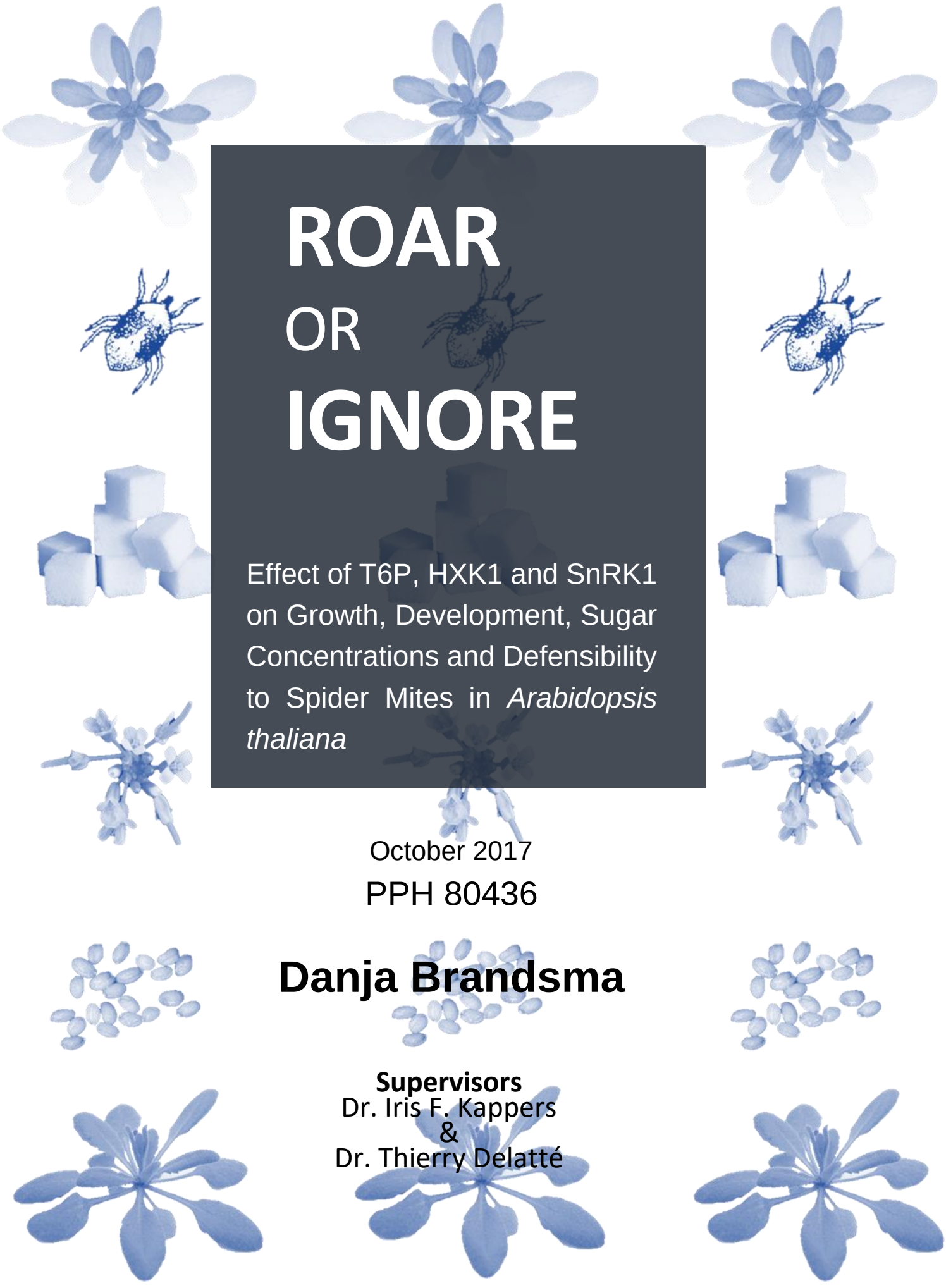

ROAR OR IGNORE

Effect of T6P, HXK1 and SnRK1 on Growth, Development, Sugar Concentrations and Defensibility to Spider Mites in *Arabidopsis thaliana*

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ABSTRACT

Plant growth and development depends primarily on the production of sugars. Sugars produced in source tissue is distributed through the plant, and used to fuel plant growth. This process is highly regulated, not only under non-stressed conditions but also under stress. In this research I investigate the effect of three important sugar signalling agents, trehalose-6-phosphate (T6P), hexokinase I (HXK1), and SNF1-related protein kinase 1 (SnRK1), on plant growth, development, and sugar concentrations under stressed and non-stressed conditions. I subjected the plants to biotic stress, by infesting them with the Two-Spotted Spider Mite, and looked at the plants defensibility. I made use of the bacterial OtsA and OtsB gene to modulate the plants T6P levels, and a subunit of SnRK1, KIN10, to modulate SnRK1 activity in Arabidopsis thaliana. I asked myself the question whether an increased defensibility to spider mites through for example the production of secondary metabolites, has a negative trade-off to growth, hence Roar or Ignore.

In **Chapter 1**, I investigated the effect of SnRK1, T6P, and HXK1 on hypocotyl elongation, leaf expansion, seed production and sugar concentrations under non-stressed conditions. I showed that high T6P levels and AtHXK1 overexpression have an opposite effect on hypocotyl growth, inhibition and elongation respectively. Although it is believed that the effect of T6P of mostly mediated through the inhibition of SnRK1 by T6P, I showed in this chapter that there is an independent role for T6P, apart from SnRK1 inhibition.

In **Chapter 2**, I infested the plants with the Two-Spotted Spider Mite, and I investigated the effect of this biotic stress agent on leaf growth, seed production, sugar concentrations and defensibility of the plant to mites. I showed that HXK1 mutants have the lowest leaf damage, but the highest reduction in leaf growth. Plants with high T6P levels, and AtHXK1 overexpression have a decrease in relative FW, when infested with mites, but an increase in relative DW%. This shows that there is an effect of mites on the plant water content in these mutants. I also show that an increase in raffinose levels is associated with an increase in plant defensibility.

Since some phenotypic characteristics of HXK1 overexpressing plants overlap with plants with altered T6P levels, and SnRK1 activity, I investigated a possible interaction of HXK1 with T6P and SnRK1 in **Chapter 3**. I crossed HXK1 mutants and WT col, with OtsA, OtsB, KIN10 RNAi, and KIN10 OE, and used the F1 generation in my experiments. I showed by looking at leaf expansion, hypocotyl elongation, and sugar concentrations that there is no direct interaction of HXK1 with T6P. However, I also showed that HXK1 interacted with SnRK1, possibly through the inhibition of SnRK1 by the primary product of glucose phosphorylation by HXK1, glucose-6-phosphate (G6P).

In **Chapter 4** I wanted to investigate whether there is an interaction between HXK1 with SnRK1 and T6P under stressed conditions. I infested the different crossing that I made in Chapter 3 with mites, and looked at the plants response. I showed that, as in Chapter 3, HXK1 interacts with KIN10 OE. As in Chapter 2, HXK1 had a major effect on the plants defensibility, by decreasing leaf damage, but with a negative trade-off to growth. I looked at two defense metabolites in the plant, and showed that spider mites can induce the production of secondary metabolites.

With this research I underpin the importance of HXK1, T6P, and SnRK1 as sugar signalling agents in growth and development under stressed and non-stressed conditions. I showed that an increased defensibility has a negative trade-off to growth.

CONTENT

INTRODUCTION

The importance of carbon, and sugar signalling agents in plants

Plant growth and development depends primarily on the production of sugars. Sugar produced in photosynthetic source tissue is distributed through the plant, and used to fuel plant growth, respiration and the production of secondary metabolites. The allocation of sugars calls for a precise regulation in order to optimise plant growth and development under different conditions. The plant must be able to distinguish between source tissue, where sugars are produced, and sink tissue, like young growing leaves, where carbon is used. The regulation of carbon allocation for plant growth and development is mainly controlled by sugar signals. Three important sugar signalling agents are trehalose-6-phosphate (T6P), SNF1-related protein kinase 1 (SnRK1), and Hexokinase 1 (HXK1) (Smeekens et al. 2010; Li & Sheen 2016; Xiao et al. 2000; Tsai & Gazzarrini 2014; Granot et al. 2014; O'Hara et al. 2013; Lastdrager et al. 2014). In this research I will investigate the role of these three sugar signals with respect to plant growth, development, and resistance to biotic stress.

T6P reflects sucrose availability

One of the main sugars allocated through the phloem of *Arabidopsis thaliana* plants is sucrose (Maeda et al. 2006; Riesmeier et al. 1994). It was found that the concentration of sucrose in plant tissue is positively correlated with T6P levels. An increase in sucrose results in an linear increase of T6P content. Therefore T6P reflects sucrose availability in plant tissue (Cátia Nunes et al. 2013). T6P is formed from UDP-Glucose and G6P, a reaction that is catalysed by the enzyme trehalose-6-phosphate synthase (TPS). T6P is then dephosphorylated by trehalose-6-phosphate phosphatase (TPP) into trehalose, which is then hydrolysed by the enzyme trehalase into two glucose molecules (Figure 1: T6P pathway) (Reviewed in Tsai & Gazzarrini 2014). The production of T6P is important, because T6P functions as a signalling molecule, to 'inform' the plant about its carbon status. High T6P levels are a signal of high carbon availability, and low T6P levels for energy deprivation. High T6P levels will result in, for example, starch accumulation (Kolbe et al. 2005). This 'sugar sensing' system of is conserved in several organisms, and because this system is highly regulated in plants, researcher make use of the

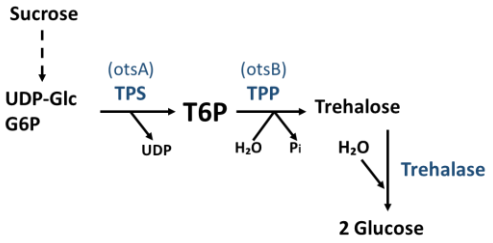


Figure 1: T6P pathway
An increase in sucrose levels results in an similar increase in T6P concentrations. First TPS phosphorylates UDP-Glucose, and G6P into T6P. The TPP dephosphorylates T6P intro trehalose. Trehalase then hydrolyses trehalose into 2 glucose molecules. To modulate T6P levels, the TPS and TPP bacterial homologs (*OtsA* and *OtsB*, respectively) are expressed in the plant.

bacterial TPS and TPP genes, *OtsA* and *OtsB* respectively. Expression of *OtsA* will lead to increased T6P levels, and expression of *OtsB* results in a decrease concentration of T6P (Zhang et al. 2009; Wingler et al. 2012). In this research I will make use of these constructs to modify the plants T6P levels.

SnRK1: an important regulator under stress

An important role of T6P is the inhibition of SnRK1 through an intermediate factor (Zhang et al. 2009; Nunes 2014). SnRK1 regulates a wide range of genes, and partly regulates reprogramming of the transcriptome to promote plant survival under energy deprivation (Baena-González et al. 2007; Tomé et al. 2014; Polge & Thomas 2007; Ghillebert et al. 2011; Cátia Nunes et al. 2013). SnRK1 consists of 3 subunits (Figure 2). In Arabidopsis there are two α-subunits, KIN10 and KIN11 (reviewed in van Wijk 2013). Both are responsible for the kinase activity, but it was found that KIN10 has the highest contribution (Jossier et al. 2009). In this research I will look at the effect of *KIN10* overexpression (KIN10 OE), and partial silencing of *KIN10* by RNA interference (KIN10 RNAi). Since high T6P levels lead to the inhibition of SnRK1, I expect that KIN10 RNAi and KIN OE resemble the same phenotypes as OtsA and OtsB mutants respectively.

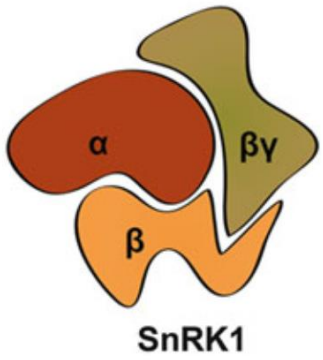


Figure 2: SnRK1 and its subunits
SnRK1 and its subunits, a picture from van Wijk et al, 2013.

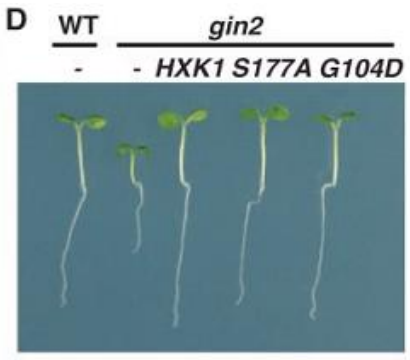


Figure 3: Hypocotyl growth
Seedlings of Arabidopsis thaliana ecotype Landsberg, and the HXK1 mutant gin2, with an insertion of AtHXK1, or the catalytic inactive HXK1 (S177A, G104D) (Moore et al., 2003).

Hexokinase: an enzyme with dual functions

Hexokinase (HXK) is an important enzyme for plant growth. HXK1 is a mitochondrion bound sugar phosphorylating enzyme with a high affinity for glucose (reviewed in Granot 2008; Granot et al. 2013). Although the major function of HXK is phosphorylating sugars and providing energy to the plant, Moore et al. (2003) showed that HXK also has a signalling role besides its catalytic function. The overexpression of catalytic inactive *HXK1*[1] led to a similar glucose signalling response, such as an elongated hypocotyl (Figure 3), leaf expansion under high light intensities, and chlorophyll repression (Moore 2003). It was shown that HXK1 can be transported into the nucleus where it can regulate the expression of multiple genes (Cho et al. 2006). An example is the repression of the CAP2 promotor, an important gene for chlorophyll production. Furthermore it was shown that HXK has a function in stomatal aperture (Kelly et al. 2013; Lugassi et al. 2015), leaf expansion as well as inhibition (Kelly et

[1] The two catalytically inactive HXK1 mutants, S177A and G104D, maintained their glucose-binding site, but both enzymes where impaired in their phosphorylation activity.

al. 2012), hypocotyl elongation (Moore 2003), leaf senescence (Swartzberg et al. 2011; Dai et al. 1999), and flowering time (Unpublished results D. Brandsma & D. Granot). The overexpression of *AtHXK1* results in hypersensitivity to ABA and sugars (Moore 2003), and a decrease in starch content (Dai et al. 1999). Some of these phenotypic characteristics strongly overlap with plants with altered T6P levels or *KIN10* expression. In this research I crossed *AtHXK1* overexpressing plants with plant with altered T6P levels and *KIN10* expression to investigate a possible interaction between these sugar signalling agents.

Scope of the research

In this research I will investigate the effect of the sugar signalling agents T6P, SnRK1, and HXK1 on plant growth, development, and resistance to biotic stress. In chapter 1 I will focuss on the effect of sugar signals under non stressed conditions. The effect of T6P, HXK1, and SnRK1 is strongly effected by the environment, therefor I first create basic knowledge of the behavoir of the mutants in my specific environment before introducing a new factor. In chapter 2 I will investigate the effect of sugar signals when I introduce a biotic stress, the Two-Spotted Spider mite. I expect that the plants respons to this stress will be different among the different mutants compared to non stressed conditions. In chapter 3 and 4 I will look at the interaction between T6P and SnRK1 with HXK1 under non-stressed conditions (CH3), or with the application of the biotic stress agent (CH4). By comparing these results with previous chapters I hope to answer the question whether there is an interaction between HXK1, with SnRK1 and T6P. In the final discussion I will try to answer the question whether increased plant defenses to biotic stress are a negative trade-off to growth, hence Roar or Ignore.

MATERIAL AND METHODS

Plant material and growth conditions

Figure 4: Arabidopsis thaliana plant
Arabidopsis *thaliana* plant, ecotype Columbia. http://bioinfolab.miamioh.edu/jnsviewer/index.html#/home

This research was conducted with Arabidopsis *thaliana* plants (Figure 4) using different eco- and genotypes. To investigate the effect of altered T6P levels, plants with the expression of the bacterial OtsA and OtsB gene, TPS and TPP respectively, in ecotype Columbia background were used. Seeds from the HXK1 overexpressing plants (Columbia background) were kindly provided by the lab of David Granot. For the overexpression of HXK1, the CaMV 35S promoter was used. For the differentially expressed KIN10 unit, plants were used that either had the RNAi contruct to reduce KIN10 mRNA levels, or the overexpression of the KIN10 gene by using 35S promotor in Landsberg erecta background. Before sowing seeds were kept in dark at 5˚C for 4 days to break seed dormancy. Seeds were germinated on Rockwool at 22/17˚C temperature in a 16/8h day/night cycle, and 65% relative air humidity (RH). Plants were watered 3 times a week with water containing plant nutrition (Hyponex 8 Japan, Osaka, Japan). For the hypocotyl elongation experiments, seeds were germinated on agar medium. Before sowing, seed were sterilized by Vapor-Phase sterilization. Seeds were placed in a closed container, in the presence of 150 mL commerical bleach, with 3 mL 37% HCl, and left for 3 hours. The seeds were sown on a 0.8% and ½ MS agar medium and grown under long day conditions. Throughout the different experiments growth conditions were kept as similar as possible. However, due to organizational reasons it was not possible to conduct each experiment under exactly the same growth conditions, and in the same growth room. Table 1 gives an overview of the different growth conditions and growth rooms for each experiment.

Table 1: Growth conditions and growth rooms

Room	Conditions	Experiment
Tissue Culture Room	Long day: 16/8h (Day/Night), 24˚C/18˚C (D/N), RH ≈ 100%	• Hypocotyl elongation
Greenhouse 7.4	Long day: 16/8h (D/N)	• Crossing transgenic lines • Biotic stress and seed production
Growth room B9	Long day: 16/8h (D/N), 22˚C/17˚C (D/N), RH≈65%	• Sugar analysis • Leaf growth • Plant defensibility • Metabolite production

Biotic stress and Spider mite performance

Plants were subjected to biotic stress, by infesting them for 1 week with 10 adult spider mites per plant. The spider mites were collected from the mite hatchery in the Plant Physiology lab, where a mite population is grown on bean plants. Adult mites, that are distinctive by their brown colour and larger size, were selected from bean leaves. To prevent the mites from migrating to a different genotype, plants from the same genotype were kept in a plastic container. All plants were kept in insect tents for safety reasons. The plants defensibility to mites was determined by looking at leaf damage. Leaf damage was scored in 5 different categories according to Table 2.

Figure 5: Severely damaged leaves
Example of severely damaged leaves. Plants with several of these leaves were scored with a 4.

Table 2: Damage score

0	No damage
1	A few spots
2	Clearly visible damage but still not much
3	Clearly damaged several leaves
4	Severely damaged (Figure 5)

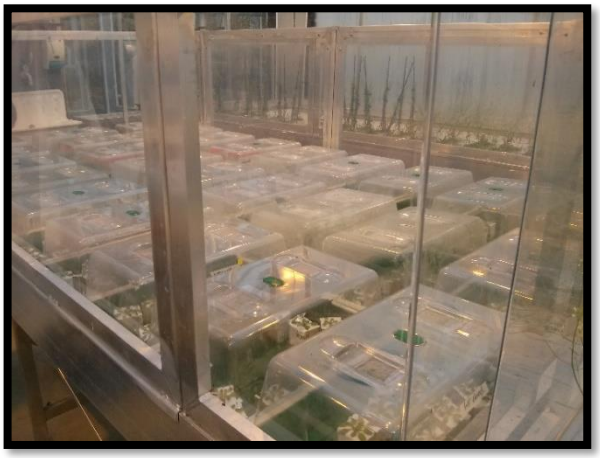


Figure 6: Plants growing in plastic containers
The growth environment of the plants for 1 week after they were infested with spider mites.

Seeds collection experiment

For the seed collection experiment, plants were grown for 4 weeks, and then subjected to biotic stress, sprayed with JA to induce a stress response, or kept as a control. For the systemic induced stress response, plants were sprayed with a thin layer of Jasmonic acid solution. The solution contained 1mM JA, 0.5% EtOH and a drop of tween 80, to improve the uptake of JA by the plant. The spider mite infested treatment and the control treatment were sprayed with a solution of 0.5% EtOH and a drop of tween 80. The plants were kept in plastic containers for safety reasons (Figure 6). Because of the humid environment inside, watering of the plants was done only at the start of the treatment. After 1 week all plants were sprayed with insectides to kill the spider mites. Plants were removed from the plastic containers, and kept in the growth room till they reached their final stage of scenecense and seed maturation. Once the first silliques started to brown, the plants were place in paper bags, to prevent loss of seeds. Finaly plants were harvested, and final plant and seed dry weight was measured. Florescence architectures was determined by counting the number of branches from the base of the plant.

Crossing transgenic lines

To analyse the interaction between T6P and SnRK1, with HXK1, crossings of the different transgenic lines were made according to Table 3. Crossing of the genotypes was done as following: Flowerbuds that were close to opening, but were anthers did not reach the stigma so selfpolination did not take place, were use. Flowerbranches with 2-4 flowerbuds at the right stage were selected, and all other flowers and buds were removed. The remaining buds were carefully opened, and all anthers and petals were removed till only the stigma was left. The flowers were polinated, by carefully 'dipping' an anther of a plant with the desired genotype on the stigma till the stigma was completely filled with pollen. The polinated flower was marked with a collored thread and harvested after seed maturation. The F1 generation of each cross was used in the experiments. The cross was regarderd succesful when the expected phenotype was observed. This was done by looking at hypocotyl length, rosette size, leaf collor, and flowering time.

Table 3: Crossing transgenic lines

Mother line		Father line
WT col	X	WT ler
WT col	X	KIN10 RNAi (ler background)
WT col	X	KIN10 OE (ler background)
WT col	X	OtsA (col background)
WT col	X	OtsB (col background)
HXK31	X	WT col
HXK31	X	WT ler
HXK31	X	KIN10 RNAi (ler background)
HXK31	X	KIN10 OE (ler background)
HXK31	X	OtsA (col background)
HXK31	x	OtsB (col background)

Sugar extraction

For the sugar extraction from the leaves, 10-15mg freeze dried plant material was used. The plant material was carefully grinded and 1 mL of 80% MeOH with 0.4M melezitose was added to the samples. The samples were kept for 15 min at 76˚C. After the extraction the samples were placed in a Speedvac to evaporate MeOH. The material was dissolved again into 1mL mQ water, and after vortexing centrifuged for 5 min at 14 000 rbp. The cleared supernatant was diluted 10 times and stored at -80 ˚C till analysis. The sugar concentrations were analysed with HPLC (DIONEX) as described in (Sergeeva et al. 2000) using a CarboPac PA 1 column (4×250 mm, Dionex, Sunnyvale, CA, USA) and 100 mM NaOH as eluent. Sugar concentrations in the sample were given in mg/L, and adjusted to the internal standard, 0.4M melezitose. Final sugar concentrations are given in µg per mg plant dry weight.

Secondary Metabolite analysis

For the metabolite analysis, 50µg of frozen leaf tissue was grinded, and 200µL of 94% MeOH with 0.125% FA was added. The tissue was mixed with the solution by vortexing, and then centrifuged for 5 min at 14 000 rbp. Aliquots of 10 µl of each sample were combined to make a quality control sample (QC), that was analyzed multiple time throughout the analysing sequence in order to be able to correct to shifts in measurements. and analysed by ultra-performance liquid chromatography coupled to Time of Flight mass spectrometry (Orbitrap UPLC-MS). Metalign software was used for baseline correction, mass spectra extraction and mass signal alignment. To explore differences in metabolite composition multivariate analysis on the relative abundances of individual metabolites (percentage of total concentrations) was performed using unsupervised Principal Component Analysis (PCA) to obtain an overview of the whole dataset using MetaboAnalyst after log2 transformation and noise subtraction. PLS Discriminant Analysis (PLS-DA) was performed to obtain maximum separation among classes in order to understand which variables carry the class separating information. Individual metabolites were analysed for significant changes among genotypes and treatments using a two-sided Student's t-test. Selected endogenous metabolites were putatively identified according to the accurate molecular weight using KNApSAcK database (http://kanaya.naist.jp/KNApSAcK/, Shinbo et al., 2006).

Image-J

ImageJ for Windows, Java 64 bits (https://imagej.nih.gov/ij/download.html) was used to measure leaf area and hypocotyl length. For the leaf area measurements, pictures were taken with a camera (Canon PowerShot SX120 IS) at a fixed distance, and uploaded into the ImageJ software. The scale in the software was set by using the ruler that was photographed together with the plants. Leaf area was measured in cm^2 by selecting a colour threshold that darkened the entire leaf area. This black area was then selected with the Wand (tracing) tool and automatically measured by the software. For hypocotyl elongation, agar plates were scanned, and pictures were uploaded to ImageJ. The scale was set by using the ruler that was scanned with the agar plates. Hypocotyl length was then measured using the *Straight* tool, with which the hypocotyl was selected by hand and measured according to the scale.

Statistics

To test the significance of the results a two-sided student t test was used in Excel 2016 for Windows 7. The significance level for all tests was $P<0.1$. In all experiments, except the seed experiment (10 repetitions), there were 5 repetitions. Correlation between different variables was tested with excel using the regression function. Significance level for all correlation tests was $P<0.1$. Standard error (SE) was calculated in excel with the following formula: STDEV.P(range)/SQRT(number of repetitions).

CHAPTER I

Growing up with sugars

The effect HXK1, T6P, and SnRK1, on sugar content, growth, and development of Arabidopsis thaliana under non-stressed conditions

INTRODUCTION

From germination to leaf expansion

Important steps of seedlings growth after germination are; hypocotyl elongation, the unfolding and greening of the cotyledons, and finally root growth (Figure 7). This process is called photomorphogenesis, and once the hypocotyl stops elongating, and the cotyledons are open and green, the plant is de-etiolated. The de-etiolation of a seedling requires light, and it is believed that photomorphogenesis is mainly regulated by the plants photoreceptors (Neff & Chory 1998; Lorrain et al. 2009; Franklin & Quail 2010). Recent publications, however, showed that the elongation of the hypocotyl was not only influenced by light, but that sugars also effect final hypocotyl length. Application of sucrose to the media, resulted in longer hypocotyls (Stewart et al. 2011; Liu et al. 2011; Zhang et al. 2010). It was shown that the sugar signalling agents HXK1, T6P and SnRK1 all effect hypocotyl elongation (Moore 2003; Schluepmann et al. 2004; Delatte et al. 2011). This suggests that sucrose dependent hypocotyl elongation is mediated through HXK1, T6P and SNRK1, and shows the importance of sugars, and sugar signalling agents already in the first stages of plant development.

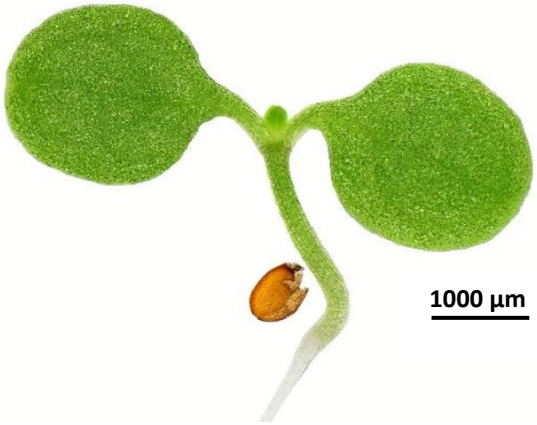


Figure 7 *Arabidopsis thaliana* seedling
A 7 days old *Arabidopsis thaliana* ecotype Colombia seedling. The hypocotyl is fully elongated, and the first true leaves are visible. Picture taken with binocular.

After hypocotyl elongation and the unfolding of cotyledons the first true leaves appear. Young growing leaf tissue is an important sink for carbon, and it was shown that growth of young leaves is partially mediated through T6P. In *Nicotiana tabacum* the overexpression of the *E. Coli* derived *TPP* gene, and thus a decrease in T6P levels, caused an increase in leaf area, while the overexpression of *TPS* result in the opposite (Pellny et al. 2004). Furthermore, the overexpression of *AtHXK1* was also shown to effect leaf growth (Kelly et al. 2012). These findings underpin the importance of sugar signalling agents in plant growth from germination to leaf expansion.

Figure 8: Flowers of Arabidopsis thaliana
https://inbotanicalmood.wordpress.com/tag/arabidopsis-thaliana/

Seed production for plant survival

Arabidopsis thaliana is a pioneer species with a relatively short lifespan. As an annual plant its survival depends primarily on seed production and dispersal. Therefor the allocation of carbon towards the production of seeds, in the final stages of its life is an important process. It is was shown that T6P and SnRK1 interact to regulate the seed filling process (Martínez-Barajas et al. 2011; Lawlor & Paul 2014; Griffiths et al. 2016). Furthermore it is known that HXK1, SnRK1, and T6P are all involved in the plants transition from the vegetative to the generative state, and influence flowering time (Gómez et al. 2010; Shin et al. 2017; Wahl et al. 2013).

Soluble sugars

With sugars as primary energy source, the amount of sugars available to the plant are an important determinant for plant growth and seed filling. It was shown that T6P, HXK1, and SnRK1 are important regulators of this process. It was shown that these sugar signalling agents can mediate the signalling function of sugar, and in turn effect the levels of soluble sugars in the plant (Lastdrager et al. 2014; O'Hara et al. 2013; Smeekens et al. 2010; Li & Sheen 2016). Apart from effecting the concentration of soluble sugars, it was also shown they effect starch levels, and thus the storage of sugars (Figure 9) (Kolbe et al. 2005; Baena-González et al. 2007; Y. M. Kim et al. 2013). High T6P levels result for example in starch accumulation in the leaves, and high SnRK1 activity promotes starch degradation.

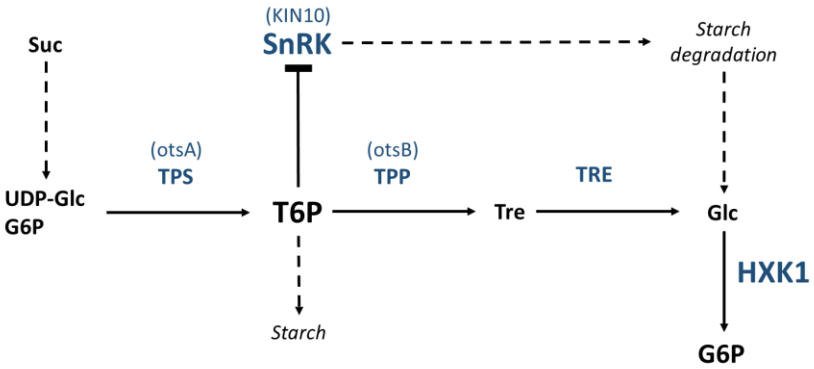


Figure 9: Sugar signalling agents
Sucrose levels effect T6P levels. TPS phosphorylates UDP-Glucose, and G6P into T6P. The TPP dephosphorylates T6P into trehalose. Trehalase then hydrolyses trehalose into 2 glucose molecules. T6P inhibit SnRK1 which promotes starch degradation. HXK1 phosphorylates glucose into glucose-6-phosphate.

In this chapter I will in investigate the effect of T6P, HXK1, and SnRK1 on leaf growth, hypocotyl elongation, seed production and sugar concentrations under non-stressed conditions. In all experiments Arabidopsis plants where grown under long day conditions, with 16h light and 8h dark.

RESULTS

Hypocotyl length

Recently a special role for sucrose was attributed to hypocotyl elongation (Stewart et al. 2011; Liu et al. 2011; Zhang et al. 2010). Seedlings grown on a medium containing sucrose showed an increase in final hypocotyl length. This raised the question whether sugar signalling agents, like T6P, SnRK1 and HXK1, are involved in hypocotyl growth of a seedling. In this chapter I investigated the effect of these sugar signalling agents on hypocotyl elongation under long day conditions. Arabidopsis seedlings where grown on agar medium (0.8% agar, ½ MS), and 1 week after germination, hypocotyl length was measured (Figure 10). As reported before, *AtHXK1* overexpression resulted in a strong increase in hypocotyl elongation (Moore 2003). Seedlings of the HXK43 and HXK31 lines had almost 3 times longer hypocotyls compared to WT col. Seedlings overexpressing OtsA, and thus containing higher T6P levels (OtsA), were significantly smaller compared to the WT col. This is consistent with the previously reported inhibition of hypocotyl length by trehalose in the dark, which is believed to be due to a positive feedback of trehalose to T6P levels (Schluepmann et al. 2004; Delatte et al. 2011). Even though the hypocotyl length of OtsB seedlings seemed to be longer, this was not significant compared to WT col.

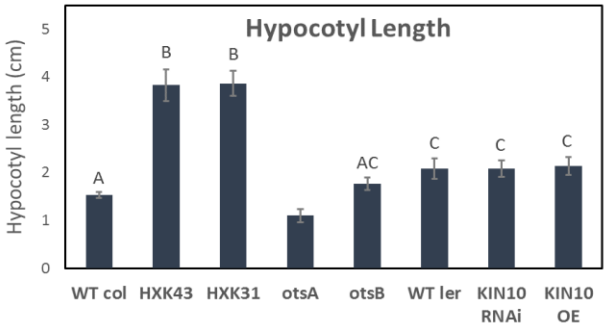


Figure 10: Hypocotyl length
Hypocotyl length of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE in cm. Hypocotyl length was measured 1 week after gemination. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

It was reported by Delatte et al. (2011) that the negative effect of trehalose on hypocotyl elongation in the dark could be rescued by the overexpression of KIN10. The overexpression of KIN10 should counteract the inhibition of SnRK1 by T6P. However, under these conditions I did not find significant differences between KIN10 OE, KIN10 RNAi and WT ler, which suggest that there is no effect of KIN10 on hypocotyl elongation under my experimental conditions.

Leaf area and growth

It was reported that HXK1 and T6P levels influence leaf growth (Pellny et al. 2004; Kelly et al. 2012). To investigate the effect of T6P, SnRK1, and HXK1 on leaf growth under long day and non-stressed conditions, Arabidopsis plants where grown for several weeks, and in week 2, 4 and 5 leaf area was measured (Figure 11). The relative growth rate (RGR) in week 2-4 and week 4-5, and the final leaf area before harvest (week 5) was measured.

As reported before in Tobacco plants the RGR in week 2-4, was significantly lower in OtsA mutants compared to WT col (Pellny et al. 2004). Although I expected OtsB plants to behave opposite to OtsA, and have a faster RGR compared to WT col, there was no significant difference between the two. The

RGR in week 2-4 of HXK43 was significantly faster than the RGR of WT col. This is consistent with previous observations (Unpublished results, D. Granot & D. Brandsma), however HXK31 did not show any significant difference with WT col. WT ler had a higher growth rate compared to WT col. The major function of T6P is the inhibition of SnRK1 (Zhang et al. 2009; Nunes 2014). There for I expected that mutants with different expression of the SnRK1 subunit KIN10 behaved in a similar way to mutants with genetically modified T6P levels. Consistent with the behaviour of OtsB and OtsA mutants there was no significant difference between KIN10 OE and WT ler, but the RGR of KIN10 RNAi was significantly lower compared to KIN10 OE and WT ler.

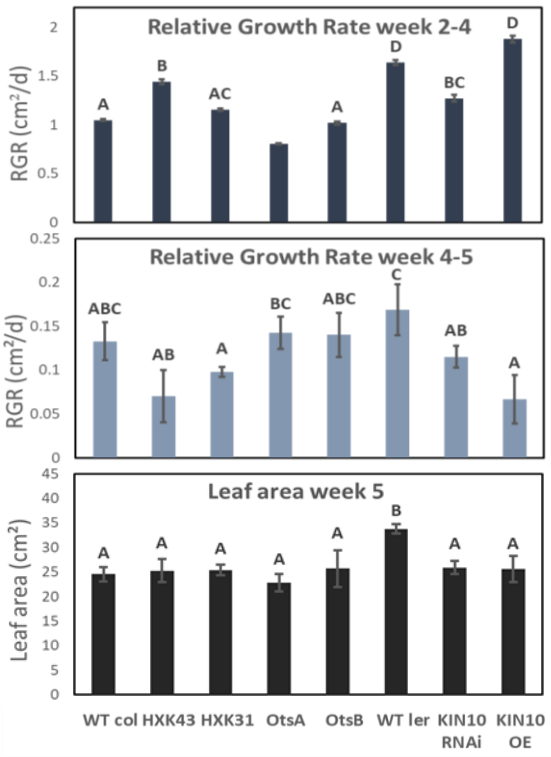


Figure 11: Leaf area and growth
Leaf area (LA) in week 5 after germination and RGR ($(LA_{week\ t=1}-LA_{week\ t=0})/LA_{week\ t=0}$) in $cm^2\ d^{-1}$ in week 2-4 and week 4-5 of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE was measured. Bars with the same letter are not significantly different (P<0.1). Error bars present SE.

The RGR in the last week before harvest, was strongly reduced compared to the RGR in week 2-4. A reduction of almost 10 fold or more for each mutant or ecotype was observed. In the final week the RGR was not much different between the different mutants and ecotypes. However the RGR of HXK43 and HXK31 was significantly lower compared to OtsA. The RGR in week 4-5 was highest in WT ler, but not significantly different from WT col. KIN10 RNAi, and KIN10 OE had a significantly lower RGR compared to WT ler, but this was not significantly different from each other.

Even though RGR where different between the mutants and ecotypes, the final rosette area before harvest (week 5) of WT col, HXK43, HXK31, OtsA, and OtsB, where not significantly different. The leaf area of WT ler was significantly larger compared to WT col, and the leaf area of KIN10 RNAi and KIN10 OE were significantly lower compared to WT ler. There was no difference between KIN10 RNAi and KIN10 OE.

Seed production

It was described that sugar signalling agents do not only influence the plants development and growth in early stages, but also effect seed filling (Martínez-Barajas et al. 2011; Lawlor & Paul 2014; Griffiths et al. 2016). In this research I investigated the seed production in the different mutants. After plants were fully grown and reached their final stage of senescence, total plant DW and seed weight was measured. The weight of the aerial part of the plant without the seeds, the seed weight, and the percentage of total plants weight partitioned into the seeds is given (Figure 12).

The total plant weight of HXK43, HXK31, and OtsA was significantly higher, while OtsB mutants had a significantly lower weight, compared to WT col. The total weight of WT ler plants, was significantly lower from KIN10 RNAi, and KIN10 OE. Surprisingly almost all mutants partitioned about 45% of their total weight into the seeds. Only OtsB partitioned 28% of total weight into seed production, which was significantly lower compared to all other mutants. HXK31 and OtsA partitioned significantly less weight into seeds compared to WT col, but the difference with HXK43 was not significant (P<0.100). The difference in percentage dry weight partitioned into seeds, was not significant between WT ler, KIN10 RNAi, and KIN10 OE.

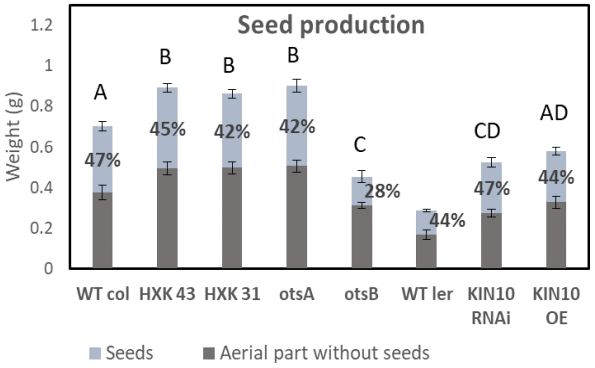


Figure 12: Seed production
Plant weight of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE. The weight was measured after the plant reached its final stage of maturation. Bars represent total plant weight in which the lower part is the aerial part without the seeds, and the upper part is the seed weight. The percentage of dry weight partitioned into the seeds is given. Plants with the same total weight share the same letter (P<0.1, N:10 plants). Error bars present SE.

Sugar content

To investigate the effect of T6P levels, KIN10, and HXK1 on plant soluble sugar content, plants were grown for 5 weeks, and harvested at noon. The content of sucrose, trehalose, glucose, fructose, and raffinose was measured from freeze dried leaf tissue (Figure 13). The most remarkable effect on sugar content was found in OtsB overexpressing plants. Plants overexpressing the bacterial TPP gene OtsB had an almost 10 fold increase in sucrose, and 7 fold higher trehalose content compared to WT col. The increase in sucrose concentration in OtsB plants is consistent with previous findings (SOURCE). Plants overexpressing the SnRK1 subunit KIN10 contain a significantly more sucrose and trehalose compared to WT ler, however this was not as pronounced as in the OtsB mutants. Glucose and fructose levels in OtsB were higher compared to WT col however this difference was not significant (P>0.100). This was also true for KIN10 OE plants compared to WT ler. There was no significant difference in sucrose and trehalose content in HXK43, HXK31, OtsA and WT col. Glucose levels, however were significantly lower in HXK43 plants, compared to WT col and OtsB, but not compared to HXK31 and OtsA overexpressing plants. Surprisingly glucose levels in HXK31 were not significantly different from glucose concentration in WT col. The concentration of fructose was not different in WT col, HXK43, HXK31 and OtsB, but was significantly lower in OtsA. WT ler and KIN10 RNAi had a higher sucrose content compared to WT col, but were not significantly different from each other. The concentration of raffinose was similar in WT col, HXK43, HXK31, OtsA, OtsB, and WT ler. Raffinose concentration in KIN10 RNAi was, however, significantly lower compared to all other genotypes, and KIN10 OE had significantly lower raffinose levels compared to HXK43, and HXK31. The hexose/sucrose ratio was highest in WT col, HXK43, HXK31, and OtsA. In OtsB plants hexose/sucrose ratio was significantly lower compared to all other genotypes. Even though the ratio was lower in the WT ler ecotype, there was no significant difference between WT ler, KIN10 RNAi, and KIN10 OE.

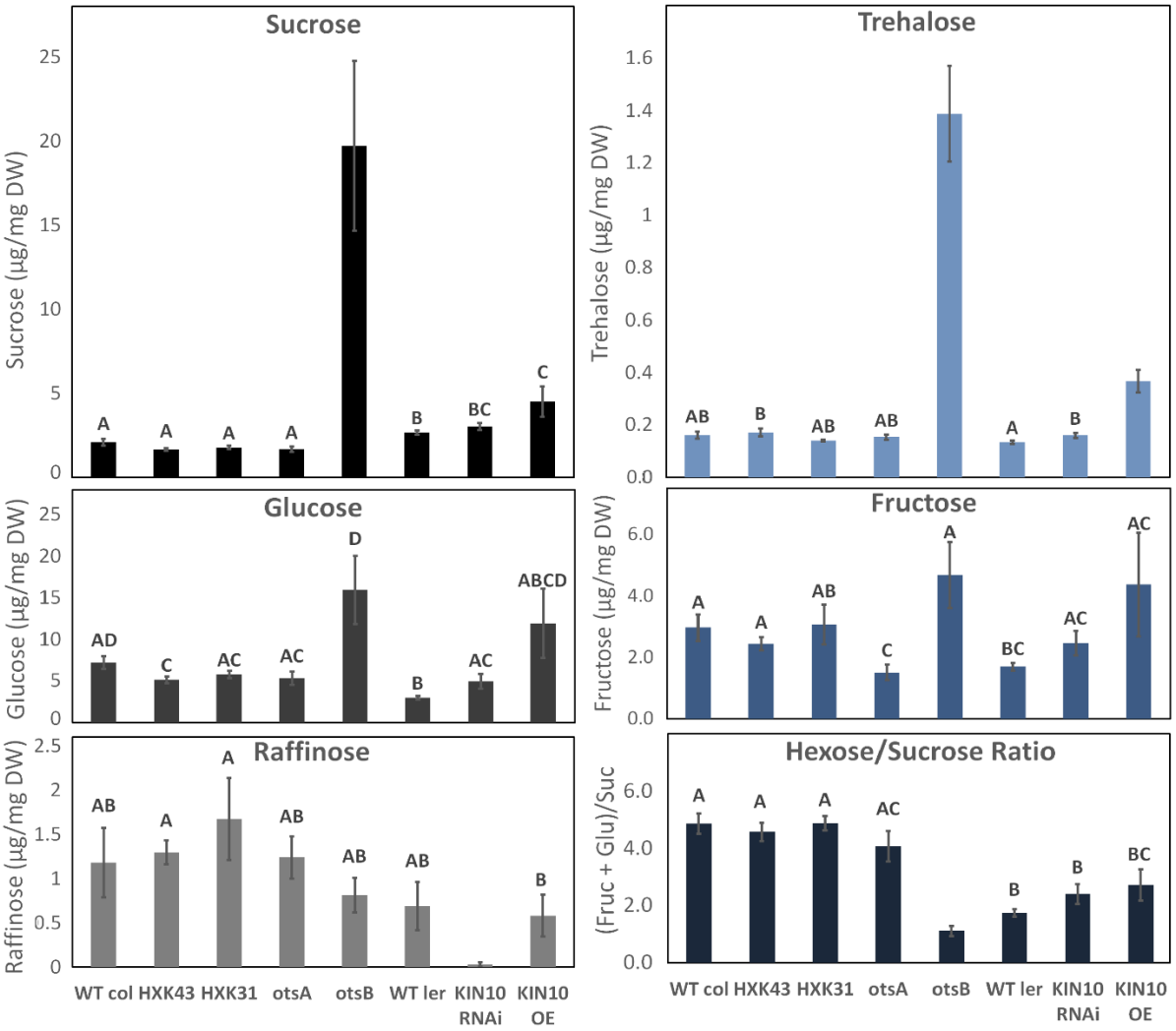


Figure 13: Sugar concentration

The concentration of sucrose, trehalose, glucose, fructose, raffinose (in µg/mg DW) and the hexose/sucrose ratio (glucose + fructose / sucrose) in Arabidopsis plants grown for 5 weeks, and harvested at noon. Bars with the same letter are not significantly different (P<0.1), and error bars show SE.

DISCUSSION

In the introduction of this chapter it was explained how sugar signalling agents effect several stages of development, and how it influences growth. As mentioned before the regulation of development and the involvement of sugars is a highly regulated process, which is very much influenced by the environment. It was therefore necessary to assess the effect of the different sugar signalling agents in this specific environment, before applying the biotic stress or looking at the effect of the different crossings. In the discussion of this chapter I will address the effect of the different sugar signalling agents under long day conditions.

Sugar signalling agents effect hypocotyl elongation

Hypocotyl elongation is an important process for the seedling to reach light for photosynthesis and ensure plant growth. Many factors influence this elongation process, under which several light regulated genes and proteins (Chory et al. 1996; Lorrain et al. 2009; Franklin & Quail 2010). It was shown that sucrose, and sugar signalling agents effect this elongation process as well (Stewart et al. 2011; Ma et al. 2011; Zhang et al. 2010; Moore 2003; Schluepmann et al. 2004; Delatte et al. 2011). My results confirm that sugar signalling agents are involved in hypocotyl elongation. OtsA mutants had significantly shorter hypocotyls compared to WT col while HXK31 and HXK43 mutants had elongated hypocotyls.

It is interesting to argue why HXK1 and T6P effect hypocotyl elongation in an opposite manner. The answer may lie in findings from resent publications showing that sucrose promotes the transcription of the PHYTOCHROME INTERACTING bHLH factor 4 (PIF4). PIF4 is an important regulator of hypocotyl growth (Bernardo-Garc??a et al. 2014; Zhang et al. 2013; Li et al. 2015). It regulates a wide variety of genes of which some are involved in hypocotyl elongation, and it is also known that the PIF family is partly regulated through phytochromes. Since T6P reflects sucrose availability (Cátia Nunes et al. 2013), we might expect increased T6P levels to coincide with an increase in PIF4 expression, when seedlings are growing on sucrose. In this way seedlings of the OtsA mutant should have an increase in hypocotyl length, however, the length was shorter compared to the WT. Furthermore it was found that OtsA seedlings had a decreased PIF4 transcription (Paul et al. 2010), instead of the expected increase of PIF4. This suggests that the sucrose induced PIF4 transcription and therewith hypocotyl elongation is not mediated through an increase in T6P levels, but T6P rather has the opposite effect. This is interesting for the following reason: T6P can sense sucrose produced by photosynthesis. Therefor T6P could function as a signal to the seedling that it reached a height sufficient to capture enough light for photosynthesis, and thereby inhibiting hypocotyl elongation (Figure 14). This hypothesis is supported by the finding that the elongation response to sucrose was found to be concentration depend. Seedlings grown on 1% sucrose had the longest hypocotyls. A concentration higher than 1% resulted in a decrease of hypocotyl length, and higher than 3% resulted in hypocotyls even shorter than that of seedlings grown without sucrose (Unpublished results, D. Granot & D.

Brandsma). This means that sucrose, and thus increased T6P levels, can inhibit hypocotyl elongation, but this depends on the sucrose concentration. It is interesting to note, that mutants with a different expression of KIN10 and low T6P levels (OtsB), did not show any effect on hypocotyl elongation. This suggests that the effect of high T6P levels on hypocotyl elongation is not mediate through the inhibition of SnRK1, and asks for a better understanding of the effect of T6P apart from the inhibition of SnRK1 (Recommendation 1).

Still the question remains how the sucrose induced hypocotyl elongation is mediated. Interestingly, and consistent with previous findings, HXK43 and HXK31 mutants showed a strong increase in hypocotyl length (Moore 2003). Furthermore is was shown that *AtHXK1* overexpressing seedlings have an increase in PIF4 transcription, and that the effect of sucrose on hypocotyl elongation can be counteracted by applying NAG, an inhibitor of HXK, to the growth media (Unpublished results, D. Granot & D. Brandsma). This suggests that HXK1 is the main mediator in sucrose induced hypocotyl elongation.

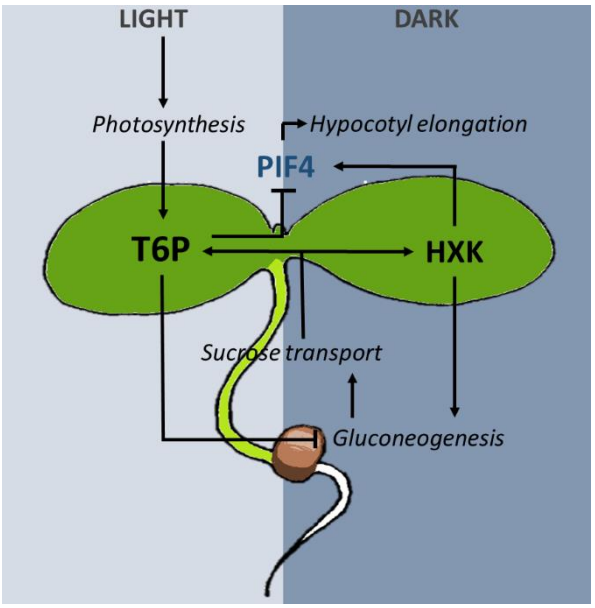


Figure 14: Sugar regulated hypocotyl elongation
The proposed regulatory function of T6P and HXK1 in hypocotyl elongation, with T6P and HXK1 having opposite effects. T6P levels increase due to sucrose production by photosynthesis in light. It then inhibits gluconeogenesis, and PIF4 transcription, that both have a positive effect on hypocotyl elongation. HXK, has the opposite effect to T6P and promotes gluconeogenesis and PIF4 transcription, thereby stimulating hypocotyl growth.

It is believed that hypocotyl elongation is primarily fuelled by gluconeogenesis from lipids stored in the embryo and endosperm (Cornah et al. 2004). Agdhasi (2007) proposed a role for T6P in the control of this lipid remobilization for hypocotyl growth in the dark, where increased T6P levels result in less remobilization of carbon and thereby inhibiting hypocotyl elongation (Mahnaz Aghdasi 2007) (**Fout! Verwijzingsbron niet gevonden.**). This is supported by Martin et al., who showed that seedling growth on sucrose almost completely blocked storage lipid breakdown (Martin et al. 2002). This is consistent with the hypothesis that T6P informs the plant it has enough energy coming from photosynthesis to sustain itself, and that there is no need for import of sugars from the endosperm.

In contrast to T6P, sugars coming from photosynthesis can bypass HXK1, and it is believed that the role of HXK1, is mainly restricted to night time, and in sink tissue (Rojas et al. 2008). Kim et al. (2013) showed that a reduction in HXK1 expression in Tobacco plants resulted in more starch. Their hypothesis was, that a reduction in HXK1 activity resulted in accumulation of glucose coming from starch degradation. In turn, this would be a feedback system to the plant to inhibit starch degradation (Y. M. Kim et al. 2013; Veramendi et al. 1999). In this way, the activity of HXK1 could determine the sink strength of plants tissue. It might be possible that opposite to high T6P levels the overexpression

of *AtHXK1* promotes gluconeogenesis. The primary product of gluconeogenesis, is glucose the substrate of HXK1. It could be that HXK1 has the same effect on gluconeogenesis as on starch degradation, and thereby explains part of the hypocotyl elongation. This however, has never been mentioned before, and more research is needed to fully understand the effect of HXK1 and T6P on gluconeogenesis and hypocotyl elongation (Recommendation 2).

Growing tissue as a strong sink

In this research I show that all three sugar signalling agents; T6P, HXK1 and KIN10 effect the RGR of Arabidopsis in week 2-4. Consistent with earlier findings, OtsA mutants have a lower RGR compared to WT col (Pellny et al. 2004; Schluepmann et al. 2003). This is interesting, because high T6P levels are associated with a high carbon availability. In fact, T6P is often referred to as a 'feast' signal to the plant. You might expect that under these favourable conditions there is plenty enough sugar to fuel plant growth, and that high T6P levels would promote growth. However, in OtsA mutants, 'access' sugars were shown to be stored as starch (Baena-González et al. 2007; Kolbe et al. 2005), and Martins et al. (2013) showed that T6P can inhibit starch degradation (Martins et al. 2013). This starch storage could explain why the sink tissue is limited in sugars, and thus results in growth reduction. However Delatte et al. (2011) showed that starchless mutants have the same decrease in growth, which eliminates the option of growth reduction because of starch accumulation (Delatte et al. 2011). In OtsA mutants T6P levels are more 'fixed' compared to WT col, and even when there is low availability of sucrose, it still senses high sucrose levels. So, even when plant tissue becomes a sink, the plant still recognizes this as tissue with enough sugar available. The reduction in RGR in OtsA mutants, could therefore be explained by an imbalance in source/sink ratio, and this would underpin the importance of T6P in the source/sink balance of the plant. To illustrate this I would like to refer to a common practise in horticulture. The grower can manipulate plant growth, by adjusting the temperature. Low temperatures, for example, result in very compact plants with thick leaves. This is caused by an imbalance of the source/sink ratio. Temperature is an important determinant for the speed of plant development. The higher the temperature the faster new organs are formed, and thus a higher sink demand. If there is enough light and CO_2 for photosynthesis to meet this demand, the source/sink

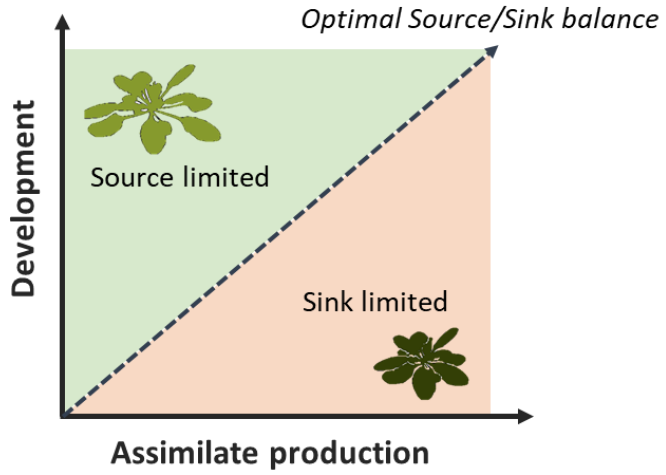


Figure 15: Optimal source/sink balance
The source sink balance in Arabidopsis. With a right balance between plant development (the formation of new organs), and plant assimilate production, there is an optimal source/sink balance. Source limited plants are characterized by their thin leaves, and larger leaf area, while sink limited plants are characterized by thick small leaves.

ratio is balanced (Figure 15). If we now apply this information on OtsA mutants, we can understand why OtsA plant have a lower growth rate. Even though there are enough assimilates, the plant does not recognize the sinks to distribute the assimilates to: OtsA plants are 'sink limited'. It is interesting to note that OtsB plant do not show the opposite phenotype to OtsA plants. The reduction of T6P levels in OtsB mutants is a signal to the plant that it requires energy. OtsB mutants are so to speak source limited, and we would expect an increased RGR. This would also be consistent with other findings (Pellny et al. 2004; Schluepmann et al. 2003). However under these conditions OtsB mutants were not significantly different from WT col. This could be explained by the growth conditions. It could be that the long day, and non-stressed conditions are not effecting OtsB mutants that much, because there is an non-limited energy supply.

The signal of T6P is believed to be primarily generated through the inhibition of SnRK1 (Zhang et al. 2009; Nunes 2014). Consistent with this, the KIN10 RNAi mutants also showed a lower RGR compared to WT ler, while KIN OE was not significantly different. This shows that, indeed, the effect of T6P on growth is mediated through the inhibition of SnRK1. However, it would be interesting to see the effect of high T6P levels, in KIN10 OE plants. Can the overexpression of *KIN10* counteract the negative effect of T6P on RGR? (Recommendation 1)

Since HXK1 mutants also show a higher RGR, it is interesting to link this to source and sink balance as well. As described before HXK1 is believed to be mainly active in the dark, and in sink tissue (Rojas et al. 2008). Kim et al. (2013) showed that a reduction in HXK1 expression in Tobacco plants resulted in more starch, due to a reduction in sink strength (Y. M. Kim et al. 2013; Veramendi et al. 1999). If HXK1 overexpression can increase the sink strength, it could explain, why HXK43 mutant have a larger RGR, and this would be consistent with the findings of hypocotyl elongation in *AtHXK1* overexpressing mutants.

Seed filling and inflorescence architecture

The production of flowers and seeds is an very important step in development. By the production and dispersal of seeds the plant ensures its survival. Since seeds are a very strong sink in plants, it is likely that the partitioning of assimilated into seed production is influenced by sugar signalling agents. In this research I looked at dry weight partitioning into the seeds, and showed that indeed sugar signalling agents influence this stage of development. There is a clear difference between plants with high T6P levels (OtsA), and low T6P levels (OtsB). Way less weight is portioned into seed production in the OtsB mutants. This is opposite to previous findings in maize, where the overexpression of TPP in maize ears resulted in an up to 49% higher yield, under non-stressed conditions (Nuccio et al. 2015). Even though maize is a monocot, and completely different from Arabidopsis, this could not be the only reason why my results are opposite. It is good to mention that OtsB mutants had a delay in flowering (Supplementary data S2) and seed maturation, and at the time of harvest OtsB plants were not yet in their final stage of senescence. This could have effected total seed weight. If I would have harvested them in a later stage, there would have been more time for seed filling, and probably a

higher seed weight. But even though the results are opposite to previous findings, this still points towards an important function of T6P in the seed filling process which is consistent with the previous articles (Martínez-Barajas et al. 2011; Lawlor & Paul 2014).

KIN10 expression did not seem to have much effect on seed filling. Even though total plant weight was significantly higher compared to WT ler, the DW partitioning into the seeds was not significantly effected.[2] It was expected that, due to the interaction of T6P with SnRK1, differentially expressed KIN10 would result in a similar phenotype to mutants with altered T6P levels. The interaction of T6P with SnRK1 during the seed filling process was also mentioned in a review paper of Griffith et al. (Griffiths et al. 2016). Since KIN10 RNAi and KIN10 OE do not have similar phenotypes to OtsA and OtsB respectively, this could point towards an independent role for T6P and SnRK1 in the seed filling process. This would be consistent with the results of hypocotyl elongation, in which KIN10 RNAi and KIN10 OE did not show the same behaviour as OtsA and OtsB as well. However, it would be good to repeat this experiment, because unfavourable growth conditions might have effected the outcome (Recommendation 4). The plants were kept in a small container the first few days of inflorescence growth. The upper part of the inflorescence reached the top of the container, and after removal of the box, the plant aborted the first flowers. This could have influenced the growth of the inflorescence and finally seed weight.

AtHXK1 overexpressing plants behaved in a similar way as OtsA mutants. Although not much is known about the effect of HXK1 in the seed filling process, it is known that prior to seed filling, there is an accumulation of glucose, the primary substrate of HXK1 (reviewed in H. Weber 1997). It was also shown that the highest expression of HXK1 was found in the flower of *Nicotiana tabacum* (Y. M. Kim et al. 2013). This does suggest an important role for HXK1 in seed development. However to understand the role of HXK1 in this process, more research needs to be done.

It is interesting to note that sugar signals seem to effect inflorescence architecture as well (Supplementary data S3). A clear example is that HXK1 overexpressing plants had more branches compared to WT col (Figure 16). It is mentioned before that HXK1 and T6P levels effect inflorescence architecture (Barbier et al. 2015). A difference in shoot architecture could explain why plant weight of HXK43, HXK31, and OtsA was significantly higher compared to WT col, while dry weight partitioning into seeds was lower

Figure 16: Inflorescence
Branches of HXK31 (left) and WT col (right), 6 weeks old plants. A clear difference between the size of the inflorescence.

[2] WT ler started to flower early, and due to experimental limitations the upper part of the inflorescence branch was damaged. This resulted in very small inflorescence and possibly effected the total amount of flowers (see also supplementary data S3)

Soluble sugars

With sugars as primary energy source, the amount of sugars available to the plant are an important determinant for plant growth and seed filling. It was shown that T6P, HXK1, and SnRK1 can effect the levels of soluble sugars in the plant (Lastdrager et al. 2014; O'Hara et al. 2013; Smeekens et al. 2010; Li & Sheen 2016). In this research I show that the overexpression of the bacterial TPP gene (OtsB) causes a strong increase in sucrose, glucose and trehalose concentrations in the plant. The sucrose concentration in OtsB plants exceeded such high levels, that the hexose/sucrose ratio was more than 4 times lower compared to WT col. Sugar accumulation in OtsB plants was reported before, and it shows the importance of low T6P levels as a feedback signal to the plant for sucrose mobilization (Wingler et al. 2012). However, this high concentration of sugars in OtsB plants did not positively effect seed weight (Figure 12). This could suggests that sugars are not able to move between plant tissue, because the whole plant is recognized as sink. It would be interesting to investigate the sugar concentrations in the phloem, to see if sugars are still transported, or that they just accumulate in the leaf tissue (Recommendation 5). Or the plant is not able to utilize the sugars as described before by Delatte et al. (2011). It was reported that the signal of T6P is partially regulated through the inhibition of SnRK1 (C??tia Nunes et al. 2013; Tsai & Gazzarrini 2014; Wingler et al. 2012). It is, however, remarkable that KIN10 OE plants do not contain the same extreme sucrose concentrations as OtsB plants. Together with the results from hypocotyl elongation, and seed production this strongly suggests that T6P has different regulatory functions besides the inhibition of SnRK1.

It was expected that the overexpression of HXK1, would lead to reduced glucose levels, however this was only the case for HXK43. This could be explained by the proposed role of HXK1, that it might only be needed in sink tissue, and at night (Granot 2008). This could explain why in this research, where all measurements were done at noon, under non-stressed conditions, does not show a large effect of HXK1. It would be worth to look at sugar concentrations over time (Recommendation 6).

CONCLUSION

In this chapter I wanted to investigate the effect of HXK1, T6P, and SnRK1, on sugar content, growth, and development of *Arabidopsis thaliana* under non-stressed conditions. I showed that sugar signalling agents have an important role in hypocotyl elongation, seed production, inflorescence architecture, and soluble sugar concentrations. High T6P levels reduce hypocotyl elongation, and RGR of the plant, while HXK1 promotes hypocotyl elongation and growth. The effect of T6P on RGR is mediated through SnRK1. However differentially expressed KIN10 did not result in similar phenotypes as plants with altered T6P levels, with respect to hypocotyl elongation and seed filling. This suggests an independent role for T6P in early and late development of the plant. All together these results, indicates an important role for T6P, SnRK1, and HXK1 in development and growth of the plant, and underpins the importance of this research. In the next chapter I will investigate whether these signals are still important when the plant is under stress.

CHAPTER II

Introducing biotic stress

The effect HXK1, T6P, and SnRK1, on plant growth, development and sugar content of Arabidopsis thaliana plants infested with the Two-Spotted Spider mite

INTRODUCTION

Spider mites

Plants are subjected to an ever changing environment. As sessile organisms, plants have multiple survival strategies to cope with unfavourable conditions. These strategies differ among plant species and dependent on the origin of stress. The Two-Spotted Spider Mite, *Tetranychis urticae* Koch is pest to a wide variety of economical important crops (Gore et al. 2013; Archer & Bynum 1993). The spider mite is a generalist that feeds on more than 1100 plant species, adapts easily to pesticides, and can survive extreme and changing environments. This makes it difficult to manage this pest. The spider mite is a cell content feeding herbivore, and it uses its stylet to pierce trough the epidermis, without causing damage to the epidermal cells. There it feeds on the cell content of palisade and spongy mesophyll cells. Spider mite feeding results in a decreased photosynthetic gene activity, leaving chlorotic spots, which negatively effects plant yield (Landeros et al. 2004; Bensoussan et al. 2016).

Figure 17: Two-Spotted Spider Mite
A picture of the Two-spotted spider mite in all growth stages on a leaf. Adults, nymphs and eggs.

The costs of plant defence

A survival strategy of the plant in response to the spider mites is the production of defense metabolites. These metabolites are either used for direct defense by the production of toxic compounds, or indirect defense, like the production of volatiles to attract natural enemies of the pest (Bennett & Wallsgrove 1994). Examples of defense metabolites produced during spider mite attack in Arabidopsis are glucosinolates and anthocyanins (Zhurov et al. 2014; unpublished results Kappers & Brandsma). The production of defense compounds requires energy. It was for example shown that 15% of the sugars produced by photosynthesis was partitioned into the production of glucosinolates,

under non-stressed conditions alone (Bekaert et al. 2012). And in several articles induced defences by herbivory is described as a cost for plant growth (reviewed in Schwachtje & Baldwin 2008; Cipollini et al. 2003; Purrington 2000). This shows that biotic stress to the plant can create strong sinks, with a negative trade-off to growth. This increase in sink strength of the plant, is particularly interesting in relation to my findings in chapter 1. In chapter 1 I showed, that T6P, SnRK1 and HXK1 can effect the source/sink balance, and thereby plant growth. It would be interesting to investigate the effect of biotic stress, and thus the increase of sink strength, on the same mutants, and see how they behave when dealing with herbivory stress.

Herbivory and seed production

Another effect of herbivores is carbon partitioning towards the roots. It was shown that SnRK1 plays an important role in this process under stress (Ferrieri et al. 2013; Schultz et al. 2013; Schmidt et al. 2015; Zhou et al. 2015; Havko et al. 2016). It was hypothesised that this carbon storage in the roots is used for later regrowth, or seed production. However this has never been shown in Arabidopsis plants. Since Arabidopsis is an annual plant species, it could be possible that this defense response is too costly for these short-lived plants. However, it would be interesting to study the effect of herbivory on seed production in Arabidopsis without the negative effect of leaf damage cause by mites. It is known that spraying JA to the plant triggers a defence response that mimics the response to herbivory (Thaler 1999; Dicke et al. 1999; Shoresh et al. 2004; Clarke et al. 2000). It was shown that JA related genes are upregulated by spider mites, and JA is associated with resistance to this pest (Zhurov et al. 2014; Miyazaki et al. 2014). By spraying Methyl Jasmonate (MeJA), the methylated form of jasmonic acid, the effect of plant defense induction can be studied without the negative effect of leaf damage by herbivory. In chapter 1 I showed sugar signalling agents effect seed production. In this chapter I would like to see the effect of the mite and MeJA induced defense responses on seed production in the different mutants.

Sugar signalling agents and the defense response

In the first chapter I studied the effect of the sugar signalling agents on plant growth and development. I found that T6P, HXK1 and SnRK1 are important in the plants source/sink balance, and thereby effect hypocotyl length, leaf growth, seed filling, and sugar concentrations. In this chapter I introduce a biotic stress, the two-spotted spider mite, to create an additional sink. In this way I want to see if the sugar signalling mutants behave differently to an lower source/sink balance. And if the effect of herbivory, results in a negative trade-off to growth.

RESULTS

In order to investigate the effect of mites on development and growth in different sugar signalling mutants, all results are relative values to the control treatment. In this way, I am able to study the magnitude of the response, normalized for the different genetic backgrounds.

Growth reduction

To investigate the effect of herbivory on leaf growth, 4 weeks old *Arabidopsis thaliana* plants were infested with mites. Leaf area was measured at T=0, the time of infestation and T=7, 1 week after infestation, and plant fresh weight (FW) and dry matter percentage (DM%) was measured at T=7 and compared to the control treatment. The relative growth rate of the plant (RGR) was calculated by dividing the increase in leaf area between T=0 and T=7, by the initial leaf area (T=0) (Figure 18). As expected, the RGR was lower for plants infested with mites for 7 days compared to the control treatment in all mutants. However this decrease was only significant for HXK43, HXK31, OtsA and in WT ler plants (indicated by *). The most pronounced effect of spider mite infestation on leaf RGR was found in HXK43 plants. In these plants the RGR was almost 5x as small compared to the control treatment. Interestingly total plant FW of HXK43 in week 5, was not significantly lower compared to the control treatment, while this was significantly reduced in HXK31, OtsA, WT ler, and KIN10 RNAi (P<0.100). Interestingly HXK43, HXK31, OtsA, and KIN10 RNAi mutants all had a significantly increase in DW% compared to the control treatment, while the DW% of WT col, OtsB, WT ler, and KIN10 OE was not significantly effected by spider mite infestation.

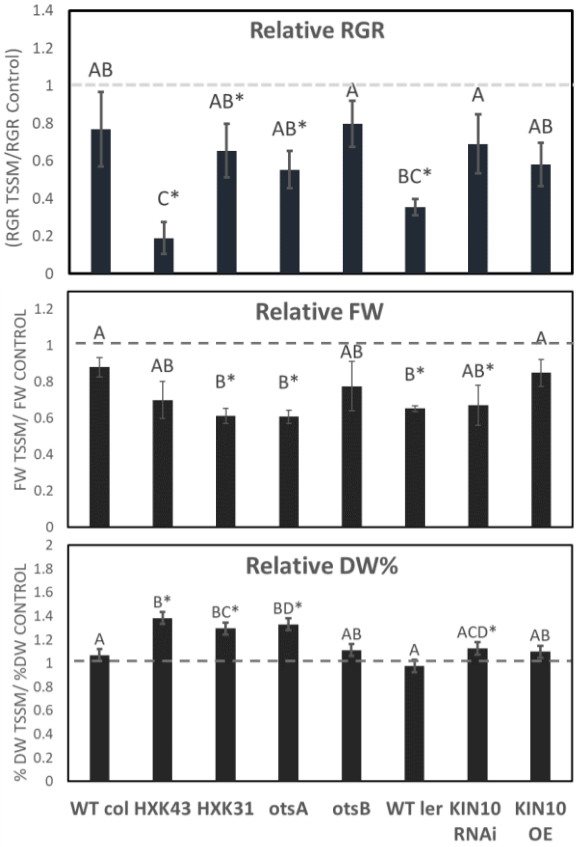


Figure 18: Effect of SPIDER MITE on RGR, FW, and DW%
The RGR (($LA_{week\ 5}$-$LA_{week\ 4}$)/$LA_{week\ 4}$), plant FW and DW% in week 5 of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE was measured. Bars with the same letter are not significantly different (P<0.1). A significant difference from the control treatment (the dotted line) is indicated by a *. Error bars present SE.

Flowering time and seed production

It is hypothesized that herbivory can induce the transition to the generative phase and enhance seed production. To test this hypothesis Arabidopsis plants where grown under long day conditions, and before bolting, treated with MeJA, or infested with spider mites. After 1 week all plants were sprayed with pesticides to kill the mites, and left in the growth room till the final stages of senescence and seed ripening. Plant weight of plants sprayed with MeJA was significantly lower in WT col, HXK43, HXK31, OtsA, OtsB, and KIN10 RNAi compared to the control treatment (Figure 19). This decrease was most pronounced in OtsA. Plant weight of WT ler and KIN10 OE, was not significantly different when treated with MeJA. Infestation with spider mites led to a decrease in plant weight, compared to the control treatment in HXK31, OtsA, OtsB, and KIN10 RNAi. Weight of WT col, HXK43, WT ler, and KIN10 OE, was not significantly different between the control treatment and the treatment with spider mites. The difference in response between systemic induced resistance with MeJA and infestation with spider mites was only significant in WT col, HXK31, and OtsA (indicated with ▼). In all three genotypes MeJA application resulted in a higher weight reduction compared to plants infested with mites. Seeds weight in MeJA treated plants was significantly lower in WT col, HXK43, HXK31, OtsA, and KIN10 RNAi compared to the control treatment. In WT ler the seed weight was significantly higher compared to the control treatment, and in OtsB andKIN10 OE, it was not significantly different. HXK31, OtsA, KIN10 RNAi and KIN10 OE plants infested with mites, had a reduction in seed weight compared to the control treatment. Seed weight of WT col, HXK43, OtsB, and WT ler of mite infested plants was not different from the control. Seed weight of MeJA treated WT col, and HXK31 plant was significantly lower compared to plants infested with mites, and seed weight of KIN10 OE plants was higher in MeJA treated plants, compared to mite infestation. The relative % of total weight partitioned into seeds was increased in HXK31 and OtsA with MeJA application, compared to the control treatment, and in

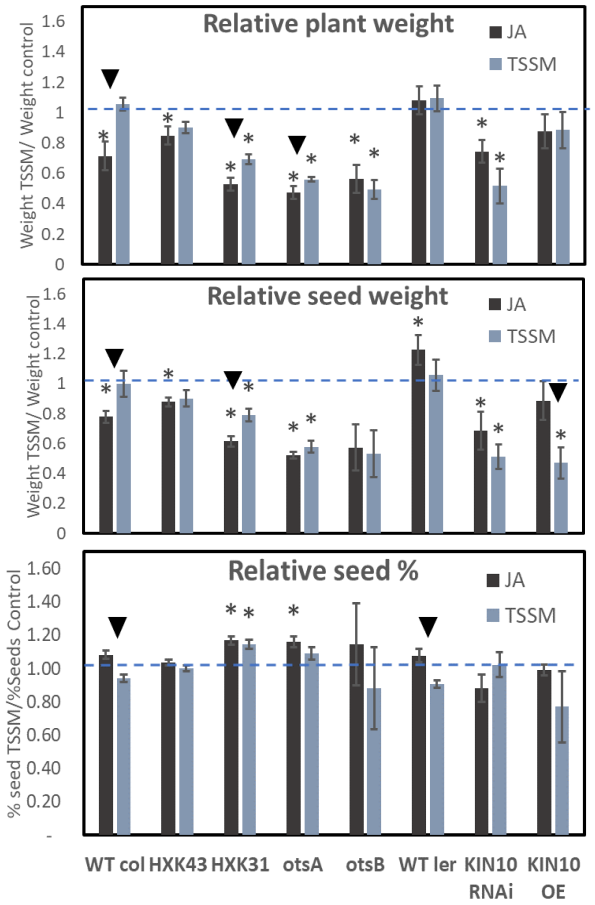


Figure 19: Relative plant weight, seed weight, and seed weight %

Plant weight, seed weight and percentage of total plants weight partitioned into seeds of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE infested with spider mites or treated with MeJA was measured relative to the control treatment. Plants were harvested after plants completed their final growth stage of seed filling and senescence. Significant differences between MeJA treated and spider mite infested plants is indicated by ▼, differences between MeJA and spider mite infested plants with the control treatment (the dotted line) are indicated with *(P<0.1, N:10 plants). Error bars present SE.

HXK31 also mite infestation resulted in more partitioning into the seeds. WT col and WT ler plants partitioned less weight into the seeds, when infested with mites, compared to MeJA application. The relative percentage of dry weight partitioned into seeds of OtsB mutants has a very high SE. This is probably due to the late development of OtsB plants. OtsB mutants had a delay in flowering (Supplementary data S2) and seed maturation.

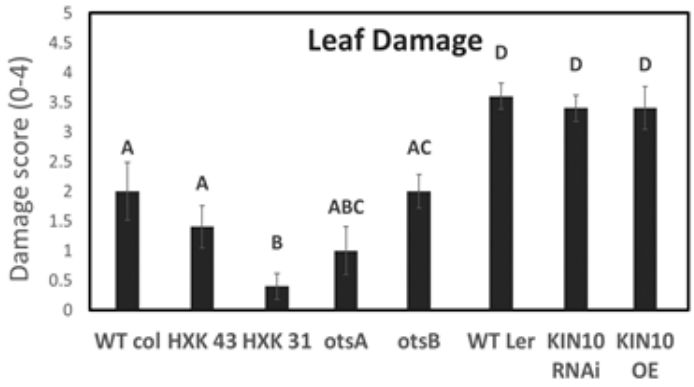


Figure 20: Leaf damage
Leaf damage of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE plants infested with spider mites. The severity of the damage is scored with a mark between 0-4. 0 is no damage, and 4 is given to severely damaged plants. Bars sharing the same letter are not significantly different. Error bars present SE.

Leaf damage by mites

To investigate whether there is a difference in plant defensibility to spider mites between the different mutants, it was investigated whether Arabidopsis rosettes have a difference in leaf damage after 1 week exposure to mites. The severity of leaf damage was scored by giving plants a damage score from 0-4. A score of 0 indicates an undamaged rosette, whether a damage score of 4 indicates a severely damaged rosette. The highest damage was found in WT ler plants, and no effect of KIN10 expression was found (Figure 20). Remarkably the HXK31 line, which had the highest reduction in leaf fresh weight compared to the control treatment (Figure 18), had significantly less leaf damage compared to WT col. There was a slight reduction in leaf damage in HXK43, and OtsA mutants, however this difference was not significant. There was no significant difference in leaf damage between OtsB and WT col.

Effect of spider mites on sugars

To investigate the effect of mite infestation on sugar content in the plant, *Arabidopsis thaliana* plants were grown for 4 weeks under long day conditions and infested with SPIDER MITE for 1 week. In week 5 all plants were harvested at noon, and sugars were extracted and analysed with HPLC. The sugar content is given in Figure 21, relative to the control treatment. Sucrose and glucose concentration were significantly higher compared to the control treatment in HXK43, HXK31, and OtsA ($P<0.1$). While sucrose ad glucose levels, did not differ in WT col, OtsB, WT ler, and KIN10 RNAi, and was significantly reduced in KIN10 OE, upon mite infestation. Interestingly, while glucose and sucrose levels were different, fructose levels were not significantly different in any of the genotypes compared to the control treatment. Trehalose was not significantly different from the control treatment in WT col, HXK43, OtsB, WT ler, and KIN10 RNAi. However, in HXK31, and OtsA trehalose was significantly higher, and in KIN10 OE, trehalose was significantly lower compared to the control. The most fascinating result was found in the raffinose concentration of HXK43, HXK31, and OtsA. All three genotypes had a

significantly higher raffinose content in mite infested plants compared to the control treatment. In KIN10 OE raffinose was significantly lower, and as described before (Chapter 1: Figure 13) raffinose was undetectable in KIN10 RNAi mutants. The hexose/sucrose ratio was increased in mite infested HXK43, HXK31, and OtsA plants, compared to the control treatment, but in the other genotypes there was no significant difference.

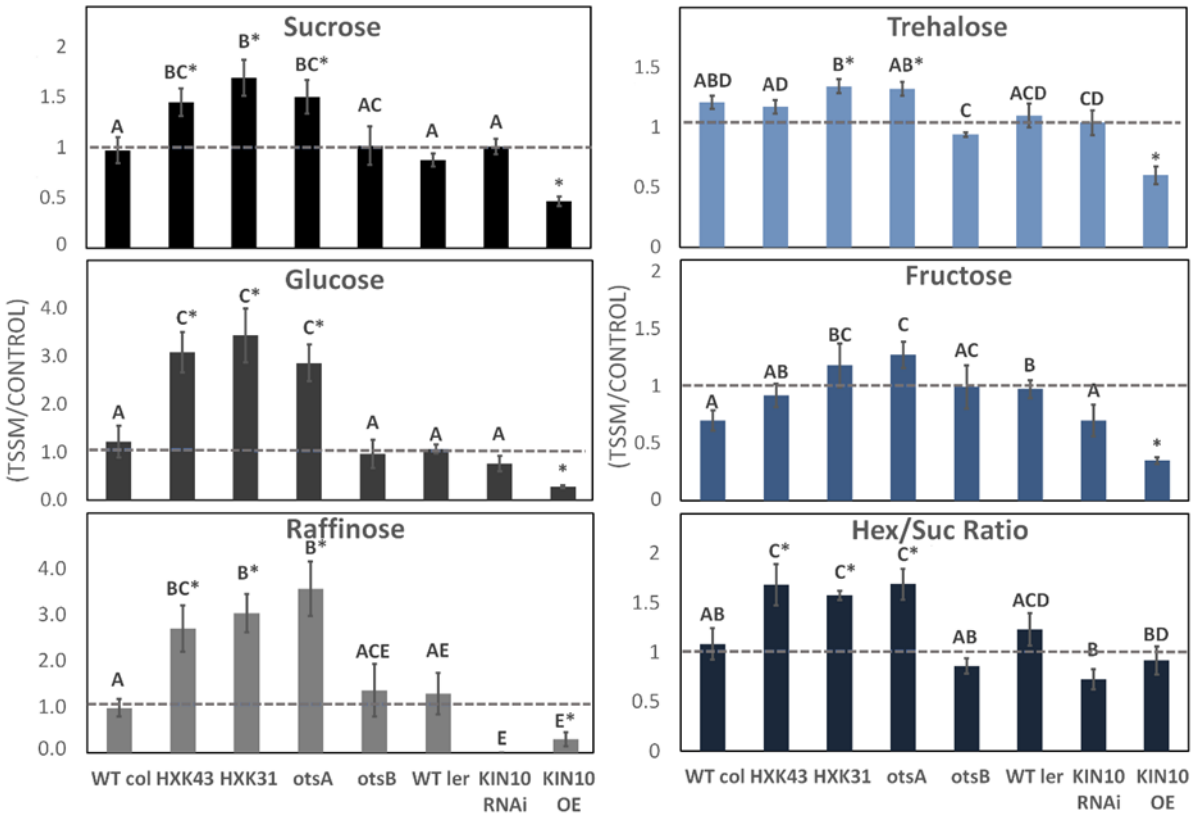


Figure 21: Relative sugar concentrations in spider mite infested plants
The concentration of sucrose, trehalose, glucose, fructose, raffinose (in µg/mg DW) and the hexose/sucrose ratio (glucose + fructose / sucrose) in Arabidopsis plants infested with spider mites relative to the control treatment. WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE plants were grown for 4 weeks, infested with spider mites, and in week 5 harvested at noon. Bars with the same letter are not significantly different (P<0.1), and error bars show SE. The dotted line represents the relative concentration of the control treatment, and significant differences are indicated with a *.

DISCUSSION

Trade-off to growth

As a response to herbivory the plant produces secondary metabolites to defends itself (Bi & Felton 1995). It is believed that this has a negative effect on plant growth by partitioning more assimilates into defense metabolites, instead of using it for plant growth (Schwachtje & Baldwin 2008; Purrington 2000; Cipollini et al. 2003). In chapter 1 I showed that T6P, HXK1, and SnRK1 are important sugar signalling mediators in distinguishing between source and sink. In this research I looked at the effect of mite infestation on plant RGR of these mutants. I showed that, as expected, in each case the average RGR declined when plants were infested with spider mites. This was, however, not significant for each genotype. Probably infesting the plants with mites for one week, shortly before bolting, does not have a very apparent effect on RGR, and it would be interesting to investigate the effect of herbivory on plant growth, in an earlier developmental stage (Recommendation 7). However, in HXK1 overexpressing plants, plants with high T6P levels, and in WT ler, relative RGR was significantly lower compared to the control treatment. It is interesting to argue whether this is due to an acceleration to the generative state when plants are infested with mites. HXK31 and HXK43 plants had an accelerated transition to the generative state when infested with mites or with MeJA application (Supplementary data S2). This reduction of the vegetative state could have influenced the RGR in a negative way.

Biotic stress and plant-water relations

An in interesting finding in this chapter is the FW of HXK43, HXK31, OtsA and KIN10 RNAi, that is significantly reduced in plants infested with spider mites, while DW% was increased. This suggests that leaf water content was significantly lower in leaves infested with mites. Although studies about leaf water content reduction by herbivory are hard to find, it was reported before that a low water content in the leaves negatively effects herbivore performance, especially for sap feeding herbivores (Scriber 1977; Huberty 2004; Tabashnik 1982). A spider mite has a similar feeding behaviour to phloem feeding herbivores, such as aphids. The mite uses its stylet to pierce through the leaf tissue (Bensoussan et al. 2016) (Figure 22). Their feeding behaviour might be effected by lower cell turgor in water deprived plants. However there was only a very low correlation between leaf DW% and leaf damage, and it was not negative (R=0.307, P=0.003) (Supplementary data S4). Furthermore the DW% in the WT plants was not effected by mites, which suggest that Arabidopsis plants are normally not responding to herbivory by a reduction in water content.

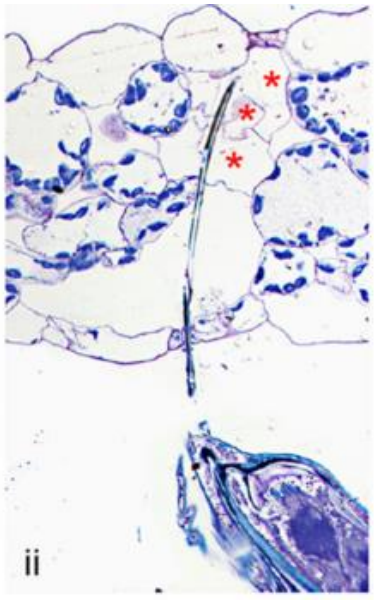


Figure 22: Cross section of spider mite feeding on bean
Picture from the article of Bensoussan et al., depicting a spider mite piercing through the stomata of the plant (Bensoussan et al. 2016)

It is interesting to argue why OtsA, KIN10 RNAi, and HXK1 mutants have such an altered DW% in response to herbivory. It is known that T6P and HXK1 are able to effect plant-water relations, by influencing stomatal aperture. The stomata facilitates the uptake of CO_2 for photosynthesis, and at the same time loses water to the surrounding environment. Because of this constraint for CO_2 uptake, it is no surprise that sugar signalling agents effect the stomatal aperture of the stomata. It is believed once the plant is high in sugars, and there is no more need for photosynthesis, stomata close in order to minimize water loss. It was found that HXK1 and high T6P levels both effect stomatal aperture (Gómez et al. 2010; Van Houtte et al. 2013; Kelly et al. 2012; Lugassi et al. 2015). However, HXK1 overexpression leads to stomatal closure, while high T6P levels result in stomatal opening. So, even though this shows the involvement of sugar signalling agents in plant-water relations, it can still not explain why HXK1 and T6P behave in a similar way to herbivory stress. HXK1 is also involved in water transport by downregulating important members of the plasma membrane intrinsic proteins (PIPs) (Kelly et al. 2014). PIPs are an important class of aquaporins that regulate water transport through the cell membrane, and are important for the plant response to abiotic stress (Afzal et al. 2016). A lower expression of PIPs, result in a lower osmotic water permeability of mesophyll protoplasts (Kelly et al. 2017). This could effect cell turgor, especially under high osmotic stress. Considered the fact that upon herbivory stress there is a accumulation of sugars in HXK1 mutants, and thus a higher osmotic value, it could very well explain, why there is a decrease in water content upon herbivory stress. Even though there is not much known about aquaporins in relation to herbivory, there is an article that describes the increased expression of specific members of aquaporins upon herbivory stress in maize (Lawrence et al. 2013). If this would be a similar phenomenon in Arabidopsis, it could explain why WT plants still have the same percentage of DW, while in HXK1 DW% is reduced. If there is an increased expression of aquaporins in WT plants when effected by herbivores, the water transport can still take place, and cell turgor maintains the same. In HXK1 plants, the expression of aquaporins is inhibited, and this could explain the decrease in cell turgor. Unfortunately there is nothing known about aquaporins in relation to T6P and SnRK1, and therefore more research is needed to get a better understanding of plant water relations under herbivore stress. It would for example be interesting to look at the expression of important aquaporin members in the different sugar signalling mutants, with and without biotic stress (Recommendation 8).

Seeds for survival

It was questioned whether herbivory increased seed production of the plants to ensure survival. Therefore plants were infested with spider mites or sprayed with MeJA to investigate the effect of herbivory on seed production. It was clear that spider mites and MeJA effected total seed weight in almost all genotypes. This is consistent with earlier reports of for example spring wheat and Nicotiana attenuate were the application of MeJA resulted in a lower seed weight (Heil et al. 2000; Van Dam & Baldwin 2001). It is unlikely that damage caused by spider mites, causing a reduction in photosynthetic tissue, was responsible for the reduction in seed weight. It was described before that removal of 50% of the leaves of an Arabidopsis plant shortly before bolting did not effect seed production (Akiyama & Ågren 2012), and in this experiment MeJA induced the same response on seed weight as infestation with mites. This suggests that the reduction in seed weight is rather caused by an induced defense

response that redistributes assimilates to defense related processes, such as the production of defense metabolites.

Interestingly the highest significant reduction in seed weight was found in HXK1 overexpressing plants, and plants with high T6P levels. This might indicate a negative trade-off to seed production, once these plants are infested with mites. However, the relative DW partitioning (%) into the seeds was higher in HXK31 and OtsA plants, which shows that relatively more assimilates were distributed to the seeds. This suggests that indeed, the plant increases assimilate distribution towards seed production to ensure survival under stress. However, WT plants did not show an increase in relative percentage of DW in the seeds, which does not confirm this mechanism under biotic stress.

Even though this experiment points more toward a negative effect of herbivory on seed production it would still be interesting to investigate whether the reduction in total seed weight also resulted in a reduction in the number of seeds produced (Recommendation 9). Till now there are various reports about the effect of herbivores on the number of seeds, however all of them report different results, and are investigated in different plant species (Smith & Hough-Goldstein 2014; Lucas-Barbosa et al. 2013; Garrido et al. 2016; Stowe & Marquis 2010; Bekaert et al. 2012; van Dam & Baldwin 1998).

Glucose levels under biotic stress

Apart from the increase in sucrose, HXK1 overexpressing plants and OtsA mutants showed a strong increase in glucose levels when infested with spider mites. Because of the increased sucrose levels, higher glucose levels could be explained by an increased invertase activity. Invertase converts sucrose into glucose and fructose. It is described before that biotic stress can cause a strong increase in cell wall invertase transcription (Sonnewald et al. 2008; Appel et al. 2014; Castrillón-arbeláez et al. 2012; Ferrieri et al. 2013). However, other articles point towards a decrease in invertase activity (Machado et al. 2015; Seaton et al. 2015). Since fructose levels are unchanged it is unlikely that the increase in glucose concentrations are due to high invertase activity, and in the WT plants glucose and fructose levels are unchanged, so the glucose increase is specific for HXK1 overexpressing plants and plants with high T6P levels. In animal cancer cells a very high activity of HXK is found. Interestingly the application of MeJA, an anti-cancer agent, results in the detachment of HXK from the mitochondrion. This detachment depend on the level of mitochondrion bound HXK, which is characteristic for cancer cells, containing high levels of mitochondrion bound HXK, and are therefore targeted by MeJA (Goldin et al. 2008). This mechanism might also be present in plant, but this has never been shown (Xiang et al. 2011). The HXK1 overexpressing plants also overexpress mitochondrion bound HXK. It would be interesting to investigate whether MeJA indeed is able to detach HXK1 in HXK1 overexpressing plants (Recommendation 10). Since mites trigger JA production in the plant, this could explain the increase in glucose levels in HXK1 overexpressing plants infested with mites. However this still does not explain the same effect of mites on glucose levels in OtsA plants. → Why TPS? Interaction with JA?

It was mentioned that under normal conditions in WT plants grown on 6% glucose leads to an almost 2 fold increase of HXK1 expression. However in TPS1 overexpressing plants, HXK1 expression was not effected by glucose, and stayed the same. So, apparently TPS1 overexpression, and thus high T6P levels effect the expression of HKX1. This article also report a decreased expression of TPS1 when plants are grown on glucose. (Avonce et al. 2004)

Raffinose and trehalose in stress resistance

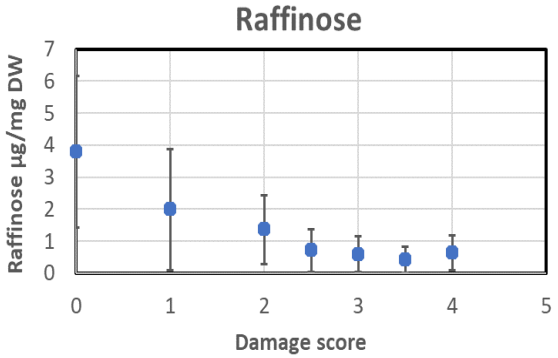


Figure 23: Raffinose levels in plants with different damage scores

In this chapter I showed that raffinose levels were increased in HXK1 overexpressing plants, and plants with high T6P levels when plants were infested with mites. These plants also had lower leaf damage caused by spider mites (Figure 23), and there was a weak negative linear relation found between raffinose concentrations and leaf damage (R=-0.523, P=0.000) (Supplementary data S4). But by taking the natural logarithm (Ln) of raffinose levels, the negative correlation became stronger (R=-0.700, P=0.000). It was reported before that raffinose has an effect on plant resistance to abiotic stress, by protecting the cells from oxidative damage (Nishizawa et al. 2008). These results point towards increased resistance of plants with high raffinose concentrations to biotic stress as well. While there is only a limited amount of information about raffinose, it is known that it is involved in phloem-loading, and effects long distance transport of sugars (reviewed in Braun et al. 2013). This could promote the reallocation of sugars to important sink tissue. Trehalose was also reported to be involved in biotic stress resistance (Govind et al. 2016). HXK31 and OtsA overexpressing plants, with the lowest leaf damage, did not only have increased raffinose levels, but also higher trehalose concentrations when infested with spider mites. However, there was no significant correlation found between trehalose and leaf damage (R=0.041, P=0.702) (supplementary data S4).

The regulator of several genes related to trehalose and raffinose biosynthesis is the basic region-leucine zipper transcription factor 11 (bZIP11). bZIP11 is shown to be involved in the low-energy response and systemic induced resistance (Ma et al. 2011; Radchuk et al. 2010; Delatte et al. 2011; Baena-González et al. 2007; Wang et al. 2016). Moreover the expression of bZIP11 is inhibited by sucrose and bZIP11 inhibits growth (Hanson et al. 2008). It was shown that there is an interaction between T6P and KIN10 with bZIP11. First of all, the overexpression of bZIP11 leads to increased T6P levels when plants are grown on trehalose (Delatte et al. 2011). Secondly, bZIP11 transcription is increased in KIN10 mutants (Baena-González et al. 2007). This interaction between bZIP11, T6P, SnRK1, in relation to herbivory stress could explain why raffinose and trehalose levels are changed upon mite infestation.

Defensibility and leaf damage

In this chapter I showed that the introduction of biotic stress had a negative effect on growth in HXK1 overexpressing plants, and plants with high T6P levels or low KIN10 expression. In the introduction I described that it is believed that herbivores have a negative effect on growth. This could be caused by redistribution of assimilates towards the production of defense metabolites, instead of using them for plant growth (Schwachtje & Baldwin 2008; Purrington 2000; Cipollini et al. 2003). It is therefor expected that HXK1 overexpressing plants, OtsA and KIN10 RNAi mutants, that have less growth, have an increase in defense metabolite production. Which, then results in a higher defensibility to mites. In this research I looked at plant defensibility, by scoring the amount of leaf damage. And indeed, HXK1 overexpressing plants and OtsA mutants had lower leaf damage, which shows a higher defensibility. However, I also showed that KIN10 did not effect leaf damage, which would not support the hypothesis of a trade-off between growth and defensibility.

More research needs to be done to conclude on the effect of different sugar signalling agents on resistance to mites. Especially because different result have been reported. Unpublished results show that OtsB plants are less susceptible to mites (Unpublished results Kappers & Brandsma), and this was also found in plants grown in tissue culture where I found less spider mite offspring on OtsB and KIN10 OE plants, and increased susceptibility of HXK31 plants (Supplementary data S5).

CONCLUSION

In this chapter I wanted to look at the effect of HXK1, T6P, and SnRK1 on plant growth, development and sugar content under stressed conditions. I showed that there was a difference in response to spider mite infestation in the different sugar signalling mutants. The overexpression of HXK1 result in the lowest leaf damage, but also has the highest reduction in RGR and plant weight once subjected to biotic stress. Furthermore spider mites can induce the accumulation of sugars in HXK1 overexpression plants and OtsA mutants, while they reduce the soluble sugar levels in KIN10 OE. Mite infestation also lead to lower seed production. However in HKX1 and OtsA mutants a higher percentage of DW is partitioned into the seeds upon herbivory. The highest negative correlation with leaf damage was found with raffinose levels, which suggest that higher raffinose levels, result in a higher plant defensibility. As described before (Chapter 1), the behaviour of plants with different expression of KIN10, did not resemble plants with different T6P levels, and this shows that T6P has a role in plant development and growth independent of SnRK1.

CHAPTER III

Interaction between signalling agents

Is there an interaction between T6P and SnRK1 with HXK1 under non-stressed conditions?

INTRODUCTION

Overlapping phenotypes

The phenotypic characteristics of plants with altered T6P levels or different KIN10 expression show strong similarities with HXK1 overexpressing plants. All three sugar signals were shown to influence stomatal aperture, starch and anthocyanin content, hypocotyl elongation, the ABA and glucose response, and the expression of similar genes (Table 4) (Gómez et al. 2010; Van Houtte et al. 2013; Paul et al. 2010; Avonce et al. 2004; Wingler et al. 2012; Kelly et al. 2012; Lugassi et al. 2015; Dai et al. 1999; J. I. Kim et al. 2013; Moore 2003; Cho et al. 2006).

Table 4: Phenotypes of Arabidopsis plants with high T6P levels and or low SnRK1 activity compared to plants overexpressing HXK1

High T6P and/or low SnRK1 activity	Hexokinase1 overexpression
Open stomata (Gómez et al., 2010; van Houtte et al., 2013)	**Closed stomata** (Kelly et al., 2012; Lugassi et al., 2015)
High starch content (Lunn et al., 2006)	**Low starch content** (Dai et al., 1999; Kim et al., 2013)
Short hypocotyl (Paul et al., 2010)	**Elongated hypocotyl** (Moore et al., 2003)
Insensitive to ABA and Glc (Avonce et al., 2004; Gómez et al., 2010)	**Hypersensitive to ABA and Glc** (Moore et al., 2003)
High expression photosynthetic genes (Avonce et al., 2004)	**Low expression photosynthetic genes** (Moore et al., 2003; Cho et al., 2006)
High anthocyanin content (Wingler et al., 2012)	**Low anthocyanin content** (Own observations)

Table 4 suggests that there is an opposite effect of HXK1 and high T6P levels on many plant processes. This is also confirmed by my results in Chapter 1. High T6P levels and overexpression of *AtHXK1* had an opposite effect on hypocotyl elongation and RGR.
Several attempt have been made to explain these overlapping or contrasting phenotypes, but still an interaction between T6P and SnRK1 with HXK1 was never shown, and the similar phenotypes are still not fully understood.

Do sugar signalling agents interact?

It is known that in yeast T6P can inhibit HXKII by binding to the catalytic site (Blazquez et al. 1993). The opposite phenotypes of HXK1 overexpressing plants, and plants with high T6P levels, could therefore be explained by the inhibition of HXK1 by T6P. However, this inhibition has never been shown to be conserved in the plant kingdom (Eastmond et al. 2002). Besides the lack of evidence for an interaction between HXK1 and T6P, I showed in chapter 1 and 2 that in later developmental stages HXK1 overexpressing plants show more similarities to plants with high T6P levels (OtsA), then with low T6P levels (OtsB). Also under stress HXK1 overexpressing plants have a similar response to plants with high T6P levels.

In this chapter I will look into a possible interaction of T6P and SnRK1 with HXK1 by looking at hypocotyl elongation, Leaf area, and sugar concentrations. I crossed OtsA, OtsB, KIN10 RNAi, and KIN10 OE, with WT col or HXK31 according to Table 3. The F1 generation was used to investigate a possible interaction between the sugar signalling agents. This means that only one allele of each gene was represented in the different backgrounds.

RESULTS

How to read the graphs

The main focus of this chapter is to investigate the interaction of HXK1 with T6P levels and SnRK1 activity. This interaction is studied by looking at hypocotyl length, leaf growth, and sugar content in crossings of WT col and HXK31 mutants with OtsA, OtsB, KIN10 RNAi, KIN10 OE, and WT ler. All results described in this chapter are from the F1 generation of the crosses, and are relative values to the same cross with WT col. In this way it is possible to study the effect of one allele HXK1 overexpression in different backgrounds. For example, Figure 24A shows the hypocotyl length of WT col, and the cross of WT col with HXK31, WT col with OtsA, and of HXK31 with OtsA. To investigate the effect of HXK31 in WT col and OtsA background, the length of the cross of HXK31 with WT col is divided by WT col and the cross of HXK31 with OtsA is divided by the value of the cross of WT col with OtsA. From this Figure 24B is formed which shows that HXK31 has the same effect on hypocotyl length in OtsA background compared to WT col. Furthermore it shows that the cross with HXK1 is causing longer hypocotyls compared to the cross with WT col. In this chapter I will use similar graphs to Figure 24B to look at the interaction of HXK31 with T6P and SnRK1 (unless mentioned otherwise).

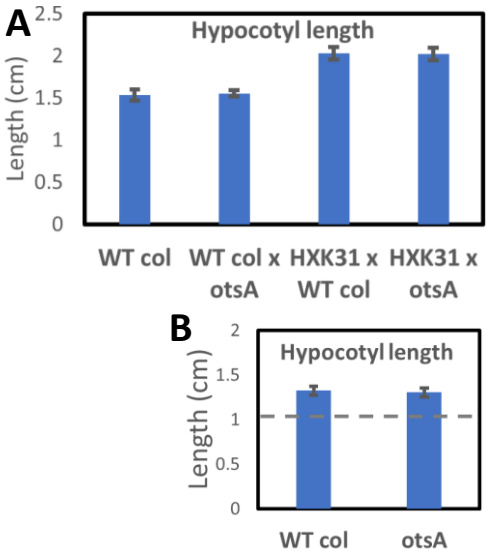


Figure 24: Example graph
Example graph of how the results in this chapter will be presented. In graph A the actual length is given, while in figure B the relative length of the cross with HXK31 is given compared to the same cross with WT col.

Hypocotyl length

In chapter 1 I showed that HXK1 and OtsA effected the hypocotyl length in opposite manner, and OtsB, KIN10 RNAi, and KIN10 OE, did not effect hypocotyl elongation. To investigate a possible interaction of HXK1 with T6P levels, and SnRK1, hypocotyl length of crossings of different mutants was measured 1 week after germination (Figure 25). Consistent with the results of chapter 1, in which HXK1 overexpression resulted in longer hypocotyls, the cross of HXK43 and HXK31 with WT col resulted in significantly longer hypocotyls. While OtsA was shown to negatively effect hypocotyl elongation, crossing OtsA with HXK31 partly restored the negative effect of OtsA, compared to the same cross with WT col. However HXK1 did not have a similar effect when crossed with OtsB, because crossing HXK43 and HXK31 with OtsB did not result in longer hypocotyls, compared to the cross of OtsB with WT col. HXK43 and HXK31 crossed with WT ler, resulted in longer hypocotyls compared to the cross with WT col. Which is consistent with the

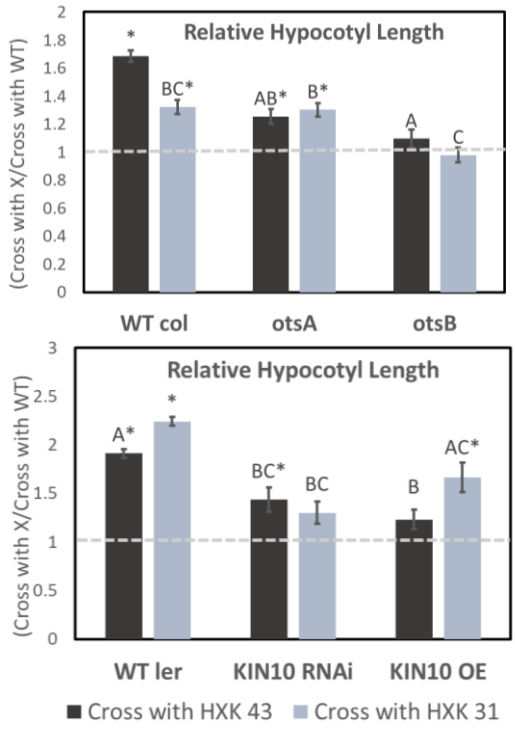


Figure 25: Hypocotyl length

Hypocotyl length of the cross of HXK31 and HXK43, with OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE relative to the same cross with WT col (cm). Hypocotyl length was measured 1 week after gemination. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE. Significant differences from the cross with WT col (dotted line), are indicated with a *.

results from the cross of HXK1 mutants with WT col. Crossing HXK43 and HXK31 with KIN10 RNAi and KIN10 OE did not result in consistent results. While line HXK43 crossed with KIN10 RNAi resulted in a significantly longer hypocotyls compared to the cross with WT col, line HXK31 only resulted in significantly longer hypocotyls when crossed with KIN10 OE.

Relative growth rate and leaf area

In chapter 1 it was shown that T6P, SnRK1 and HXK1 effect leaf area and RGR. To investigate the interaction of T6P and SnRK1 with HXK1 in leaf growth, leaf area of the crossed mutants was measured in week 2, 4 and 5, and from this the RGR was measured. The leaf area of week 5 (Figure 26) and the RGR between week 2-4 and week 4-5 (Figure 27) are given for the cross of WT col, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE with WT col or HXK31.

As mentioned in chapter 1 the final leaf area was not much different between WT col, OtsA and OtsB. Crossing these mutants with HXK31 did not result in any significant differences, compared to the same cross with WT col, in final leaf area (week 5). In chapter 1 KIN10 RNAi and KIN10 OE did not have a difference in final leaf area. However, here I show that crossing these mutants with WT col, there is a significant reduction in final leaf area in KIN10 RNAi and a significant increase of leaf are in KIN10 OE. This difference is even larger when crossed with HXK31.

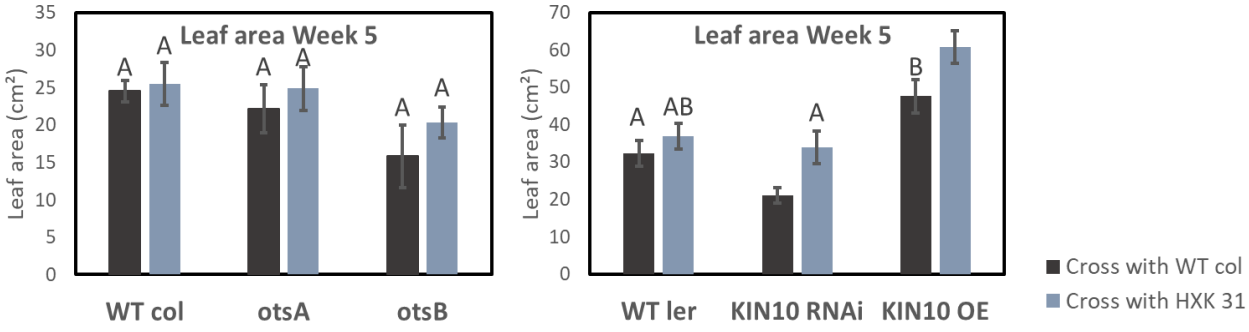


Figure 26: Leaf area in week 5

Rosette area of the cross of WT col and HXK31, with OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE in cm^2. Leaf area was measured after 5 weeks. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

I showed that OtsA mutant had a reduction in RGR compared to WT col, and that the RGR of OtsB was not significantly different from WT col. In this chapter I show that in week 2-4 the cross of OtsA and OtsB with WT col have a similar effect on RGR. However in week 4-5 the RGR of the cross with OtsB was significantly reduced. Like with final leaf area HXK31 did not effect the RGR of the cross with WT col, OtsA and OtsB compared to the same cross with WT col.

As described in chapter 1 KIN10 RNAi and KIN10 OE had the same effect on RGR compared to OtsA and OtsB mutants, respectively. In this chapter I show a similar response as in chapter one, where the cross of WT col with KIN10 RNAi results in a decreased RGR in week 2-4 and the cross of WT col with KIN10 OE had the same RGR compared to the cross of WT col with WT ler. Also in week 4-5 the RGR was similar to that of OtsA and OtsB crossed with WT col. HXK31 did not effect RGR in week 2-4 when crossed with KIN10 RNAi and KIN10 OE, compared to the same cross with WT col. Remarkably, it did significantly increase the RGR in week 4-5 when crossed with KIN10 OE.

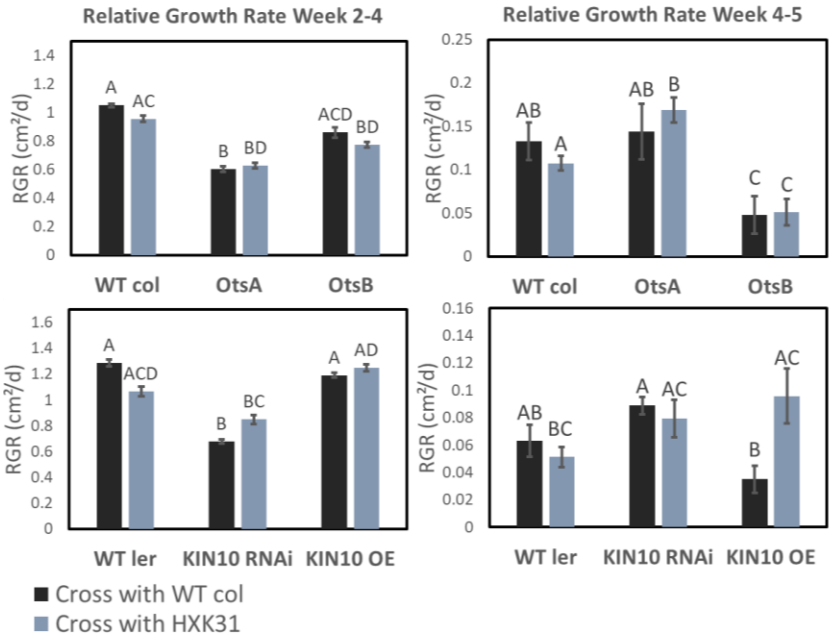


Figure 27: Relative growth rate
RGR ($(LA_{week\ t}-LA_{week\ t-1})/LA_{week\ t-1}$) in week 2-4 and week 4-5 of the cross of WT col and HXK31, with OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE. Leaf area was measured in week 2, 4 and 5 after germination. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

Sugars

To find out if the overexpression of HXK1 combined with genetically modified T6P levels, or different KIN10 expression effects sugar levels, I analysed sugar content of Arabidopsis plants that were grown for 5 weeks and then harvested at noon. HXK1 overexpression had a significant effect on sucrose, fructose, trehalose, and raffinose levels in OtsB background (Figure 28). The levels of raffinose were significantly higher compared to the cross with WT col, and sucrose, fructose and trehalose levels were significantly lower. Although glucose levels also seemed to be effected by HXK1, it was not significantly lower

compared to the cross with WT col. Glucose levels were, however, significantly lower in the cross of HXK31 with WT col. Furthermore hex/suc ratio was significantly lower in the cross with HXK31 and WT col and significantly higher when crossed with OtsB. Crossing WT ler and KIN10 RNAi with HXK31, did not cause any significant change in sugar levels. It did however cause a significant decrease in glucose, trehalose and Hex/Suc ratio when crossed with KIN10 OE.

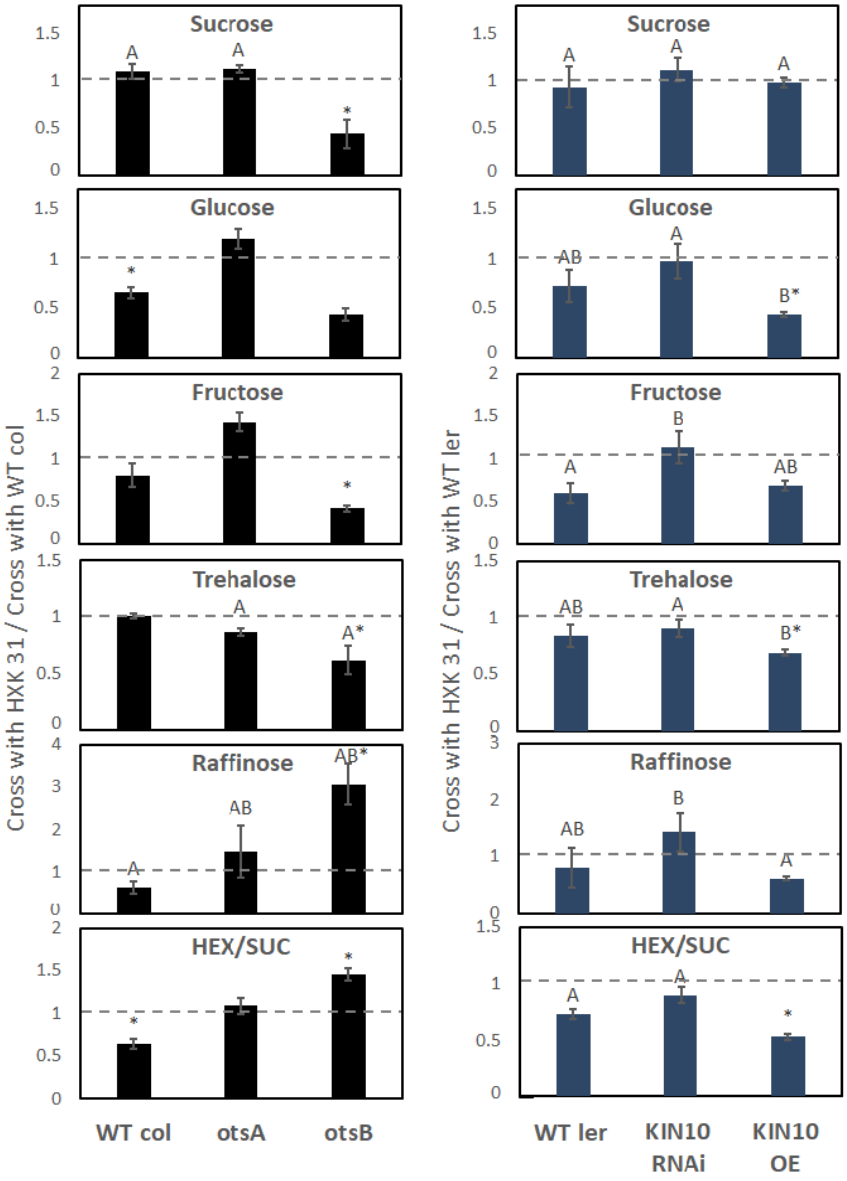


Figure 28: Relative sugar concentrations
The sucrose, trehalose, glucose, fructose, raffinose and hexose/sucrose ratio (glucose + fructose / sucrose) of HXK31 plants crossed with WT col, OtsA, OtsB, WT ler, KIN10 RNAi, d KIN10 OE relative to the same cross with WT col. Plants were grown for 5 weeks and harvested at noon. Bars with the same letter are not significantly different (P<0.1), and error bars show SE. The dotted line represents the relative value of the cross with WT col, and significant differences are indicated with a *.

DISCUSSION

Interaction of HXK1 and T6P during hypocotyl elongation

For long it has been questioned whether HXK1 and T6P interact. To investigate this, a cross was made between HXK1 overexpressing plants, and plants with a genetically modified increase in T6P (OtsA) or decrease in T6P levels (OtsB). A possible interaction between these sugar signalling agents was made by looking at hypocotyl elongation on sucrose. It was shown that application of sucrose to the growth media below a concentration of 1% stimulates hypocotyl elongation. Once sucrose levels exceed the threshold of 1% it negatively effects hypocotyl elongation in HXK1 overexpressing plants (Unpublished results Granot & Brandsma). It was therefore suggested that this inhibition was though an increase in T6P levels with higher sucrose concentrations. Since T6P was not shown to inhibit HXK (Eastmond et al. 2002), but it has an effect on starch accumulation (Wingler et al. 2000; Kolbe et al. 2005), it was hypothesized that increased T6P levels by sucrose feeding might redirect the sugars to starch storage and bypass HXK1. However in this research I show that there was no effect of HXK1 when crossed with OtsB, and thus low T6P levels. This suggests that sucrose mediated hypocotyl reduction is not due to starch storage. Furthermore the hypocotyl elongation in the cross of HXK1 and OtsA was not completely abolished.

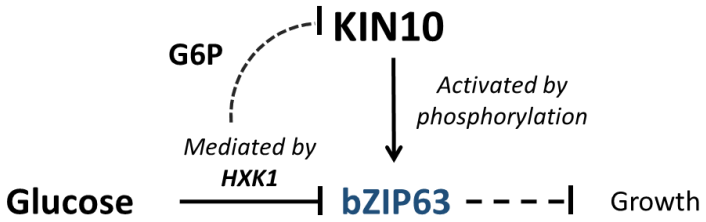
In chapter 1, I showed that plants with high T6P levels have the opposite phenotype in hypocotyl elongation compared to HXK1 overexpressing plants. As shown in the results, the effect of HXK1 on hypocotyl elongation was still apparent in OtsA background (Figure 25). This shows that HXK1 can at least partly restore the negative effect of T6P on hypocotyl elongation. In OtsB background HXK1 did not have any additional effect on hypocotyl elongation. However OtsB crossed with WT col resulted in the same hypocotyl length as WT col crossed with HXK31 (Supplementary data S7), and this suggests that plants with low T6P levels are also able to induce hypocotyl elongation. In Chapter 1 I propose a special role for T6P levels and HXK1 in hypocotyl elongation by effecting gluconeogenesis and PIF4 transcription (Figure 14), and that T6P and HXK1 have an opposite effect in this process. Combined with the results of the cross of HXK31 with OtsB it is very likely that HXK1 and T6P do effect the same pathway, but possibly not by a direct interaction with each other. To get a better understanding of how these sugar signalling agents effect hypocotyl elongation it would be interesting to see what happens to hypocotyl length, starch content, and PIF4 transcription in these crossings when grown on different sugar concentrations (Recommendation 11). This can give more insight into a possible interaction of HXK1 with T6P in hypocotyl elongation.

HXK1 stimulates growth

In chapter 1 I showed that HXK1 overexpression resulted in an increase in RGR, and that a reduction in KIN10 expression and high T6P levels decreased the RGR. To study the interaction of HXK1 with T6P and SnRK1 during leaf expansion, I looked at the RGR of the crossing made between the different sugar signalling mutants. I show that even though it was reported in chapter 1 that HXK1 overexpression increased the RGR, this was not the case for HXK31 mutants crossed with WT col. Since this cross only contains 1 allele of the HXK31 mutant, the abolishment of the effect of HXK31 on the RGR might be caused by a lower expression level of *AtHXK1*. There was also no effect of HXK1 when combined with 1 allele of OtsA or OtsB.

Even though it is not apparent that there is an interaction of HXK1 with T6P in relation to plant growth, I did find an interaction of HXK1 with KIN10. In this chapter I show that in week 4-5 KIN10 OE mutants show a reduced RGR, which can be fully restored when this mutant is crossed with HXK31. This interesting because it is believed that SnRK1 induces the basic region-leucine zipper transcription factor 63 (bZIP63). bZIP63 was shown to be involved in the energy starvation response and has a negative effect on growth (Mair et al. 2015; Carvalho et al. 2016). bZIP63 can be inhibited by glucose and it was shown that HXK1 was necessary for this inhibition. The inhibition of bZIP63 was probably through a process downstream of glucose phosphorylation by HXK1, and not by the HXK1 signalling function itself (Kunz et al. 2015) (Figure 29). This could explain why RGR is higher in KIN10 OE when crossed with HXK31.

Figure 29: Inhibition of growth by bZIP63
Plant growth is inhibited by bZIP63. KIN10 activates bZIP by phosphorylation. Glucose inhibits bZIP63, and this is mediated through the phosphorylating activity of HXK1. Here I propose that this bZIP63 inhibition is due to inhibition of KIN10 by G6P, the primary product of glucose phosphorylation by HXK1.



This indirect interaction of HXK1 and KIN10 also reflects in the results from sugar concentrations. I showed that the cross of HXK31 with KIN10 OE resulted in lower glucose and trehalose levels. In chapter 2 I already proposed that sugar signalling agents effect trehalose levels through in interaction with bZIP11, and that SnRK1 could promote the expression of bZIP11 (Delatte et al. 2011). In earlier work it was concluded that HXK1 does not interact with SnRK1 (Baena-González et al. 2007). However, they only assessed plants with an impaired expression of HXK1. Arabidopsis plants are known to have 3 different HXK genes, and it was reported that HXK2 has the same contribution to glucokinase activity as HXK1 (Karve et al. 2008). If the interaction of HXK1 with KIN10, is not mediated through the signalling activity of HXK1, but by the phosphorylation activity, this could explain, why earlier articles state that there is no interaction of HXK1 with KIN10. This effect of the phosphorylating activity of HXK1 on the interaction with KIN10 was proposed

before. It is known that the primary product of glucose phosphorylation by HXK, G6P, is able to inhibit SnRK1 (C??tia Nunes et al. 2013). Nagele et al., showed this with a mathematical model. By changing the catalytic activity of HXK it reduced the activity of SnRK1 (Nägele & Weckwerth 2014). It would be interesting to look at the SnRK1 activity in plants of the cross of KIN10 OE with HXK31 (Recommendations 12)

CONCLUSION

In this chapter the main question was whether HXK1 interacts with T6P and SnRK1. By looking at hypocotyl elongation in the different crosses I conclude that there is no direct interaction of HXK1 with T6P. Because of the pronounced effect of HXK31 crossed with KIN10 OE, I conclude that there is an interaction of HXK1 with KIN10 OE during leaf expansion, and that this is primarily through the phosphorylation activity of HXK1, and not because of its signalling role.

CHAPTER IV

Roar or Ignore

Is there an interaction between T6P and SnRK1 with HXK1 under stressed conditions, and does this effect the defense response of Arabidopsis thaliana to the Two-spotted Spider Mite

INTRODUCTION

From previous chapters

In the first two chapters I showed the effect of T6P, HXK1 and SnRK1 on plant growth, development and sugar concentrations under stressed and non-stressed conditions. In the third chapter I looked at the interaction of HXK1 with T6P and SnRK1. I showed that there is no direct interaction of HXK1 with T6P. I also concluded that there is an interaction of HXK1 with KIN10 OE, but not through the signalling function of HXK1, but by its catalytic activity (Figure 29). In this chapter I want to investigate whether the introduction of biotic stress effects these interaction in a different way.

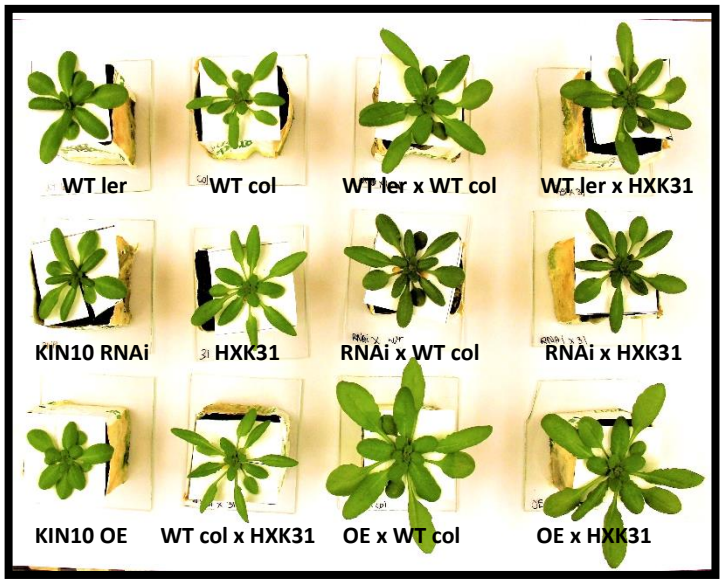


Figure 30: Rosettes of crossings
A picture of WT ler, KIN10 OE, KIN10 RNAi plants crossed with either WT col or HXK31, and all the parental lines. The plants are 4 weeks old.

Metabolite production

The cross of WT col and HXK31 with WT ler and KIN10 OE resulted in a remarkable leaf area increase. In week 5 the rosette area of the cross of HXK31 with KIN10 OE was almost 2.5 times as big as WT col and 2 times as big at WT ler (Supplementary data S8) (Figure 30). In this report I want to investigate whether induced defenses redirect assimilates to the production of defense metabolites and result in a negative trade-off to growth. Because of the interesting increase in leaf area I decide to infest these plants with mites,

and see if this negatively effect growth. I performed an untargeted LC-MS on an extraction of the leaf tissue of these crossings to investigate the metabolite profile. In this way I want to see whether mites increase the production of defense metabolites. One of the most important toxic compounds produced by the plant, of which the production is induced by spider mites are the Indole Glucosinolates (Zhurov et al. 2014; Mewis 2006; Kim & Jander 2007; Clay et al. 2009). Glucosinolates can be cleaved by myrosinase, which in presence of water cleaves off the glucose group which results in either a isothiocyanate, nitrile, or thiocyanate which are toxic to herbivores. To prevent plant damage glucosinolates and myrosinase are stored in spate compartments, and only when a cell is damaged these two compounds collide (Halkier 2016). Interestingly indole glucosinolates have a shared precursor with auxin biosynthesis, and a T-DNA insertion in the CYP83B1 gene, that encodes an important cytochrome for the glucosinolate biosynthesis pathway, leads to auxin overproduction (Naur et al. 2003; Bak et al. 2001; Bak 2001; Mikkelsen et al. 2004). Auxin is an important hormone involved in cell division and expansion (Ljung & Bhalerao 2001). Because of this metabolic branch point between the growth related Auxin and Indole glucosinolates I will look at the production of metabolites related to this pathway.

RESULTS

In order to investigate the effect of mites on development and growth in crossings of different sugar signalling mutants, all results are relative values to the control treatment. In this way, I am able to study the magnitude of the response, normalized for the different genetic backgrounds.

Relative growth rate

The effect of spider mites on RGR was measured to investigate the interaction of T6P and SnRK1 with HXK1 under stressed conditions. Rosette area was measured in week 4, at the time of mite infestation, and in week 5. From this, the RGR was calculated, and divided by the RGR of plants that were not infested (Figure 31) (For an explanation on how to read the graphs, see Chapter III). In chapter 2 it was reported that RGR was decreased in OtsA mutants upon mite herbivory, while RGR was not significantly different in WT col and OtsB (Figure 18). The results from this chapter are consistent with this finding. OtsA mutants crossed with WT col, did have a decreased RGR when infested with mites, while the RGR of the cross of WT col with OtsB was not effected. Interestingly under stress there is no additional effect of HXK 31 combined with OtsB, OtsA or WT col, while it was reported in Chapter 2, that HXK31 mutants had a decreased RGR when infested with mites (Figure 18).

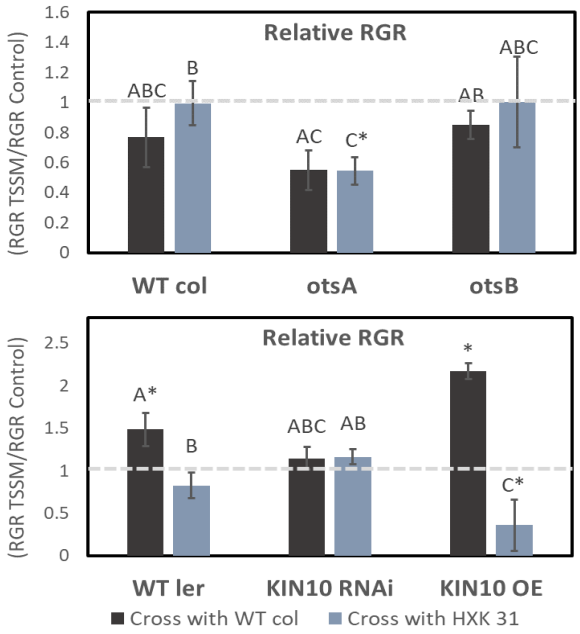


Figure 31: Relative RGR
The RGR (($LA_{week\ 5}$-$LA_{week\ 4}$)/$LA_{week\ 4}$) of the cross of WT col and HXK31, with OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE infested with spider mites for 1 week relative to the RGR of the control treatment. Leaf area was measured in week 4 and 5 after germination. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

In chapter 2 I showed that there was a significant reduction of RGR in WT ler, and no effect on RGR in KIN10 RNAi and KIN10 OE upon mite infestation. Surprisingly the RGR was significantly increased when WT ler or KIN10 OE were crossed with WT col. Consistent with previous findings, the RGR of KIN10 RNAi was unchanged. While HXK31 showed a strong decrease in RGR upon mite infestation (Chapter 2), this effect on RGR was only apparent when HXK31 was crossed with WT ler, or KIN10 OE, and did not effect KIN10 RNAi.

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Leaf damage

To see whether the overexpression of HXK1 effects the susceptibility of WT col, OtsA, OtsB, WT ler, KIN10 RNAi and KIN10 OE plants to spider mites, the crossings were infested with mites for 1 week and leaf damage was scored. This was done by looking at feeding spots of mites, and scored according to Table 2. The graph presents the relative leaf damage of the cross with HXK31 relative to the same cross with WT col (Figure 32).

In Chapter 2 I showed that HXK31 mutants had less leaf damage, but also a reduction in RGR (Figure 18Figure 20). In this chapter I already showed that HXK31 crossed with WT ler or KIN10 OE negatively effects the RGR of the plant Figure 31. Interestingly these same crosses, also have less leaf damage, while the cross with KIN10 RNAi, that did not have a change in RGR, didn't have reduced leaf damage (Figure 32).

In Chapter 2 I also showed that OtsA and HXK1 plants have a reduction in RGR, and leaf damage. However in this chapter there was no additional effect of OtsA crossed with HXK1 on RGR reduction, but there was additional reduction in leaf damage. There was no significant effect of HXK1 on plant leaf damage when HXK31 was crossed with WT col and OtsB.

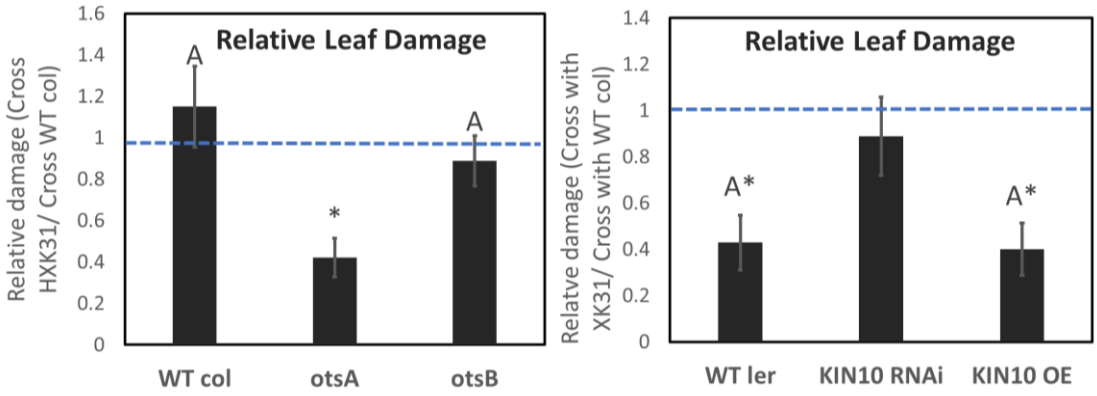

Figure 32: Relative leaf damage
Leaf damage of the cross of HXK31 with WT col, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE plants relative to the same cross with WT col (dotted line). Plants were infested with spider mites for 1 week. Bars sharing the same letter are not significantly different. Significant differences from the cross with WT col are indicated with a *. Error bars present SE.

Effect on sugars

The effect of spider mites on sugar concentrations was measured to investigate the interaction of T6P and SnRK1 with HXK1 under stressed conditions. The effect of HXK1 was most apparent in the cross with KIN10

OE. The effect of spider mites on sugar concentration change of sucrose, glucose, fructose, trehalose, and raffinose was significantly higher relative to the same cross with WT col (P<0.1) (Figure 33). The cross of HXK31 with KIN10 RNAi only resulted in significantly lower raffinose levels, but furthermore did not effect any other sugar compared to the same cross with WT col. HXK1 only effected the spider mite induced change in sucrose levels in WT col, and raffinose levels in OtsB background, which were significantly decreased upon mite infestation compared to the same cross with WT col.

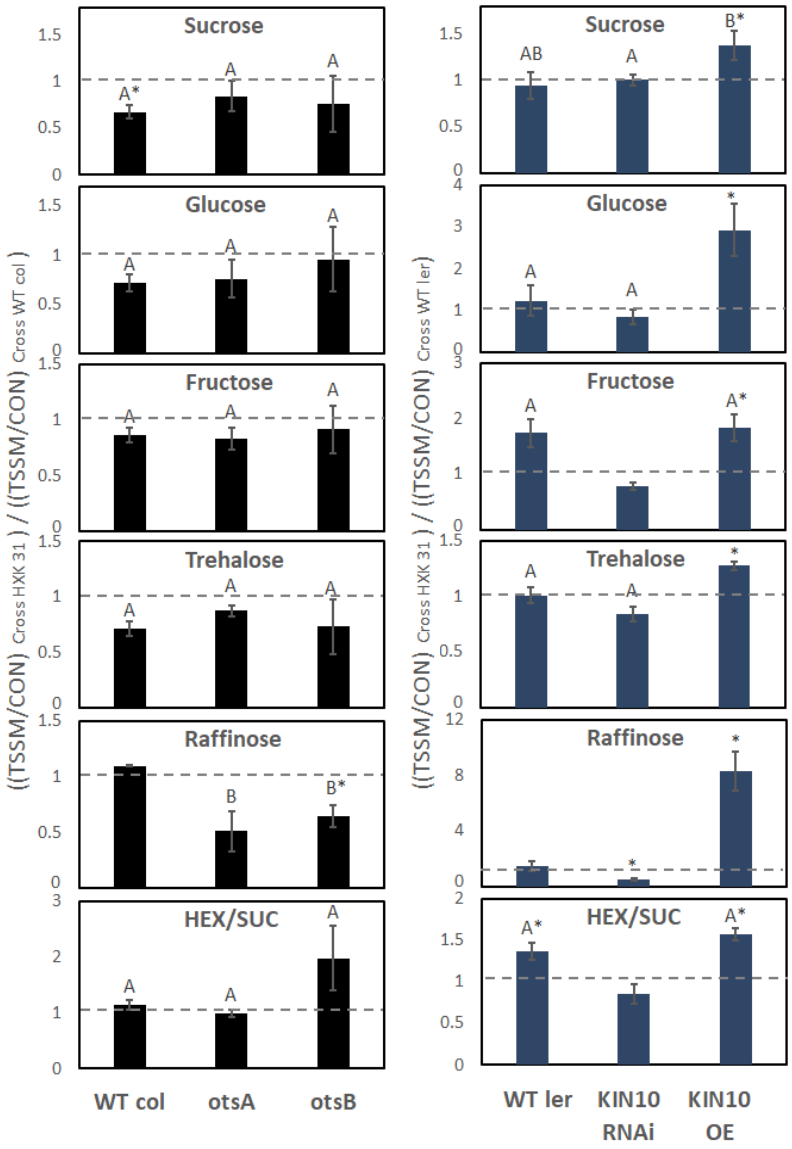

Figure 33: Relative Sugar concentration change induced by spider mites
The effect of spider mite infestation on sucrose, trehalose, glucose, fructose, raffinose and hexose/sucrose ratio (glucose + fructose / sucrose) of HXK31 plants crossed with WT col, OtsA, OtsB, WT ler, KIN10 RNAi, KIN10 OE relative to the same cross with WT col. Plants were grown for 5 weeks and harvested at noon. Bars with the same letter are not significantly different (P<0.1), and error bars show SE. The dotted line represents the relative value of the cross with WT col, and significant differences are indicated with a *.

Metabolite production

Because of the interesting RGR increase upon spider mite infestation in the cross of WT col with WT ler and KIN10 OE, untargeted LC-MS was done to investigate the metabolite production in these crossings. Two metabolites were selected, indolyl-3-methyl-glucosinolate (Figure 34), and an unknown Flavonoid that was only present in plants infested with spider mites (Figure 35). In the control treatment (Figure 34A), without mites, the highest concentration of indolyl-3-methyl glucosinolate was found in the cross of WT col and HXK31 with KIN10 OE, and the lowest concentration in WT ler. There were no significant differences between the cross with WT col and the cross with HXK31 with WT ler, KIN10 RNAi and KIN10 OE ($P<0.1$). Spider mites triggered a significant increase in Indolyl-3-methyl glucosinolates in WT ler and WT col (Figure 34). Even though HXK31 plants infested with mites also seemed to have higher glucosinolate levels, this difference was not significant.

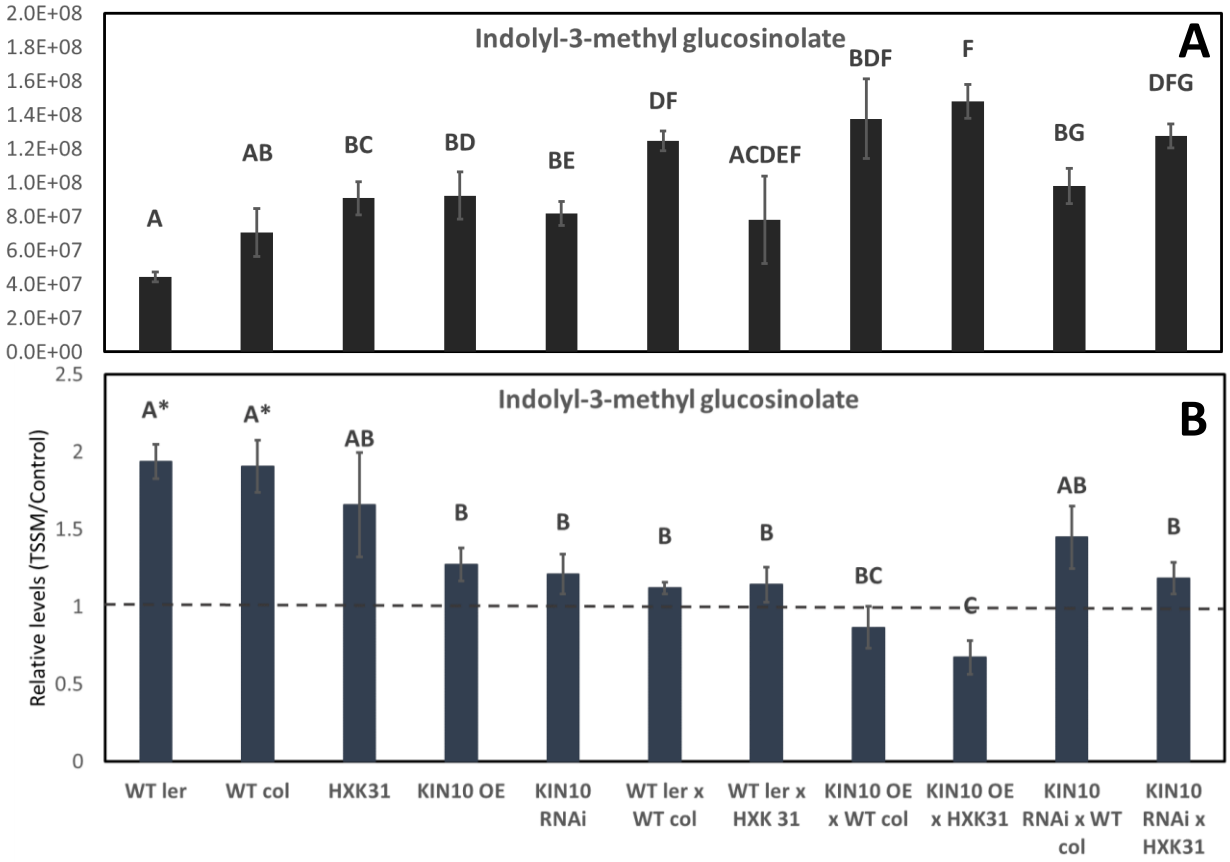

Figure 34: Indolul-3-methyl glucosinolate

Indol-3-methyl glucosinolate levels in WT ler,WT col, HXK31, KIN10 OE, KIN10 RNAi, and the crosses of WT col and HXK31, with WT ler, KIN10 OE, and KIN10 RNAi in the control treatment (A), and infested with mites relative to the control treatment (B). Plants were either infested with spider mites for 1 week, or kept as a control. Bars that share the same letter are not significantly different ($P<0.1$). In figure B significant differences between the spider mite infested and the control treatment (dotted line), are indicated with a *. And error bars present SE.

The unknown flavonoid was detected in the highest concentrations in HXK31 plants. Although the difference was significant from all other genotypes it was not significantly different from WT col. The lowest levels of the flavonoid was found in WT ler.

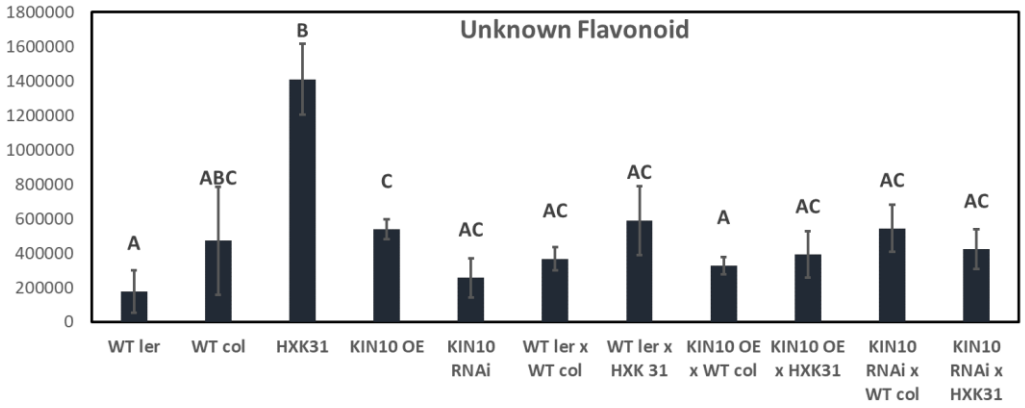

Figure 35: Unknown flavonoid

Levels of an unknown flavonoid in WT ler,WT col, HXK31, KIN10 OE, KIN10 RNAi, and the crosses of WT col and HXK31, with WT ler, KIN10 OE, and KIN10 RNAi infested with mites. Plants were grown under long day conditions, and infested with spider mites for 1 week. Bars that share the same letter are not significantly different ($P<0.1$). And error bars present SE.

DISCUSSION

HXK1 and SnRK1 under biotic stress

As proposed in Chapter 3 HXK1 and SnRK1 interact with each other through the catalytic activity of HXK1. In this chapter I show that under biotic stress conditions this interaction is still apparent. The cross of HXK31 with KIN10 OE resulted in a lower RGR, and leaf damage, and higher sugar concentration. As concluded in chapter 3 HXK1 and SnRK1 interact in the growth response of the plant. It could be questioned whether SnRK1 and HXK1 interact to mediate the defense response, since the effect of HXK31 on leaf damage was abolished in KIN10 RNAi mutants. However, HXK1 was also able to reduce leaf damage in OtsA plants, which points towards an independent role for HXK1 in plant defensibility to mites. However, the overexpression of HXK1 is often connected to abiotic stress resistance, and not much it known about the effect of HXK1 in biotic stress. It was reported that HXK1 and HXK2 expression are upregulated in Arabidopsis leaves after infection with Pseudomonas syringae (Wang et al. 2016), and this could indicate the involvement of HXK1 in the biotic stress response.

Increased RGR upon mite infestation

Interestingly the cross of WT col with WT ler and KIN10 OE resulted in a higher RGR when plants were infested with mites. In Chapter 2 I showed that, although not significant for every genotype, RGR is reduced upon mite infestation. To investigate interesting trait of the cross of WT col with WT ler and KIN10 OE, I conducted a second experiment in which I investigated the effect of MeJA on leaf growth (Supplementary data S8). Consistent with the findings for spider mite infestation, the RGR of the cross of WT col with KIN10 OE was increased. However the cross with WT ler, did not have a change in RGR upon MeJA spraying. Furthermore, the cross of HXK31 with KIN10 RNAi resulted also in a higher RGR, when sprayed with MeJA. This results are not consistent with the previous experiment.

Secondary metabolite production for defense

It is very interesting that the increased defense of HXK1 plants coincide with a decrease in RGR. This would show that there is indeed a negative trade-off of increased defenses to growth. As mentioned in Chapter 2 that the production of glucosinolates, an important defense metabolite, requires 15% of the sugars produced by photosynthesis, under non-stressed conditions (Bekaert et al. 2012). This would indicate that an increased production of glucosinolates, by for example mite infestation, would result in a negative trade-off to growth. Furthermore, glucosinolates share a precursor with the growth regulating hormone Auxin, and this point towards a trade-off of glucosinolate production versus growth (Naur et al. 2003; Bak et al. 2001; Bak 2001; Mikkelsen et al. 2004). Interestingly Glucose is known to induces the biosynthesis

of several glucosinolates. And it was shown that HXK1 was needed to mediate this effect (Miao, 2013). In this research I showed that spider mites induced the production of indolyl-3-methyl-glucosinolate, a precursor of indole-glucosinolate production, in WT ler WT col and HXK1. However the concentration of indolyl-3-methyl-glucosinolate in HXK1 did not have any significant difference with the WT. And this suggests that the increased plant defense is not due to an increased production of glucosinolates. I also found an unknown flavonoid that was only upregulated in spider mite infested tissue, and very pronounced in HXK1 overexpressing mutants. Flavonoids are found to be important secondary metabolites for the defense response of the plant (Morkunas & Ratajczak 2014). However more research is needed to identify this unknown flavonoid, to draw any conclusions. It would be interesting to do a more targeted LCMS to investigate secondary metabolite production in the different sugar signalling mutant (Recommendation 13).

CONCLUSION

In this chapter I wanted to investigate interaction of HXK31 with T6P and SnRK1 under stress. I also wanted to have a better understanding of metabolite production in spider mite infested plants. Consistent with the results from chapter 3, I showed that there is an interaction of HXK31 with high expression of KIN10. Furthermore I showed that spider mites induce the production of secondary metabolites, but that a more in depth study is needed to draw any conclusions from this.

OVERAL DISCUSSION AND CONCLUSION

In this research I investigated the effect of T6P, HXK1 and SnRK1 on growth, development, sugar concentrations and defensibility to spider mites in Arabidopsis thaliana. I asked the question whether there is a negative trade-off to growth when plants have to deal with biotic stress in the different sugar signalling agents, hence Roar or Ignore. In the overall discussion and conclusion I will mention the conclusions of this research with a small discussion.

1. **T6P and HXK1 effect the plant growth in opposite or similar matter, depending on developmental stage and stress induction, but do not directly interact with each other.**

In Chapter 1 I showed that high T6P levels result in opposite effect in respect to hypocotyl elongation and RGR compared to HXK1. However in later developmental stages, or when the plant is under biotic stress, the phenotype of HXK1 and OtsA show strong overlap. This is visible in a higher seed production, RGR reduction, increased defensibility, and the increased sugar content upon mite infestation. However in Chapter 3 and 4 I concluded, that T6P and HXK1 did not have a direct interaction with each other.

2. **There is an independent role for T6P, apart from SnRK1 inhibition.**

It is proposed that the main function of T6P is the inhibition of SnRK1. However in this research I showed that the phenotypes of KIN10 RNAi and KIN10 OE did not resemble the phenotype of OtsA and OtsB respectively. Although this difference could partly be explained by a difference in background (KIN10 in WT ler, and OtsA-B in WT col) (Chapter 1), these results strongly suggest and independent role for T6P apart from the inhibition of SnRK1.

3. **There is an interaction between HXK1 and SnRK1 during leaf expansion, in which G6P, the primary product of glucose phosphorylation by HXK1, inhibits SnRK1.**

Because of the overlapping phenotypes of T6P, and SnRK1 with HXK1, I wanted to investigate a possible interaction of these sugar signalling agents. I showed in chapter 3 and 4 that the most obvious interaction between HXK1 and SnRK1 is observed during leaf expansion. I attribute this to the increased catalytic activity of HXK1, resulting in higher G6P levels. Like T6P, G6P can inhibit SnRK1.

4. **Raffinose levels are an important determinant for plant defensibility.**

I showed that leaf damage was negatively correlated to raffinose levels in the plant (Chapter 2). This showed that an increase in raffinose had a positive effect on plant defensibility, and that these levels can be effected by sugar signalling agents.

5. **HXK1 is an important factor in plant defensibility, but its increased defensibility to mites has a negative trade-off to growth.**

In both, chapter 2 and 4, I show that the overexpression of HXK1 has a positive effect on plant defensibility to mites, but that this results in a negative trade-off to growth. This effect of HXK1 overexpression is still visible when crossed with KIN10 OE, and OtsA plants. Which suggest and independent role for HXK1 in the defense response.

6. **Seed production is negatively effected by herbivory, but high levels of T6P or overexpression of *AtHXK1* can result in a higher DW partitioning into seeds upon biotic stress.**

I showed that even though seed weight was decreased in all plants upon mite infestation, still the percentage of dry weight partitioned into seeds was higher in OtsA and HXK31. This shows that herbivory stimulates the distribution of assimilates towards the production of seeds.

7. **Spider mites can induce the production of secondary metabolites, and effect sugar concentrations in plants with high T6P levels, or overexpression of *AtHXK1*.**

In the final chapter of this research I showed that spider mites induce the production of secondary metabolites. Furthermore, I showed in chapter 2 that mites effected sugar concentrations in plants OtsA and HXK31 mutants. Even though the effect of mites was different among the different genotypes.

Concluding remark

The title of this research asks a question: Roar, or Ignore? Does the plant tolerate/Ignore the attack of herbivores, or does the plant fight back/roar? In this research I show that indeed, when a plant response to herbivory, by a reduction in leaf area and DW%, and an increase in sugars, such as raffinose and trehalose, the plants has a higher defensibility. One might ask whether this response is beneficial. Especially for such short lived plants as Arabidopsis. It might be better to ignore the herbivore attack and maintain a high growth. However, since DW partitioning into seed production increased in plants that showed a higher defensibility to mites, this indicate that indeed, it is better to Roar instead of Ignore!

RECOMMENDATIONS

Recommendation 1

Does T6P have a different signalling function apart from its known function of inhibiting SnRK1?

Hypothesis: Based on the findings in this report**,** T6P has another signalling function in plant tissue, apart from its mediation of sugar signals by inhibiting SnRK1.
Approach: Cross OtsA mutants with KIN10 OE, the counteract the negative effect of high T6P levels on SnRK1 activity. Or cross OtsA mutants with mutants that overexpress KIN10 without the binding site.

Recommendation 2

What is the effect of HXK1 and T6P on lipid remobilisation and starch content in Arabidopsis seedlings, and how does it affect hypocotyl elongation.

Hypothesis: HXK1 overexpression promotes the lipid remobilisation and starch degradation and genetically modified increased T6P levels causes a reduction.
Approach: Track lipid and starch content in Arabidopsis seedlings with different expression of HXK1 and genetically modified T6P levels over time, and link this to hypocotyl growth.

Recommendation 4

What is the effect of HXK1, T6P, and SnRK1 on seed weight and number under non-stressed conditions?

Hypothesis: The sugar signalling agents will behave differently.
Approach: Grow mutants of HXK1, T6P and SnRK1 under non-stressed conditions. Wait till seed maturation and weigh and count the seeds.

Recommendation 5

Do genetically modified T6P levels effect sugar transport through the phloem?

Hypothesis: In plants with high T6P levels, less sugars are distributed through the phloem, and with low T6P levels, more sugars are transported through the phloem compared to WT plant.
Approach: Measure soluble sugar concentrations in the phloem of OtsA and OtsB mutant.

Recommendation 6

What is the effect of T6P, HXK1 and SnRK1 on sugar composition and starch levels over the day?

Hypothesis: In plants with T6P levels, more starch will be found at night time, and in HXK1 and KIN10 overexpressing plants less starch will be found. High levels of sucrose will be found in KIN10 overexpressing plants and plants with low T6P levels.
Approach: Measure soluble sugar concentrations and starch at different time point.

Recommendation 7

What is the effect of herbivory on plant growth in plants with altered T6P levels, and different expression of KIN10, or HXK1 overexpression?

Hypothesis: Herbivory has a clear effect on plant growth. It reduces the final plant growth in all different sugar signalling mutants
Approach: Invest the different mutants with mites in an early developmental stage, and track plant growth over time. Compare to the control treatment.

Recommendation 8

How do mites effect aquaporin expression in Arabidopsis plants with altered T6P levels, and different expression of KIN10, or HXK1 overexpression?

Hypothesis: The expression of aquaporin related genes is upregulated when plants are infested with mites. This expression is blocked in HXK1 mutants and plants with High T6P levels.
Approach: Infest plants with mites for several days, and check the expression of aquaporin related genes.

Recommendation 9

What is the effect of herbivory on the amount of seeds produced by a plant with altered T6P levels, or different HXK1 or SnRK1 expression?

Hypothesis: Herbivory effects the total amount of seeds produced.
Approach: Count the number of seeds produced per plant from the seed collection experiment.

Recommendation 10

Does MeJA inhibit HXK1 phosphorylation activity in Arabidopsis thaliana plants overexpressing HXK1?

Hypothesis: The application of MeJA leads to inhibition of the phosphorylation activity of HXK1, but only in the overexpressing plants.
Approach: Spray MeJA to plants and measure HXK activity.

Recommendation 11

How do T6P and HXK1 effect hypocotyl length, starch content, and PIF4 transcription when grown on different sucrose concentrations?

Hypothesis: The inhibition of hypocotyl elongation will be more pronounced with higher sucrose and T6P levels. In these plants PIF4 transcription is low and starch accumulation high.
Approach: Grown OtsA, OtsB, and HXK1 mutants on different concentrations of sucrose. Measure hypocotyl length, starch content and PIF4 transcription.

Recommendation 12

How is SnRK1 activity affected in the cross of KIN10 OE with HXK31?

Hypothesis: I expect that the increased G6P levels by glucose phosphorylation by HXK1, result in an decreased activity of SnRK1.
Approach: Check the expression of SnRK1 regulated genes.

Recommendation 13

Investigate secondary metabolite production of different sugar signalling mutants when infested with spider mites.

Hypothesis: -
Approach: Analyse the untargeted LCMS data set of the experiment described in chapter 4.

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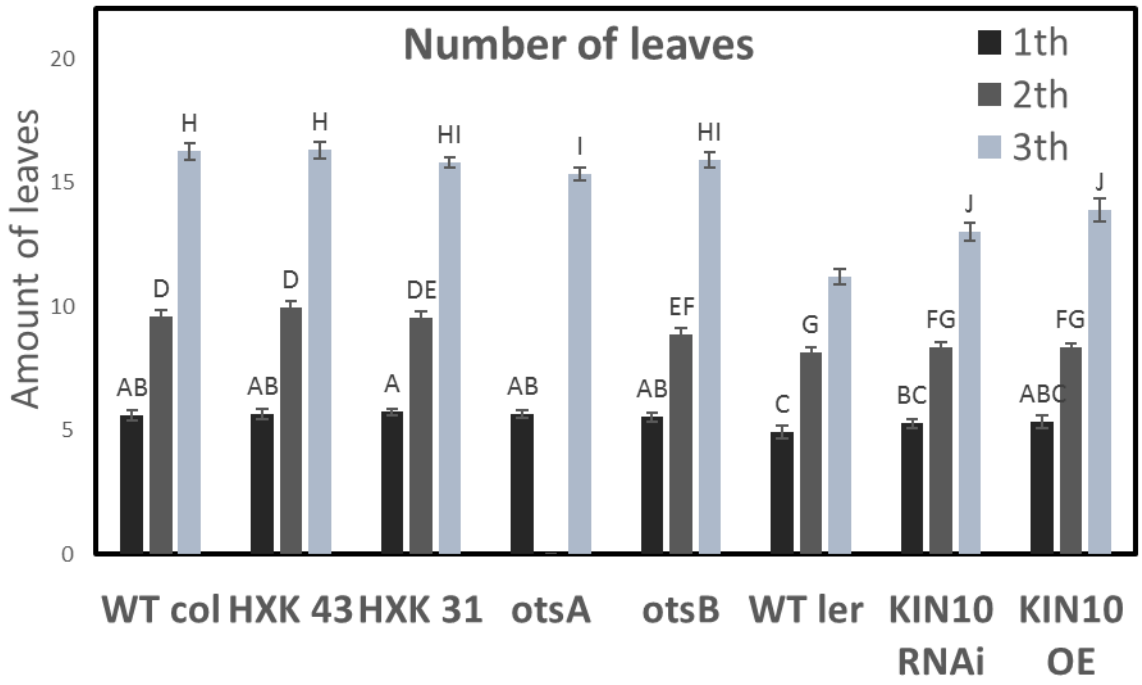
SUPPLEMENTARY DATA

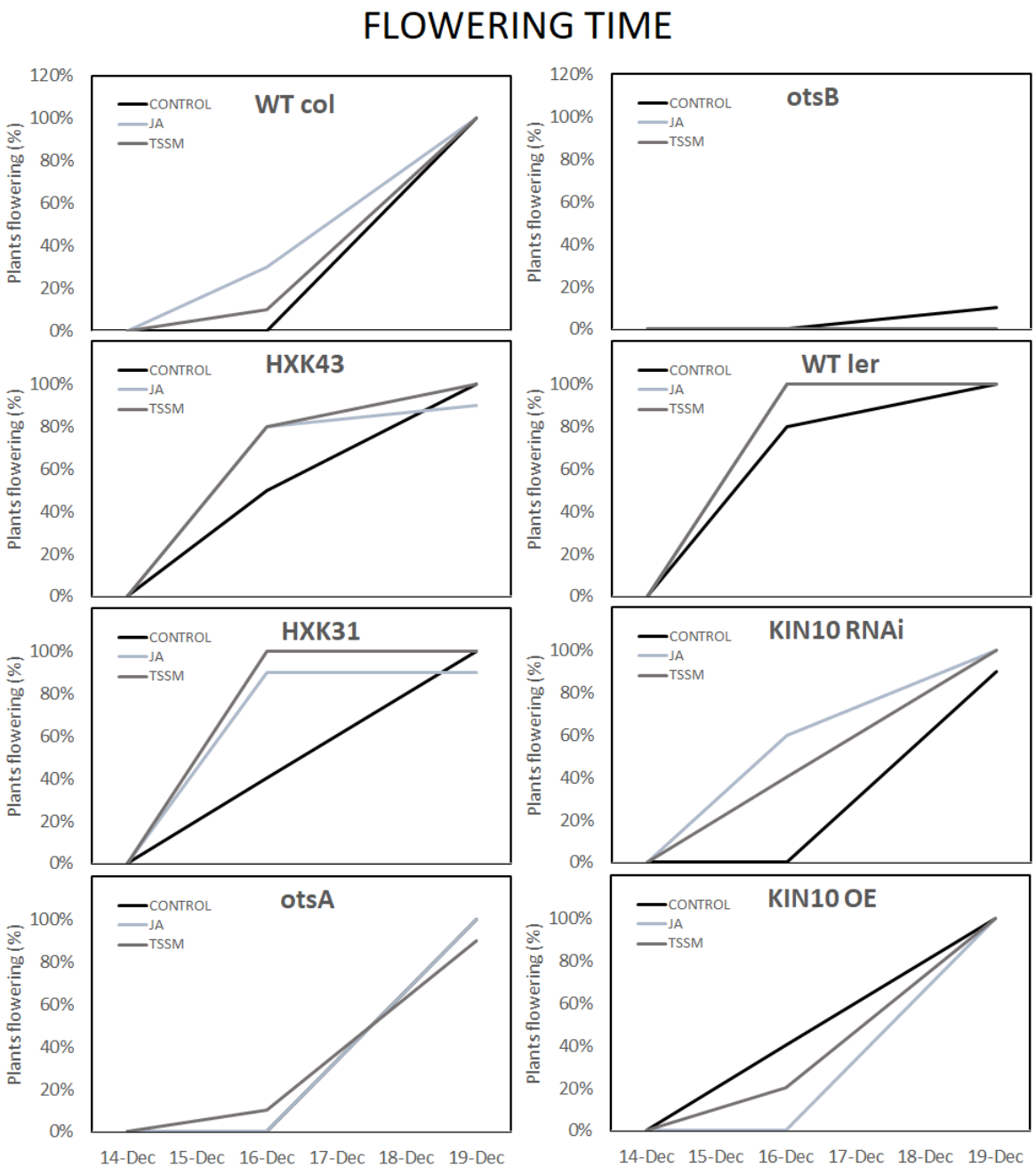
S1: NUMBER OF LEAVES



S 1: Number of leaves in different growth stages

Arabidopsis plants were grown for several weeks and in week 2 (1th), week 3 (2th), and week 4 (3th) after germination the number of leaves of WT col, HXK43, HXK31, OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE were counted. Plants were grown under long day conditions (8/16h, L/D). Bars with the same letter are not significantly different ($P<0.1$). Error bars present SE.

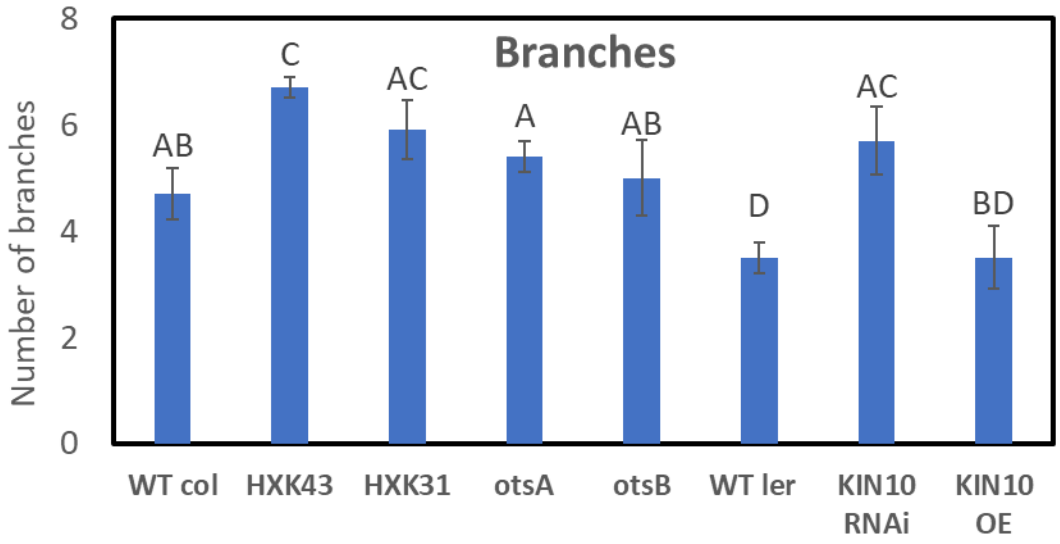
S2: FLOWERING TIME



S 2: Flowering time of different genotypes

The flowering time of WT col, HXK43, HXK31, otsA, otsB, WT ler, KIN10 RNAi, and KIN10 OE. Plants were grown under long day conditions (8/16h, L/D). The data point presents the percentage of plants flowering from a total of 10 plants. Three different treatments were given to 10 plants of each genotype. Either they were infested with spider mites, or sprayed with JA, or kept as a control.

S3: BRANCHES



S 3: Number of branches

The total number of branches counted from the base of the rosette of WT col, HXK43, HXK31, otsA, otsB, WT ler, KIN10 RNAi, and KIN10 OE. Plants were grown under long day conditions (8/16h, L/D). Bars with the same letter are not significantly different (P<0.1). Error bars present SE.

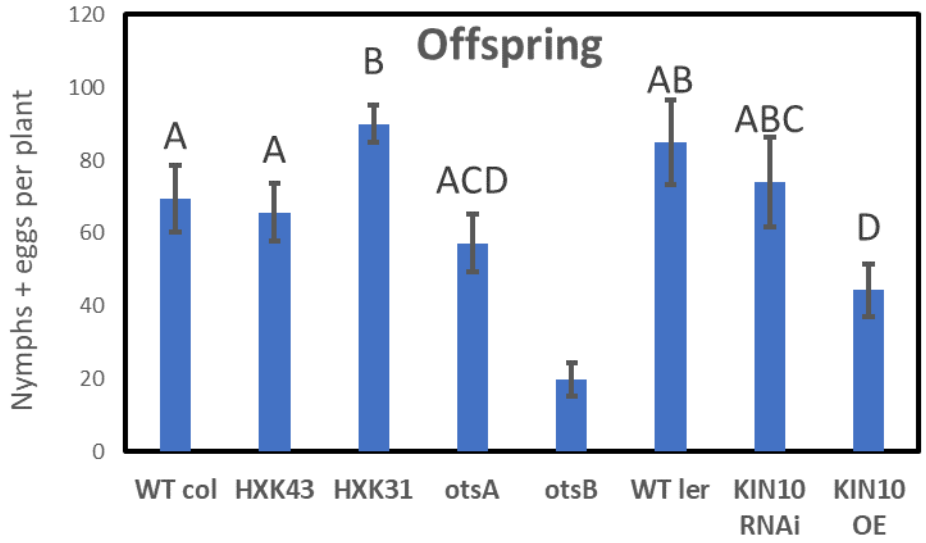
S4: CORRELATION IN MITE INFESTED PLANTS

		trehalose	glucose	fructose	sucrose	raffinose	Hex/Suc ratio	Leaf FW	Leaf area W4	Leaf area W5	Growth	RGR	%DW	Total DW	Damage
trehalose	R	-	0.551	0.848	0.957	-0.073	-0.423	-0.306	-0.298	-0.349	-0.298	-0.151	0.370	-0.261	-0.041
	P-value	-	0.000	0.000	0.000	0.492	0.000	0.003	0.004	0.001	0.004	0.152	0.000	0.013	0.702
glucose	R	-	-	0.750	0.621	0.636	0.382	-0.490	-0.410	-0.558	-0.583	-0.432	0.761	-0.313	-0.426
	P-value	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000
fructose	R	-	-	-	0.913	0.178	-0.151	-0.307	-0.265	-0.366	-0.388	-0.310	0.530	-0.197	-0.184
	P-value	-	-	-	0.000	0.091	0.152	0.003	0.011	0.000	0.000	0.003	0.000	0.062	0.081
sucrose	R	-	-	-	-	-0.035	-0.387	-0.314	-0.295	-0.359	-0.326	-0.218	0.446	-0.254	-0.070
	P-value	-	-	-	-	0.742	0.000	0.002	0.005	0.000	0.002	0.037	0.000	0.015	0.508
raffinose	R	-	-	-	-	-	0.686	-0.406	-0.337	-0.444	-0.445	-0.289	0.622	-0.227	-0.523
	P-value	-	-	-	-	-	0.000	0.000	0.001	0.000	0.000	0.005	0.000	0.030	0.000
Hex/Suc ratio	R	-	-	-	-	-	-	-0.239	-0.188	-0.262	-0.281	-0.160	0.380	-0.094	-0.402
	P-value	-	-	-	-	-	-	0.023	0.074	0.012	0.007	0.129	0.000	0.373	0.000
Leaf FW	R	-	-	-	-	-	-	-	0.923	0.971	0.683	0.084	-0.565	0.899	0.060
	P-value	-	-	-	-	-	-	-	0.000	0.000	0.000	0.430	0.000	0.000	0.573
Leaf area W4	R	-	-	-	-	-	-	-	-	0.912	0.432	-0.203	-0.524	0.826	0.057
	P-value	-	-	-	-	-	-	-	-	0.000	0.000	0.053	0.000	0.000	0.591
Leaf area W5	R	-	-	-	-	-	-	-	-	-	0.763	0.178	-0.613	0.849	0.121
	P-value	-	-	-	-	-	-	-	-	-	0.000	0.091	0.000	0.000	0.255
Growth	R	-	-	-	-	-	-	-	-	-	-	0.713	-0.525	0.566	0.175
	P-value	-	-	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.096
RGR	R	-	-	-	-	-	-	-	-	-	-	-	-0.258	0.044	0.130
	P-value	-	-	-	-	-	-	-	-	-	-	-	0.013	0.680	0.221
%DW	R	-	-	-	-	-	-	-	-	-	-	-	-	-0.235	-0.307
	P-value	-	-	-	-	-	-	-	-	-	-	-	-	0.025	0.003
Total DW	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.033
	P-value	-	-	-	-	-	-	-	-	-	-	-	-	-	0.757
Damage	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	P-value	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S 4: Correlation in mite infested plants

The correlation of Trehalose, glucose, sucrose, raffinose, Hex/suc ratio, leaf DW, leaf area W4, leaf are W5, growth increase, RGR, DW%, total DW, and damage. R giving the correlation coefficient and P the significance of the test.

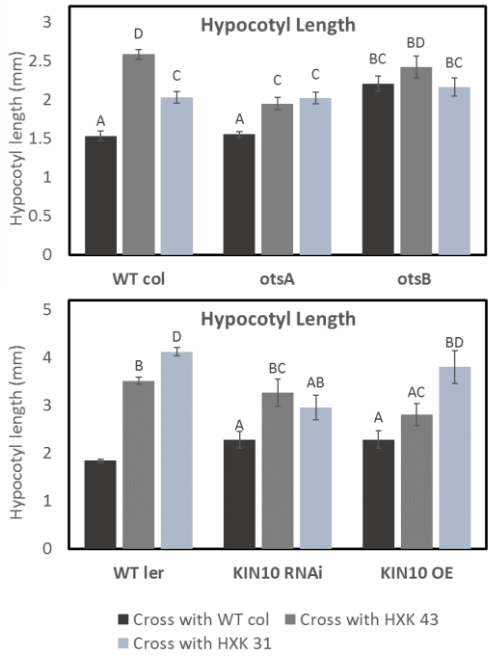
S5: OFFSPRING



S 5: Offspring spider mites

The offspring of spider mites after 1 week. Plants are grown under 12/12h D/N conditions. After 4 weeks, plant were infested with 10 mites each, and after 1 week the offspring (Nymphs + eggs) were counted per plant. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

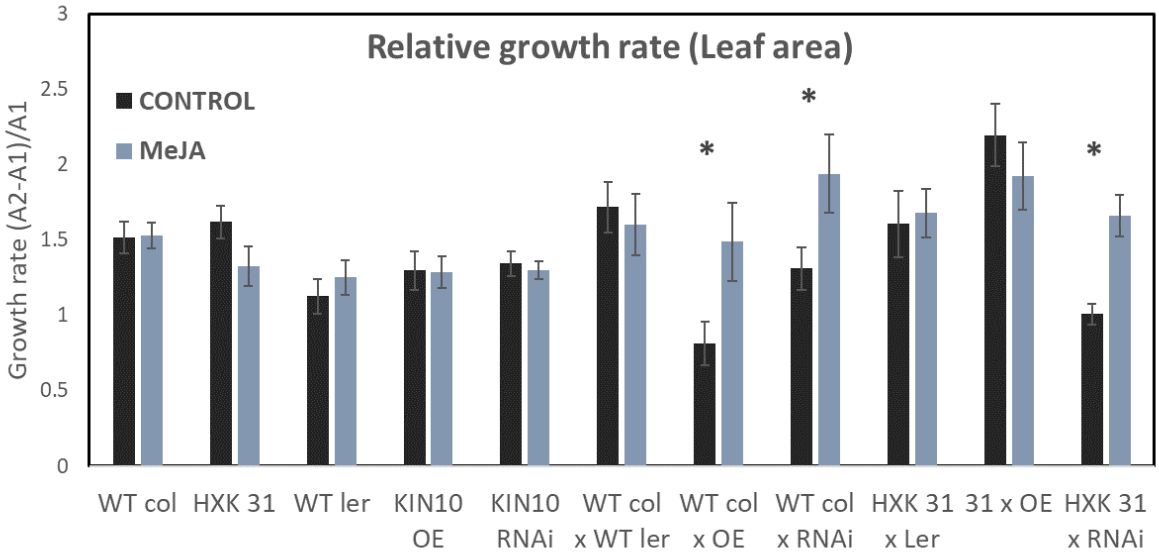
S6: HYPOCOTYL LENGTH CROSSINGS



S 6: Hypocotyl length

Hypocotyl length of WT col, HXK43, and HXK31, crossed with OtsA, OtsB, WT ler, KIN10 RNAi, and KIN10 OE in cm. Hypocotyl length was measured 1 week after gemination. Bars that share the same letter are not significantly different (P<0.1), and error bars show SE.

S7: LEAF AREA AND MEJA



S 7: Relative growth rate of plants sprayed with MeJA

WT col and HXK31 plants were crossed with WT ler, KIN10 RNAi, and KIN10 OE. After 4 weeks, leaf area was measured, and after that plants were sprayed with MeJA. After 1 week leaf area was measured again, and from this RGR was measured (A2-A1)/A1. Bars that have the * sign are significantly different from the control treatment (P<0.1), and error bars show SE.