and 4% of sucrose medium. Asterisks are indicating a significant difference of mass production within different sucrose concentration mediums ($p < 0.05$; n~10).

Primer Name	Sequence (5'-3')
SWEET1-F	CTTCTCCACTCTCCATCATGAGATT
SWEET1-R	CATCTGCAGATTTCTCTCCTTTGT
SWEET2-F	AACAGAGAGTTTAAGACAGAGAGAAG
SWEET2-R	ATCCTCCTAAACGTTGGCATTGGT
SWEET3-F	CCAACTTTTCCCTAATCTTTGTTCTTC
SWEET3-R	AACACCCTTGAAAATGTTACTATTGGA
SWEET4-F	CCATCATGAGTAAGGTGATCAAGA
SWEET4-R	CAAAATGAAAAGGTCGAACTTAATAAGTG
SWEET5-F	TGACCCTTATATTTTGATTCCAAATGGT
SWEET5-R	GCCAAGTTCGATTCCAGCATTC
SWEET6-F	GACTCGGTTACGTTGGTGAAGT
SWEET6-R	CAAACGCCGCTAACTCTTTTGTTTAA
SWEET7-F	GACCCATTCATGGCTATACCAAAT
SWEET7-R	ATCCCATAATCCGAAGTTTAATAACACT
SWEET8-F	TTGCTCTCTTCTTCATCAATCTCTCT
SWEET8-R	AGATCCTCCAGAAAGTCTTCGCT
SWEET9-F	GCAAGAGAAAGAGAGAAAAGTGAAGA
SWEET9-R	CCCATAAAACGTTGGCACTGGT
SWEET10-F	TAGAGGAAGAGAGAGGGAGAGAGT
SWEET10-R	ATATACGAACGAACGTCGGTATTG
SWEET11-F	TCCTTCTCCTAACAACTTATATACCATG
SWEET11-R	TCCTATAGAACGTTGGCACAGGA
SWEET12-F	AAAGCTGATATCTTTCTTACTACTTCGAA
SWEET12-R	CTTACAAATCCTATAGAACGTTGGCAC
SWEET13-F	CTTCTACGTTGCCCTTCCAAATG
SWEET13-R	CTTTGTTTCTGGACATCCTTGTTGA
SWEET14-F	ACTTCTACGTTGCGCTTCCAAATA
SWEET14-R	CAGTTCAACATTAAAGTCAATCACTAATTC
SWEET15-F	CAATGACATATGCATAGCGATTCCAA
SWEET15-R	GGACTCATCACGACAATACTCTTAAG
SWEET16-F	GAGATGCAAACTCGCGTTCTAGT
SWEET16-R	GCACACTTCTCGTCGTCACA
SWEET17-F	AGTGACAACAAAGAGCGTGAAATAC
SWEET17-R	ACTTAAACCGTTGCTTAAACCAACC
SUC1-F	AGAGACACAGTCGCCGGA
SUC1-R	AAAGGAGTACTGAAAGTAATAGCTAATGGG
SUC2-F	CCGGAACGGCTTCGTAAGA
SUC2-R	GATTCCGAGTAGCTGCACGTAAG
SUC3-F	CAAGAACCGCAGCCGTAATC
SUC3-R	CTTGACCGCCACCGGAAT
SUC4-F	AGTGTCAAGCGAGGAACGCATA
SUC4-R	AGTCACACGAGAAGCCATTGC
SUC5-F	GGGCTATGGGATTCCATTAG
SUC5-R	TAAAAGACAGACGACCAAGG
SUC6-F	TCCTGTCTCCGGCCTGCTT
SUC6-R	AGGCGCCCATAGCGATGA
SUC7-F	GTCTTTAAGAGACAAGCCCAC
SUC7-R	AGACTGTCTATCCACAGTCGT
SUC8-F	CTAGCTTCCATAATCTCAAGT
SUC8-R	TTGGTAAGTTTCCACCTCCAAAA

SD3: SWEET and SUC primers used for qPCR analysis

Primer Name	Sequence (5′–3′)	Tm (°C)	No. of cycle
psbA qPCR forward	TGCCATTATTCCTACTTCTGCA	60	30
psbA qPCR reverse	AGCACTAAAAAGGGAGCCG	60	30
psaA qPCR forward	GCAGGGCTACTAGGACTTGG	60	30
psaA qPCR reverse	GGCCTGTAAATGGACCTTTATG	60	30
petA qPCR forward	CAGCAGAATTATGAAAATCCACG	60	30
petA qPCR reverse	TATTAGTAGCAGGGTCTGGAGCA	60	30
ATFD2 qPCR forward	ACTTCATTCATCCGTCGTTCC	60	30
ATFD2 qPCR reverse	AAGAACCAGCACGGCAAG	60	30
LHCB1.1 qPCR forward	CCGTGTGACAATGAGGAAGA	60	30
LHCB1.1 qPCR reverse	CAAACTGCCTCTCCAAACTTG	60	30
ATBCA3 qPCR forward	CGAGTTCATAGAAAACTGGATCC	56	35
ATBCA3 qPCR reverse	AGGCAGGGGTAGTCTTGAAGT	56	35
APX1 qPCR forward	GGACGATGCCACAAGGATA	58	35
APX1 qPCR reverse	GTATTTCTCGACCAAAGGACG	58	35
GST qPCR forward	TCTATAAAACACCATACCTTCCTTCA	58	35
GST qPCR reverse	CGAAAAGCGTCAAATCACC	58	35
PP2AAC qPCR forward	GCGGTTGTGGAGAACATGATACG	*	*
PP2AAC qPCR reverse	GAACCAAACACAATTCGTTGCTG	*	*

SD4: Primers for quantifying genes involved in photosynthesis by qRT-PCR. For abbreviations one is referred to page.....

Arabidopsis gene	Other names	Substrate	Cellular localization	Organ expression[a]	Function
SWEET1	None	Glucose, galactose (weak sucrose)	Plasma membrane	Dry seed and flower	ND
SWEET2	None	2-Deoxyglucose	Vacuolar membrane	Leaf and flower	ND
SWEET3	None	2-Deoxyglucose	ND	Flower	ND
SWEET4	None	Glucose	Plasma membrane	Flower	ND
SWEET5	VEX1	Glucose	ND	Pollen vegetative cell	Possibly feeding of germ cell
SWEET6	None	Weak 2-deoxyglucose	ER	Dry seed	ND
SWEET7	None	Glucose	ND	Flower and seed	ND
SWEET8	RPG1	Glucose	Plasma membrane	Tapetum and pollen	Tapetum efflux, pollen nutrition
SWEET9	None	Sucrose (weak glucose)	Plasma membrane and TGN	nectary	Nectar secretion
SWEET10	None	Sucrose	ND	Flower and seed	ND
SWEET11	None	Sucrose	Plasma membrane	Leaf and seed	Embryo nutrition and efflux from phloem parenchyma/phloem loading
SWEET12	None	Sucrose	Plasma membrane	Leaf and seed	Embryo nutrition and efflux from phloem parenchyma/phloem loading
SWEET13	RPG2	Sucrose	ND	Tapetum, tetrads and in leaves of *sweet11;12* mutant	Tapetum efflux, pollen nutrition, primexine deposition, phloem loading?
SWEET14	None	Sucrose	ND	Flower	ND
SWEET15	SAG29	Sucrose	Plasma membrane	Seed	Embryo nutrition, possibly leaf export during senescence
SWEET16	None	Glucose, sucrose, fructose	Vacuolar membrane	Leaf, root	Increased freezing tolerance
SWEET17	None	Fructose	Vacuolar membrane	Leaf, root	Vacuolar fructose content in leaves

[a]Entries in this column derived in part from public microarray data. Abbreviations: ER, endoplasmic reticulum; ND, no data; TGN, *trans*-Golgi network.

SD5: Information of all the SWEET genes

Histologic Analysis

Subcellular localization of *SWEET11* and *SWEET12*

Cuttings from root and leaf sections of both *Arabidopsis* plants *pAtSWEET11:AtSWEET11*-GUS and *pAtSWEET12:AtSWEET12*-GUS can be seen in Figure 9. For both genes, *SWEET11* and *SWEET12*, expressions levels were detected in leaf and root vascular system.

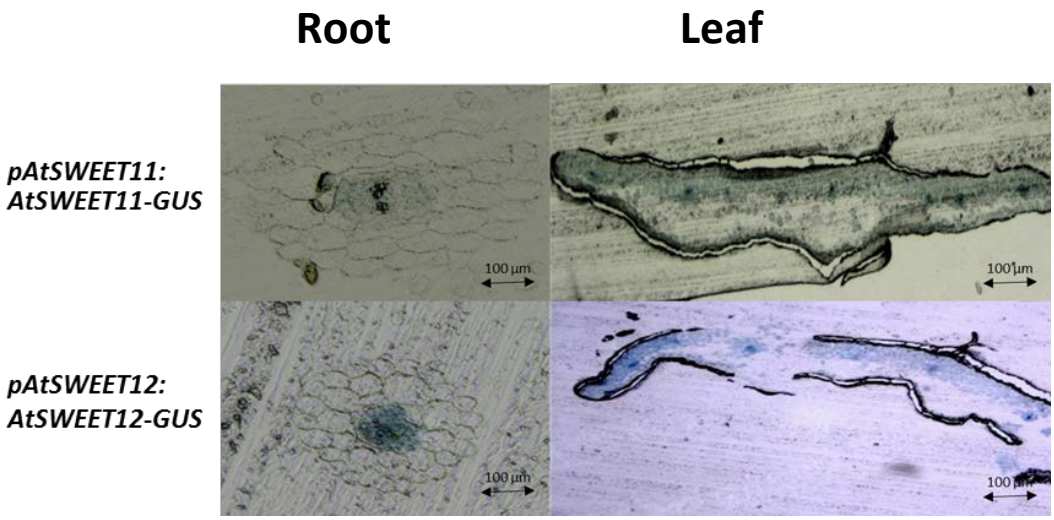


Figure 9: GUS histochemistry analysis in embedded leaves and roots placed on x-axis of transgenic Arabidopsis plants expressing translational GUS fusions of AtSWEET11 and AtSWEET12

Esculin Translocation

Esculin absorption and flow visualization were tested in roots of *Arabidopsis* transgenic plants and compared with Col-0 plants as controls. Col-0 root visualized to have a higher intensity of fluorescent blue light in comparison with the *SWEET* mutant roots. The blue colour represents the emission of the fluorescent sugar Esculin. Less emission of blue light was observed in the double mutant root's and even less in the triple mutant (Figure 10A). Quantification of the blue colour emission was performed using Image J and the results can be seen in Figure 10B. The graph from that figure confirms the above observations.

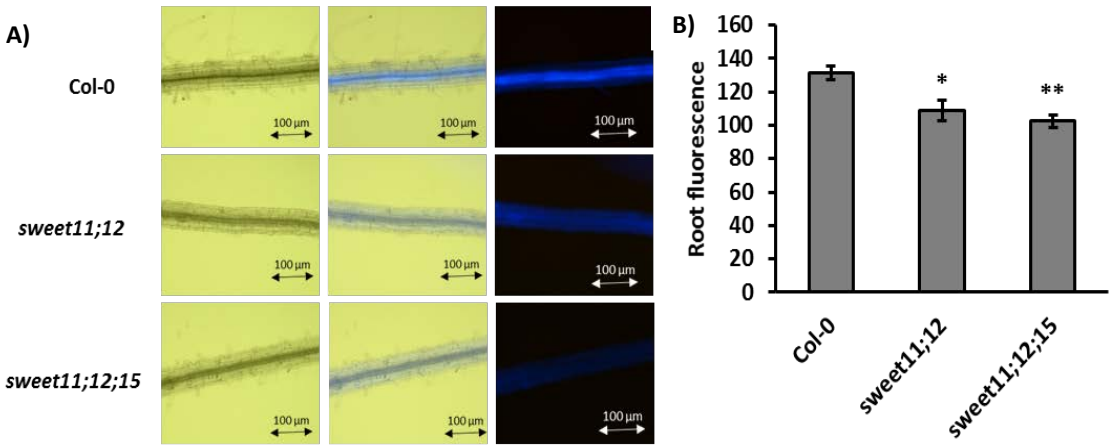


Figure 10: A) Esculin tracking using roots from Arabidopsis WT Col-0, double mutant sweet11;12 and a triple mutant sweet11;12;15. Tested lines placed on 2nM Esculin agar and then analysed under microscope. From left to right are represented pictures from microscope under normal conditions, UV microscope and UV microscope with a black background, respectively. B) Quantification of the blue colour intensity from the UV data with a black microscope using image J software. The results are presented as relative fluorescence emission. Letters represent significant differences (P<0.05) as determined by one-way ANOVA.

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