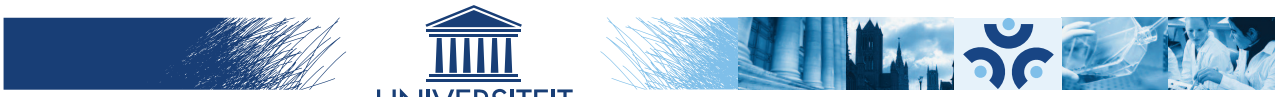

Leaf Growth Regulation by Sugars and Strobilurins in Arabidopsis

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Cause you only need the light when it's burning low…

- MICHAEL DAVID ROSENBERG

Summary

Leaves are one of the most important organs of the plant, providing energy and carbon for all plant organs during photosynthesis. Leaf growth is a tightly regulated process interconnecting genetic signaling pathways, developmental status and environmental signals. Diverse studies have demonstrated a close interaction between photosynthesis, sugar production, and leaf development. Several sugar-mediated signaling pathways regulating plant growth have been identified but how sugar signals control leaf growth is still unknown. Besides sugars, multiple other organic compounds are known to regulate plant growth. Strobilurins are agrochemical fungicides that also exert positive effects on plant physiology and growth. However, the underlying mechanisms responsible for the strobilurin-dependent growth promoting effects are still poorly understood.

First, to investigate how sugars regulate early leaf growth, we designed an experimental assay in which the sugar status is altered at a specific developmental stage during growth of Arabidopsis seedlings. At this stage, the third leaf is still fully proliferating and acts as a sugar-importing sink tissue. We found that sucrose increased final leaf size by promoting cell proliferation and postponing the transition to cell expansion. In addition, transfer to sucrose resulted in repression of plastome expression and chloroplast differentiation. A critical role for *GLUCOSE-6-PHOSPATE/PHOSPHATE TRANSPORTER2* (*GPT2*) in the sucrose-mediated effects on early leaf growth was demonstrated. *gpt2* mutant seedlings showed no stimulation of cell proliferation and no repression of chloroplast-encoded transcripts when transferred to sucrose.

Transcriptome analysis of the young growing leaves after transfer to sucrose not only resulted in a general repression of chloroplast transcription, but several other nuclear-encoded genes were affected and we selected nine for further characterization. Loss- and gain-of-function lines were generated and were preliminary screened for potential leaf phenotype. Interestingly, we found two genes, *DRM2* and *AT5G26260,* potentially involved in leaf growth regulation as their mutants produced larger and smaller mature leaves, respectively.

It is generally accepted that sugars are not only involved in primary metabolism but they can also act as signaling molecules, triggering important protein regulators to affect transcription or translation controlling growth. HEXOKINASE1 (HXK1) is a highly conserved glucose-signaling protein important for both growth promotion or inhibition depending on the environmental conditions, developmental status and plant species. A *hxk1* mutant in the Arabidopsis Col-0 background was selected and phenotyped in detail. We found that HXK1 plays a role both in cell proliferation and expansion during early development of young leaves. *hxk1* leaves contained more cells in early developmental stages and were less sensitive to sucrose to induce cell proliferation. In addition, we attempted to broaden the HXK1 protein complex by performing tandem affinity purification experiments from cell cultures continuously grown in the presence of sucrose, from sucrose-starved cells and from cells shortly after re-supplementation of sucrose. Surprisingly, proteins with diverse functions and subcellular localizations co-purified with HXK1 and we selected KINγ1 for further investigation.

Finally, we used a commercial available fungicide, Stroby, to study the positive effect of strobilurins on Arabidopsis plant growth. Stroby treatment resulted in larger rosettes and leaves due to an increase in cell proliferation. Furthermore, RNA sequencing analysis revealed differential expression of several sugar transporters, iron-related genes and the subgroup Ib basic helix-loop-helix (bHLH) transcription factors in Stroby-treated rosettes. One of these transcription factors, *bHLH039*, was found to be a key player in mediating Stroby-induced plant growth.

Samenvatting

Bladeren behoren tot de belangrijkste organen van planten. Ze zorgen voor energie en koolstof voor alle andere plant organen tijdens de fotosynthese. Bladgroei is een nauwkeurig gereguleerd proces dat genetische pathways, ontwikkelingsstadia en omgevingssignalen integreert. Verschillende studies hebben aangetoond dat er een nauwe interactie is tussen fotosynthese, suiker productie, en blad ontwikkeling. Verschillende suiker-gemedieerde signaleringswegen die de groei van planten reguleren, werden reeds geïdentificeerd maar het is nog steeds onbekend hoe suikers exact bladgroei controleren. Naast suikers zijn er tal van andere organische componenten die plantengroei kunnen reguleren. Zo bijvoorbeeld zijn strobilurins agrochemische fungiciden die de fysiologie en groei van planten positief kunnen beïnvloeden.

Om te bestuderen hoe suikers bladgroei reguleren, hebben we een experimentele assay ontwikkeld waarbij de suikerstatus kan gewijzigd worden gedurende een specifiek ontwikkelingsstadia van de Arabidopsis zaailing. Op dit tijdstip is het derde blad nog volledig aan het prolifereren en wordt het beschouwd als een ‘sink’ blad dat suikers importeert. We hebben aangetoond dat sucrose de uiteindelijke grootte van een blad kan doen toenemen door celproliferatie te promoten en de transitie naar celexpansie uit te stellen. Daarnaast resulteert de transfer naar sucrose in een algemene repressie van chloroplast expressie en differentiatie. *GLUCOSE-6-PHOSPATE/PHOSPHATE TRANSPORTER2* (*GPT2*) speelt een essentiële rol in de sucrose-gemedieerde effecten op vroege bladgroei. Mutante *gpt2* zaailingen vertoonden noch een stimulatie in de cel proliferatie noch een repressie van chloroplast-gecodeerde transcripten wanneer ze getransfereerd werden naar sucrose.

Transcriptoom analyse van jonge blaadjes na transfer op medium met sucrose resulteerde niet alleen in een algemene repressie van chloroplast transcriptie maar ook verschillende andere nucleair-gecodeerde genen worden beïnvloed. Hiervan werden negen genen geselecteerd voor verdere karakterisatie. Overexpressie lijnen en mutanten werden gegenereerd en preliminair gescreend voor een blad fenotype. Twee genen werden geïdentificeerd, *DRM2* en *AT5G26260*, die potentieel betrokken zijn in de regulatie van bladgroei aangezien hun mutanten respectievelijk grotere en

kleinere finale bladgroottes vertonen. Deze genen coderen voor eiwitten met een ongekende functie en waarvoor er nog geen rol in bladgroei regulatie is beschreven.
Tot op heden wordt het algemeen geaccepteerd dat suikers niet alleen betrokken zijn in primair metabolisme maar dat ze ook kunnen werken als signaal moleculen die belangrijke eiwit regulatoren activeren, en transcriptie en translatie beïnvloeden om groei te controleren. HEXOKINASE1 (HXK1) is een sterk geconserveerd glucose-signalerend eiwit dat belangrijk is in zowel groei-bevordering als groei-inhibitie afhankelijk van de omgevingscondities, ontwikkelingsstadia en plantensoort. We introduceerden een nieuwe *hxk1* mutant in de Arabidopsis Col-0 achtergrond en toonden aan dat HXK1 een centrale rol speelt in zowel celproliferatie als expansie tijdens de vroege ontwikkeling van jonge bladeren. *hxk1* blaadjes bevatten meer cellen vroeg in de ontwikkeling en zijn minder gevoelig aan sucrose in de stimulatie van celproliferatie. Vervolgens hebben we geprobeerd de eiwit complexen rond HXK1 verder te karakteriseren door ‘tandem affinitiy purification’ experimenten uit te voeren op celculturen die continu in de aanwezigheid van sucrose groeiden, die sucrose-gestarveerd werden en kort na sucrose toevoeging. Een groot aantal verschillende eiwitten werd geïsoleerd samen met HXK1 met verschillende functies en subcellulaire lokalisaties. We selecteerden daarvan KINɣ1 voor verder onderzoek.
In het laatste project maakten we gebruik van een commercieel verkrijgbaar fungicide, Stroby, om het positief effect van strobilurins op Arabidopsis plantengroei te bestuderen. Stroby behandeling resulteert in grotere rozetten en bladeren door een toename in cel proliferatie. Met behulp van ‘RNA-sequencing’ analyse vonden we dat verschillende suiker transporters, ijzer-gerelateerde genen en de subgroup Ib van de ‘basic helix-loop-helix (bHLH)’ transcriptiefactoren verschillend werden geëxpresseerd in Stroby-behandelende rozetten. Vervolgens konden we aantonen dat een van deze transcriptiefactoren, bHLH039, een sleutelrol speelt in de Stroby-gemedieerde bevorderende werking op plantengroei.

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List of Abbreviations

AAA	aromatic amino acid
ABA	abscisic acid
ABI	ABA-insensitive
ADP	adenosine diphosphate
ADG	ADP-glucose phosphorylase
ADPG	ADP-glucose
ATP	adenosine triphosphate
AOX	alternative oxidase
bHLH	basic HELIX-LOOP-HELIX
BR	brassinosteroids
cpDNA	chloroplast DNA
CAA	carbonic anhydrase
CAB	chlorophyll a/b binding protein
CBS	cystathionine β-synthase
CDK	cyclin-dependent kinase
CYC	cyclin
CRL	crumpled leaf
DEG	differentially expressed genes
DHAP	dihydroxy acetone phosphate
dNTP	deoxyribose nucleotide triphosphate
ETC	electron transport chain
F1,6BP	fructose-1,6-biphosphate
F6P	fructose-6-phosphate
FNR	ferredoxin-$NADP^+$ reductase
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
GBD	glycogen-binding domain
GFP	green fluorescent protein
GIN	glucose insensitive
GPT	GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER
GO	gene ontology
HXK1	hexokinase1
KM	kresoxim-methyl
LHC	light-harvesting complex
NADPH	nicotinamide adenine dinucleotide phosphate
NEP	nucleus-encoded polymerase
NO	nitric oxide
PC	plastocyanin
PCR	polymerase chain reaction
PGA	phosphateglycerate
PEP	plastid-encoded polymerase
PGM	phosphoglucomutase
PGI	phosphoglucoisomerase
PPi	inorganic pyrophosphate
PPR	pentatricopeptide-repeat
PPT	phosphoenolpyruvate translocator

PS	photosystem
PQ	plastoquinone
RBCS/L	small or large subunit of Rubisco
RuBisCO	ribulose-1,5-biphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-biphosphate
ROS	Reactive Oxygen Species
SAM	shoot apical meristem
SIG	sigma factor
Sucr	sucrose
Sucr6P	sucrose-6-phosphate
SPS	sucrose phosphate synthase
SNF1	Sucrose Non-Fermenting1
SnRK	SNF1-related kinase
SUC	sucrose transporters
T6P	trehalose-6-phosphate
T-DNA	transfer DNA
TEM	transmission electron microscope
TOR	Target Of Rapamycin
TPT	triose phosphate/phosphate translocator
uORF	upstream open reading frame
UDPG	UDP-glucose
Y2H	Yeast-2-hybrid

Scope and outline of the thesis

Leaf growth is a very complex process that is controlled by its genetic factors but also has to adjust to the environment which continuously changes the availability of water, nutrients and light. Many different molecular mechanisms regulating leaf growth have been characterized, demonstrating the involvement of a broad leaf growth-regulating network integrating environmental signals with development. The photosynthesis process in leaves produces one of the major energy sources as well as metabolic building blocks of plants: sugars. Sugars trigger conserved key regulators to signal the sugar status to the cell and monitor cellular homeostasis to modulate plant growth. However, still little is known about these sugar-responsive mechanisms and especially those that integrate the sugar status in a developmental context, such as the growth of young leaves. These so-called sink leaves depend on other leaves as sugar sources.

The sink-to-source regulation was one of the first questions we wanted to address during my PhD. I developed an experimental setup in which the availability of sucrose can be altered during the growth of the third leaf of Arabidopsis and this setup was used throughout my sugar-related research. Furthermore, one of the first indications that sugars can act as signaling molecules came from the *glucose insensitive2* (*gin2*) mutant, impaired in HEXOKINASE1 (HXK1) expression. HXK1 is a conserved glucose phosphorylating enzyme and is also known to act as glucose sensor. Major growth defects have already been described for the *gin2* mutant but it is still not clear how HXK1 exactly regulates plant growth. The second goal of my PhD was to further study the role of HXK1 during early leaf development. Finally, we selected from our transcriptome datasets several sucrose-responsive genes to find novel potential leaf growth regulators to broaden the growth-regulatory network.

Besides sugars, multiple other compounds are known to regulate leaf growth. We started a side project on different kinds of molecules, the strobilurin compounds. These compounds are worldwide used as agrochemical fungicides but also exert some interesting physiological effects resulting in enhanced crop yield. However, these positive effects on growth have only been reported in crops and the mode-of-

action was never investigated in detail. This triggered us to study the strobilurin-induced growth-promoting effects in Arabidopsis both at the cellular and molecular level to find genes putatively involved in the strobilurin-regulated control of plant growth.

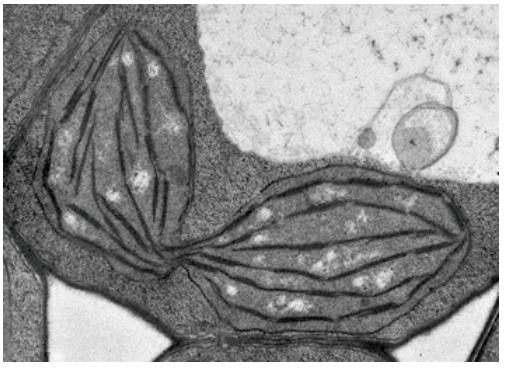
The first part of this PhD thesis consist of three introductory chapters. In the **first chapter**, I present a general introduction of sugars explaining the basic biochemistry processes by which sucrose is formed, how it is transported from source-to-sink organs and the transcriptional and translational responses known to be triggered by sugars to regulate plant growth. The **second chapter** is a review that highlights the important role of nuclear-encoded organellar proteins during plant development and the need of a more in depth characterization of these proteins at the cellular level. I finalize this introducing part with **Chapter 3**, which gives an overview of the current knowledge of strobilurins as fungicides and growth-enhancing compounds.

In the second part, my research on sugars and strobilurins is compiled in four result chapters:
In **Chapter 4**, we introduce an experimental *in vitro* setup in which sucrose can be altered during leaf development. We found that sucrose stimulates cell proliferation to increase final leaf size. Additionally, we demonstrated a central role of chloroplast differentiation, chloroplast transcription as well as the chloroplast-localized *GLUCOSE-6-PHOSPHATE TRANSPORTER2* (*GPT2*) during the sucrose-induced leaf growth.
In **Chapter 5**, nine sucrose-responsive genes were selected from the transcriptome analyses in Chapter 4, in which transcriptional responses induced or repressed by sucrose were determined in growing leaves, 3 hours and 24 hours after transfer to sucrose. Gain- and loss-of-function lines were generated and preliminary screened for a leaf phenotype.
We focus on the role of HXK1 during early leaf development in **Chapter 6**. A *hxk1* mutant in the Col-0 background is phenotyped at the rosette, leaf and cellular level. We demonstrate that *hxk1* is less sensitive to sucrose and HXK1 probably regulates sucrose-induced leaf growth independently from GPT2. To further elucidate sugar-dependent HXK1 protein partners tandem affinity purification experiments were done resulting in the identification of several interesting candidates.

Finally, in **Chapter 7** we investigated the effect of strobilurin application on the growth of Arabidopsis plants in soil. For this, we used the commercially available strobilurin, Stroby. Stroby treatment resulted in an significant increase in rosette size and transcriptome analysis on rosettes demonstrated a key role for the subgroup Ib basic HELIX-LOOP-HELIX transcription factors in the Stroby growth-promoting effect. Next, we further investigated how Stroby and loss-of-function of bHLH039 affects the development of young leaves and studied the underlying transcriptional responses during growth.

In the last part of this thesis, general conclusions and hypotheses are discussed. Future experiments are suggested to complete and elaborate the understanding of the function of GPT2 and HXK1 as well as the effects of strobilurins during plant growth.

Part 1:
Introduction



CHAPTER 1

Sugar signaling during Arabidopsis Leaf growth

Leaves as important photosynthetic sources

Leaf development in *Arabidopsis thaliana*

When plants are growing, the timely formation of leaves is crucial as they are the major source of carbon and energy that drives the growth of all other plant parts. In addition, leaves are the major sites of oxygen production fueling the atmosphere for all higher organisms on Earth. Leaves convert the energy from sunlight to chemical energy which is further processed to form carbohydrates from carbon dioxide during photosynthesis. Source leaves are the leaves that are actively performing photosynthesis producing their own energy (autotrophic) to grow and that also provide the energy for sink tissues (heterotrophic), such as flowers, roots and young proliferating leaves.

Arabidopsis dicot leaves arise as small cellular outgrowths from the shoot apical meristem (SAM) called leaf primordia. First, these primordia exclusively grow by cell proliferation (primary morphogenesis) leading to an exponential increase in the number of cells through cell division. Cell division or cell cycle progression is a strict time and space regulated process resulting in the correct separation of the genetic information in two daughter cells. The cell cycle consists of an S-phase during which DNA is replicated and the M-phase or mitosis to segregate the chromosomes. Both phases are preceded by G-phases that serve as control points and preparatory phases (Dewitte & Murray, 2003). The major regulators of the different cell cycle phases are the CYCLIN-DEPENDENT KINASES (CDKs) that interact with their regulatory cyclins (CYCs). The CDK/CYC complexes composition and activity is phase-specific, with A-type and D-type CYCs mainly involved in the G1-to-S transition and B-type CYCs mainly controlling the G2-to-M phase (De Veylder *et al*, 2007). In parallel, CDKAs are essential at both G1-to-S and G2-to-M phases and CDKBs mainly control the G2-to-M phase. Few days after emergence from the SAM, cells from the leaf primordia stop dividing and start to expand at the tip of the leaf. This progression has been well described for the third true leaf in the model organism *Arabidopsis thaliana* (Andriankaja *et al*, 2012). In optimal growth conditions all cells of the third leaf proliferate and remain relatively constant in size until nine days after stratification (9 DAS). At that time point cells at the tip of the leaf lose their capacity to

divide and a cell cycle arrest front moves down the leaf until 13 DAS, and, consequently, abruptly disappears around 14 DAS (Fig. 1). The leaf then enters its secondary morphogenesis phase and mainly grows by cell expansion (Andriankaja *et al*, 2012). Cell expansion is mainly driven by turgor pressure through enlargement of the vacuole and is also accompanied by endoreduplication (De Veylder *et al*, 2011). During this latter process, cells undergo endocycles in which their DNA is still duplicated but without subsequent cell division, increasing the nuclear ploidy levels. Next to these two major cellular processes driving growth, proliferation and expansion of the pavement cells (Fig. 2A), also the asymmetric division of meristemoids results in the formation of epidermal pavement cells and thus participates to final size control of the leaf. Besides generating pavement cells, meristemoids, dispersed in the leaf, are precursor cells for stomata guard cells (Geisler *et al*, 2000).

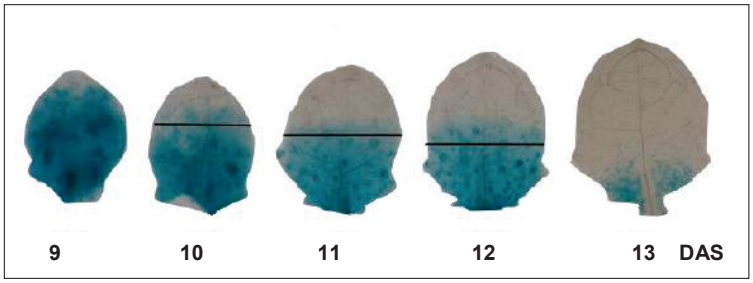


Figure 1. Expression pattern of CYCB1;1-D-box:GUS translational fusion protein during the transition phase of leaf development. Movement of the cell cycle arrest front from 9 until 13 days after stratification (DAS). Blue staining marks the dividing cells. From Andriankaja *et al*, 2012.

During development, leaf size is monitored to maximize the capacity to capture sunlight. Interestingly, the transition from cell proliferation to cell expansion in the third leaf was found to coincide with greening of the leaf, and, thus, the establishment of the photosynthetic machinery and chloroplast differentiation (Andriankaja *et al*, 2012). Just before the onset of cell expansion transcripts involved in photosynthesis are upregulated which is preceded by a strong upregulation of genes involved in chloroplast-to-nucleus or retrograde signaling (Andriankaja *et al*, 2012). Furthermore, inhibition of chloroplast differentiation was shown to affect the onset of the transition to cell expansion. These findings suggest the existence of a yet unknown signal from the chloroplasts that regulates the transition to cell expansion during leaf development.

The basics of photosynthesis

Higher plants are able to fixate carbon through three different variants of the photosynthetic process. These variants are mainly distinguished by the different carbon intermediates that are formed. Arabidopsis, rice, barley and wheat perform C3 photosynthesis that results in the formation of a three-carbon molecule. In contrast, C4 photosynthesis resulting in a four-carbon photosynthetic product happens in maize, sorghum and sugarcane and crassulacean acid metabolism, or CAM photosynthesis, also resulting in a four-carbon product, is common for plants living in arid areas. A central enzyme for these distinct photosynthetic processes is the ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) which generally catalyzes the reaction between ribulose-1,5-biphosphate (RuBP) and CO_2 but can also react with O_2. The C4 and CAM photosynthetic variants have developed specific strategies to improve the affinity of Rubisco to CO_2, by concentrating CO_2 in specific cells (bundle sheet cells versus mesophyll cells) or by opening the stomata apparatus only at night, respectively.

C3 photosynthesis in Arabidopsis leaves takes place in the chloroplasts of mesophyll cells, and consists of two types of reactions, the light-dependent reactions at the thylakoid membranes and the so-called dark reactions, the Calvin cycle or the reductive pentose phosphate cycle in the chloroplast stroma (reviewed by Stitt *et al*, 2010; Fig. 2B and C). During the light-dependent reactions, sunlight is converted in the chemical energy-storage molecule, adenosine triphosphate (ATP), and in nicotinamide adenine dinucleotide phosphate (NADPH), storage molecule of 'reducing' power, which are subsequently used in the Calvin cycle to fixate carbon dioxide to form 3-phosphoglycerate. The net photosynthesis reaction can be chemically described as follows: $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$.

The light-dependent reactions involve different protein complexes. In the first protein complex, photosystem II (PSII), light energy is used to convert water into oxygen and energized electrons (Barber 2012). This protein complex contains a photosynthetic reaction center to capture photons, consisting of two homologue proteins D1 and D2 and a special chlorophyll *a* pair (P680). Besides this center, PSII also contains a light-harvesting complex (LHC) with accessory light-harvesting

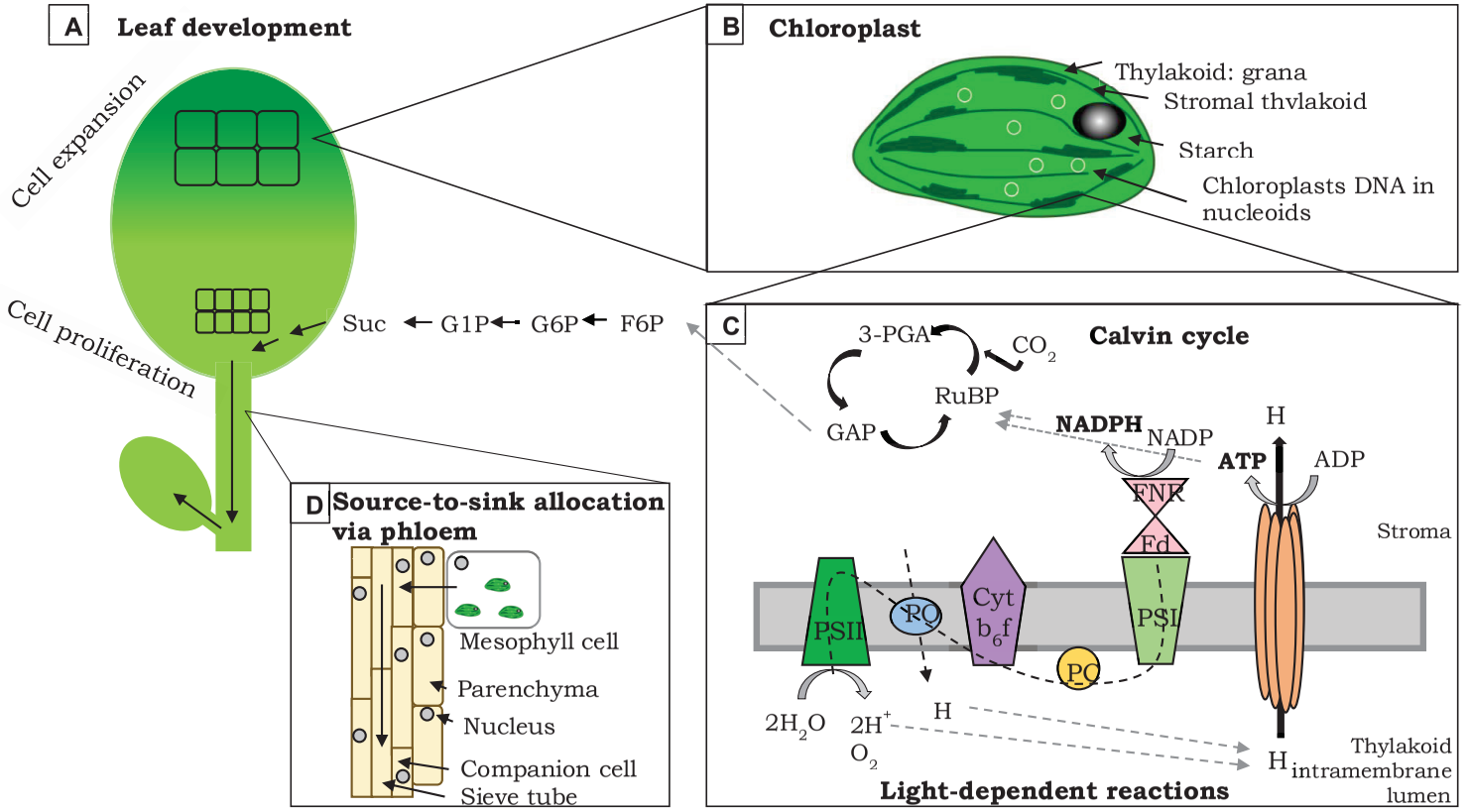


Figure 2. Photosynthetic-active source leaves and sugar transport to sink tissues. During leaf development, cells stop dividing and start to expand at the tip of the leaf (A). The transition to cell expansion coincides with the onset of chloroplast differentiation (B) and establishment of the photosynthesis machinery (C). (B) Schematic representation of a mature chloroplast. (C) Representation of the major steps of the electron transport chain during light-dependent reactions and carbon fixation in the Calvin cycle during photosynthesis. The end product of the Calvin cycle, glyceraldehyde-3-phosphate (GAP), is subsequently converted to fructose-6-phosphate (F6P), glucose-6-phosphate (G6P) and finally glucose-1-phosphate (G1P) in the cytosol or chloroplast stroma. In the cytosol G1P will be converted to sucrose to be metabolized, stored in the vacuole or transported. (D) Sucrose allocation through the phloem from the site of synthesis (source leaf) to sink tissues that are photosynthetic inactive.

proteins or antenna molecules with additional pigments, such as chlorophylls and carotenoids, to collect more light. The excited electrons are then transferred to PSI through the electron transport chain (ETC) resulting in several redox reactions. This occurs via a second protein complex, the cytochrome b_6f complex. The electrons are transported through a membrane soluble and mobile electron carrier, plastoquinone (PQ) for the transfer of electrons from PSII to cytochrome b_6f complex, and the luminal plastocyanin (PC) responsible for the transfer of electrons from the later complex to PSI (Fig. 2C). The cytochrome b_6f complex has an oxidoreductase activity and the transport of electrons establishes a proton gradient across the thylakoid membrane by pumping protons in the thylakoid lumen (Tikhonov, 2014). PSI also captures light to bring the electrons in a higher energy level to reach a 2Fe-2S-containing protein located in the chloroplast stroma, ferredoxin. The PSI complex consists of significantly more antenna proteins than PSII and also harbors a reaction center with special chlorophyll dimer that absorbs light at a higher wavelength (P700). Finally the electrons are transferred from ferredoxin to ferredoxin-$NADP^+$ reductase (FNR) to produce NADPH. The fourth and last protein complex that is part of the light-dependent reactions is the ATP synthase enzyme that generates ATP by using the energy from moving protons from the thylakoid lumen to the chloroplast stroma. Next, the reducing power of NADPH and the energy of ATP are used in the Calvin cycle (Fig. 2C). In the first step, CO_2 is incorporated in the five-carbon molecule RuBP by Rubisco to generate 3-phosphateglycerate (3-PGA). Consequently, ATP and NADPH from the light reactions are used to convert 3-PGA into the triose phosphate glyceraldehyde-3-phosphate (GAP). GAP is then used for other biosynthetic pathways or is used to regenerate RuBP to further fuel the Calvin cycle by many different enzymatic reactions and conversions. Many of these Calvin enzymes are post-translationally regulated by thioredoxin-dependent redox regulation (Buchanan & Balmer, 2005). In light, thioredoxin is reduced by the ferredoxin/thioredoxin reductase (FTR) when electrons are transferred from ferredoxin in the ETC. Consequently, the reduced thioredoxin can activate Calvin cycle enzymes and other enzymes by an interchange of reduced disulfide bridges that have stabilizing as well as regulatory roles.

From photoassimilate to starch and sucrose

The primary end product of photosynthesis is GAP which can be converted in sucrose in the cytosol or starch, as storage compound, in the chloroplast (reviewed by Stitt & Zeeman, 2012; Fig. 3). In both cellular compartments, GAP is converted in dihydroxy acetone phosphate (DHAP) by triose phosphate isomerases and both molecules are combined into a fructose-1,6-biphosphate (F1,6BP). F1,6BP is metabolized to fructose-6-phosphate (F6P) by F1,6BPase and then to glucose-6-phosphate (G6P) by phosphoglucoisomerase, PGI (Fig. 2). Next, G6P is reformed to glucose-1-phosphate (G1P) by phosphoglucomutase, PGM. G1P can be seen as the central carbohydrate precursor in both starch and sucrose biosynthesis pathways.

During the day or when enough sucrose is available, G1P reacts with ATP in the chloroplast to form ADP-glucose (ADPG). This rate-limiting step of the starch biosynthesis pathway is catalyzed by ADPG pyrophosphorylase (ADG) and liberates inorganic pyrophosphate (PPi). Consequently, ADPG is attached to the polysaccharide α-amylose by starch synthases and branching enzymes to form starch (Fig. 3). ADG is allosteric activated by 3-PGA and inhibited by inorganic phosphate (Pi) by which starch synthesis is therefore strictly coupled with the photosynthetic activity of the cell (Kleczkowski, 1999). Higher photosynthetic carbon fixation changes the production of the primary photoassimilates, such as 3-PGA, which on its turn can regulate ADG activity and thus coordinates photosynthesis with starch biosynthesis. Additionally, ADG is also redox-dependent post-translationally activated by light and sucrose through the reduction of a cysteine bridge (Hendriks *et al*, 2003). In the last decade, the classical model of starch biosynthesis in which ADG plays the essential role in producing ADPG from G1P, has been questioned. An alternative starch biosynthesis pathway has been suggested wherein sucrose synthase can also produce ADPG in the cytosol which is subsequently imported in the chloroplast (Bahaji *et al*, 2014; Munoz *et al*, 2005). Sucrose synthase is mainly involved in catabolizing sucrose into UDP-glucose (UDPG) and fructose.

At night, starch is degraded through a series of enzyme activities that phosphorylate the glucosyl residues of the starch polymers. The most important starch breakdown enzymes are β-amylases and debranching enzymes, and the main end products are

maltose and glucose. However, the major transported sugar is sucrose which can be synthesized from the starch breakdown products or is directly formed from G1P in the cytosol (Fig. 3). For the latter reaction, GAP has to be exported in the cytosol via triose phosphate/phosphate translocator (TPT) in exchange of Pi, where it will be converted to G1P. G1P is first transformed into UDPG by UDP-glucose pyrophosphorylase and, then, combined with F6P to produce sucrose-6-phosphate (Sucr6P) by sucrose phosphate synthase (SPS). Finally, sucrose is formed by sucrose-6-phosphatase and is loaded into the phloem or imported in the vacuole where it is converted by vacuolar invertases to glucose and fructose for storage. Sucrose can also be further metabolized to its hexose products in the cytosol (Fig. 3).

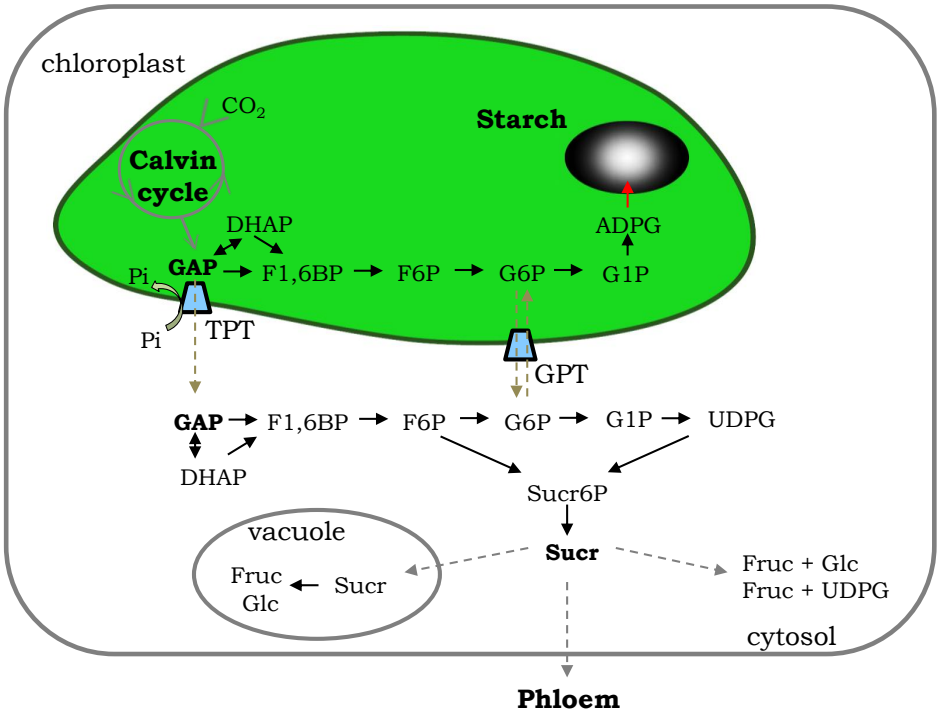


Figure 3. Starch and sucrose biosynthesis in leaves. During the day, triose phosphates GAPs are formed by the Calvin cycle in the chloroplast stroma. GAPs are converted by consecutive enzymatic reactions into starch in the chloroplast or are transported via triose phosphate/phosphate translocator to the cytosol where they are converted into sucrose. Sucrose is transported via the phloem to sink tissues, metabolized to its hexose products by cytosolic invertases, generating fructose and glucose, or sucrose synthase, generating fructose and UDP-glucose, or is stored in the vacuole, where it is also converted into its hexose products. GAP, glyceraldehyde-3-phosphates; DHAP, dihydroxy acetone phosphate; F1,6BP, fructose-1,6-biphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; GPT, glucose phosphate/phosphate transporter; UDPG, UDP-glucose; ADPG, ADP-glucose; Sucr6P, sucrose-6-phosphate.

Loading in the phloem ensures sucrose transport to sink tissues, such as flowers, roots or immature leaves that do not produce their own sugars. As in the starch synthesis pathway, sucrose biosynthesis is strictly regulated by two central enzymes, F1,6BPase and SPS (Huber & Huber, 1996). F1,6BPase is inhibited by the metabolite fructose-2,6-biphosphate (F2,6BP) which is formed when 3-PGA/Pi levels increase. SPS is allosterically activated by G6P and inhibited by Pi.

Photorespiration

The photorespiration process is a scavenging process of the plant to recover carbon and remove waste products when Rubisco interacts with O_2 instead of CO_2 (Peterhansel et al, 2010). The oxygenase activity of the enzyme is increased by warm temperatures that raise the O_2/CO_2 ratio and leads to the formation of 2-phosphoglycolate. This molecule can inhibit different enzymes involved in the Calvin cycle and therefore has to be metabolized by the cell. Three different organelles, chloroplast, peroxisome and mitochondria, are involved in the photorespiration pathway that converts 2-phosphoglycolate to glycolate and finally into glycerate which can again be used in the Calvin cycle. Glycolate is transported to the peroxisome where it is converted to glyoxylate by glycolate oxidase and subsequently to glycine by aminotransferases. Subsequently, glycine is imported in mitochondria were it gets decarboxylated and deaminated by glycine decarboxylase, and further processed to serine. Serine then goes back to the peroxisome and is converted to hydroxypyruvate and, finally, glycerate that is phosphorylated into 3-PGA in the chloroplasts. The respiration process therefore results in a net loss of some of the energy produced by photosynthesis. However, this process helps the plant cell to recycle phosphoglycolate, a harmful waste product generated by the oxygenation of RuBP and which cannot be used by the Calvin cycle.

Chloroplasts, the photosynthetic factories of the cell

Chloroplast structure and composition

Photosynthesis occurs in specialized cellular organelles, chloroplasts (Fig. 2B). Meristematic cells usually contain 10-20 colorless proplastids and light is needed to convert them to functional chloroplasts, with the synthesis of chlorophyll from its precursor, protochlorophyllide. If no light is available the proplastid will develop in an etioplast with accumulation of protochlorophyllide and the formation of a prolamellar body, i.e. a crystalline membranous structure. During leaf development, the number of chloroplasts per mesophyll cell increases to several hundred (Sakamoto *et al*, 2008). This number is positively correlated with light quantity, and, thus, with sugar production, and also with cell area during cell expansion, as shown in isolated spinach leaf discs (Possingham & Smith, 1972; Pyke & Leech, 1992). A mature chloroplast is typically lens-shaped and surrounded by an envelope consisting of two membranes: a permeable outer membrane and a more impermeable inner membrane separated by an inter-membrane lumen. The fluid inside the chloroplasts is called stroma and contains the necessary enzymes for the Calvin cycle, chloroplast ribosomes and the chloroplast DNA (cpDNA) or plastome. In addition, the stroma also contains starch granules and plastoglobuli, i.e. small lipid vesicles. Besides the envelope, chloroplasts also contain a third membrane structure called the internal thylakoid membranes organized in unstacked stromal thylakoid membranes or stroma lamellae and in stacked discs called grana (Fig. 2B). PSI is mainly located in the stromal thylakoid membranes, whereas PSII is located in the grana (Pribil *et al*, 2014). This different distribution of the PSs allows for a correct absorption of light during changes in light conditions. PSII has higher excitation energy than PSI and absorbs more light under high light conditions resulting in phosphorylation of specific components of the LHCII by the kinase THYLAKOID ASSOCIATED KINASE STATE TRANSITION 7 (STN7) (Bellafiore *et al*, 2005; Kouril *et al*, 2005). Phosphorylated LHCII will be decoupled from PSII and migrate to the PSI. Under low light conditions PSI absorbs photons faster than PSII, LHCIIs are not phosphorylated and move to the grana where they interact with PSII.

Photosynthetically-active chloroplasts contain 2000 to 3000 different proteins which are in majority encoded by the nucleus. However, most of the core components of the photosynthetic complexes are encoded by the cpDNA. Table 1 gives an overview of the major photosynthetic complexes and where their proteins are encoded. cpDNA is arranged together with DNA-binding proteins in complexes called nucleoids, as in prokaryotes, which are normally attached to the chloroplast envelop. A full developed chloroplast contains around 100 copies of its chloroplast DNA and this number of cpDNA copies remains stable during leaf development, although some contrasting results have been reported suggesting a decrease in cpDNA copy number during development (Golczyk *et al*, 2014; Oldenburg & Bendich, 2015; Zoschke *et al*, 2007). The Arabidopsis cpDNA is 154 Kbp in size and harbors 130 genes, from which 54 genes encode photosynthesis-related proteins, 45 encode rRNAs or tRNAs that mediate chloroplast translation and 31 are involved in chloroplast transcription (Lopez-Juez & Pyke, 2005; Sato *et al*, 1999). Besides these proteins, the plastome also encodes the large subunit of Rubisco, *RBCL*, whereas the small subunit, *RBCS,* is encoded by the nuclear genome. Because most of the chloroplast proteins are encoded by the nuclear genome, the nucleus and chloroplasts need to communicate to coordinate their gene expression. Nuclear control of chloroplast differentiation is called anterograde signaling, whereas chloroplast signaling to the nucleus to link nuclear gene expression with the chloroplast developmental state and/or activity is referred to as retrograde signaling (Inaba *et al*, 2011). To date, many different retrograde pathways have been found to affect nuclear expression, involving intermediates of the tetrapyrrole biosynthesis, Reactive Oxygen Species (ROS) and redox state changes during the light-dependent reaction of photosynthesis (Inaba *et al*, 2011). Furthermore, plastid gene expression and several metabolites, such as methylerythritol cyclodiphosphate, involved in isoprenoid biosynthesis, have been described to act as retrograde signals under stress conditions (Inaba *et al*, 2011). Interestingly, also sugars have been hypothesized to act as important signals during acclimation to high light (Hausler *et al*, 2014). A central player of all retrograde signaling processes is the ABA-INSENTIVE 4 (ABI4) transcription factor, involved in many different aspects during plant growth and tightly interconnected with sugar signaling during seedling development (Leon *et al*, 2012).

Chloroplast transcription

As in prokaryotes, genes on the cpDNA are organized in operons, i.e. several genes are co-transcribed from one promoter. Transcription of the plastome genes depends on two RNA polymerases, a bacterial type polymerase which is encoded by the plastome, the plastid-encoded polymerase (PEP) and a bacteriophage type polymerase which is encoded by the nuclear genome, the nucleus-encoded polymerase (NEP; Table 1). PEP consists of four core subunits, rpoA, rpoB, rpoC1 and rpoC2, encoded by the chloroplast and an additional sigma factor (SIG) encoded by the nucleus. NEP consists of one subunit encoded by three different genes: *RpoTp* for chloroplasts, *RpoTm* for mitochondria and *RpoTmp* targeted to both organelles (Hricova *et al*, 2006). Generally, early during chloroplast development, NEP is mainly active resulting in the expression of the *rpoB* operon encoding the PEP subunits *rpoB*, *rpoC1* and *rpoC2*, next to house-keeping genes (such as *clpP* and *accD*) and proteins involved in plastid translation (ribosomal proteins and RNAs). Subsequently, PEP is formed and is mainly involved in the transcription of genes encoding photosynthesis-related proteins. However, recently, NEP and PEP were described to be active at all stages during leaf development (Börner *et al*, 2015). SIG factors determine which genes are transcribed by PEP and several specific and redundant roles for individual SIG factors have been described (Chi *et al*, 2015). The Arabidopsis genome encodes six SIG factors (SIG1-6) which facilitate promoter specificity (Kanamaru & Tanaka, 2004). SIG1 and SIG5 transcripts are induced by red and blue light (Onda *et al*, 2008). SIG1 was shown to accumulate during seedling development, primarily binds to the *psaA* promoter and is suggested to be involved in the acclimation of PSI and PSII to changes in light intensities (Hanaoka *et al*, 2012). SIG5 mediates blue-light induced transcription of the *psbD/C* operon by binding the blue-light responsive promoter and is activated upon various stresses, such as high light, salt and low temperature (Nagashima *et al*, 2004; Onda *et al*, 2008). *sig5* mutants are embryo lethal and a pivotal role of SIG5 in reproduction has been reported (Yao *et al*, 2003). Additionally, it has been shown that SIG5 mediates the circadian regulation of plastome expression (Noordally *et al*, 2013). SIG2 is involved in the basic transcription of the large (*atpI/H/F/A*) and the small (*atpB/E*) operons coding for the subunits of ATP synthase (Malik Ghulam *et al*, 2012). *sig2* mutants exhibit a chlorophyll deficient phenotype with pale green leaves due to impaired

chloroplast differentiation demonstrating a central role of SIG2 during chloroplast development (Kanamaru *et al*, 2001; Privat *et al*, 2003).

Table 1. Major protein complexes involved in photosynthesis, chloroplast transcription or translation encoded by the nuclear genome (N) or chloroplast DNA (C).

Protein complex	Function	Genes	DNA
PHOTOSYSTEM II	Reaction Center	psbA	**C**
		psbD-F	**C**
		psbI	**C**
		psbL-N	**C**
	Assembly	psbJ	**C**
		psbK	**C**
		ycf9	**C**
	Oxygen splitting	psbO-R	**N**
	LHC	psbB-C	**C**
		Lhcb1-6	**N**
PHOTOSYSTEM I	Reaction Center	psaA-C	**C**
		psaD-H	**N**
		psaI-J	**C**
		psaK-L	**N**
		psaO	**N**
	LHC	Lhca1-4	**N**
	Assembly	ycf3-4	**C**
Ferredoxin/FNR		petG	**N**
		petH-I	**N**
Plastocyanin		petF	**N**
Cytochrome b6/f	Cyt f	petA	**C**
	Cyt b6	petB	**C**
	Fe-S cluster	petC	**N**
	quinone-binding protein	petD	**C**
	quinone function	petE	**C**
ATPase	α-subunit	atpA	**C**
	β-subunit	atpB	**C**
	γ-subunit	atpC	**N**
	δ-subunit	atpD	**N**
	ε-subunit	psaE	**C**
	Additional subunits	atpF-I	**C**
		atpG-H	**N**
		atpI	**C**
NADH complex	NADH dehydrogenase	ndhA-J	**C**
		psbG	**C**
RuBisCO	Small subunit	rbcS	**N**
	Large subunit	rbcL	**C**
PEP	Transcription	rpoA-B	**C**
		rpoC1-C2	**C**
NEP	Transcription		**N**
Ribosomal RNA	Translation	rrn4.3-rrn23	**C**
tRNA	Translation	trnA-V	**C**
Ribosomal proteins	Translation	rps2-19	**C**
		rpl2-36	**C**
Others	Intron splicing	matK	**C**
	Protease	clpP	**C**
	Acetyl-CoA carboxylase subunit	accD	**C**
	?	ycf1-2	**C**
	?	ycf5-6	**C**
	?	ORF77	**C**

Furthermore, transcript levels of several tRNA genes were reduced in the *sig2* mutant and one of these genes, $tRNA^{GLU}$, is involved in the first steps of tetrapyrrole biosynthesis (Hanaoka *et al*, 2003; Privat *et al*, 2003). *SIG2*-dependent regulation of $tRNA^{GLU}$ is also suggested to function in the NEP-to-PEP transcriptional switch because of the $tRNA^{GLU}$-mediated inhibition of NEP transcription (Chi *et al*, 2015; Hanaoka *et al*, 2005; Kanamaru *et al*, 2001). T-DNA insertion lines disrupting SIG3 or SIG4 function revealed regulation of *psbN* and *ndhF* transcription, respectively (Favory *et al*, 2005; Zghidi *et al*, 2007). The last and sixth sigma factor, SIG6, is involved in early chloroplast development in cotyledons (Ishizaki et al, 2005) as well as in retrograde signaling together with SIG2 (Woodson et al, 2013). SIG6 interacts with the pentatricopeptide-repeat (PPR) protein DELAYED GREENING 1 (DG1) (Chi *et al*, 2010). PPR proteins are part of a huge family of 450 proteins in Arabidopsis and are mainly involved in post-transcriptional regulation of chloroplast RNAs, i.e. splicing, cleavage and editing of the single-stranded mRNAs. Some PPRs also have a role in translation (Schmitz-Linneweber & Small, 2008).

In conclusion, a correct transcription of chloroplast genes is essential for normal plant growth and any changes in the function of the major chloroplast transcription regulators result in severe growth problems.

Sugars as metabolites and signaling molecules

Sugar transporters during source-sink translocation

The major end products of photosynthesis are triose phosphates which are converted to the disaccharide sucrose, transported via the phloem to sink tissues that do not photosynthesize or that are photosynthetically active but still need a net import of sugars to sustain their growth. First, sucrose has to be translocated through apoplastic transport, via plasmodesmata and/or through sucrose transporters from the mesophyll cells to the phloem parenchyma cells and, finally into the phloem companion cells and to sieve element complexes from the phloem where the actual long-distance transport happens (Fig. 2D). Several sugar and sugar phosphate transporters exist on the chloroplast envelop and plasma membrane to facilitate

translocation of metabolites in the cytosol and loading of sucrose in the phloem for long-distance transport (Fig. 4).

During the day, sucrose is formed from triose phosphates that are exported in the cytosol via the triose phosphate/ phosphate translocator (TPT; Schneider *et al*, 2002), whereas at night sucrose is produced from the starch degradation products, glucose and maltose, which are exported via the plastidic glucose transporter pGlcT and a maltose exporter MEX1 (Cho *et al*, 2011; Niittyla *et al*, 2004). Interestingly, *tpt-2 and pglct* single and *pglct/tpt-2* double mutants do not exhibit a growth phenotype, whereas *mex1*, *pglct-1/mex1* and *tpt-2/mex1* mutants are severely reduced in growth (Cho *et al*, 2011). This reduction in growth can be rescued with sucrose treatments, indicating that the export of starch breakdown products is essential for plant growth. In accordance, *tpt* mutants combined with starch biosynthesis mutants (*adg1-1*, *pgm1* and *pgi1-1*) also show growth retardation when grown under high light which could also be recovered by sucrose or glucose treatments (Heinrichs *et al*, 2012; Schmitz *et al*, 2014; Schmitz *et al*, 2012). These observations suggest the existence of redundantly acting sugar phosphate translocators and/or involvement of sugar signaling pathways. Interestingly, a strong induction of the *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER2* (*GPT2*) was found in the starch biosynthesis single mutants and in the *adg1-1/tpt-1* double mutant (Kunz et al, 2010). However, the *adg1-1/tpt-2/gpt2-1* triple mutant demonstrated a similar growth recovery upon sugar treatment as the double mutants, ruling out the involvement of GPT2 in rescuing the growth retardation after sugar treatments (Heinrichs *et al*, 2012). GPT2, together with its homologue GPT1, belongs to a class of phosphate translocators localized at the inner membrane of chloroplasts and involved in the G6P/Pi transport between the cytosol and chloroplast stroma (Flugge, 1999; Knappe *et al*, 2003). These glucose phosphate translocators were originally suggested to act as the main transporters involved in the import of G6P in non-green plastids of heterotrophic tissues as start product of the oxidative pentose phosphate pathway (Kammerer *et al*, 1998). This pathway is the major source of NADPH used in several biosynthetic pathways such as the fatty-acid and amino acid biosynthesis as well as in maintaining the cellular redox homeostasis in tissues that do not photosynthesize (Kruger & von Schaewen, 2003). The essential function of GPT1 in providing G6P for NADPH production was indeed demonstrated by the impaired embryo development of *gpt1* RNAi and T-DNA insertion knock-out mutants (Andriotis *et al*, 2010;

Niewiadomski *et al*, 2005). Contrastingly, under optimal growth conditions *gpt2* T-DNA insertion mutants are undistinguishable from wild type plants in growth, germination and seed yield (Athanasiou *et al*, 2010; Niewiadomski *et al*, 2005). However, *gpt2* mutants are unable to undergo dynamic acclimation, i.e. changing their photosynthetic capacity when light conditions are altered (Athanasiou *et al*, 2010). Furthermore, it was suggested that GPT2 functions as a buffer for changes in carbon metabolite contents during this photosynthetic acclimation. Mature *gpt2* mutant plants were found to accumulate less starch but synthesize the same amount of sucrose during photosynthetic acclimation (Dyson *et al*, 2015). Furthermore, mature leaves of *gpt2* mutants strongly induce the expression of photosynthesis-related genes and plastid-encoded genes and also the levels of trehalose-6-phosphate (T6P) are increased compared with wild-type plants (Dyson *et al*, 2015). Besides these findings, *gpt2* seedlings were also found to be delayed in cotyledon greening during seedling development (Dyson *et al*, 2014).

Recently, two members of a novel class of sucrose transporters, the SWEET transport family, were shown to facilitate the export of sucrose in the apoplast of the mesophyll or phloem parenchyma cells (Chen *et al*, 2012; Eom *et al*, 2015). SWEET11 and SWEET12 act redundantly because only the double mutant showed reduced growth, enhanced levels of starch, sucrose and hexoses in the leaves as well as defects in root growth.

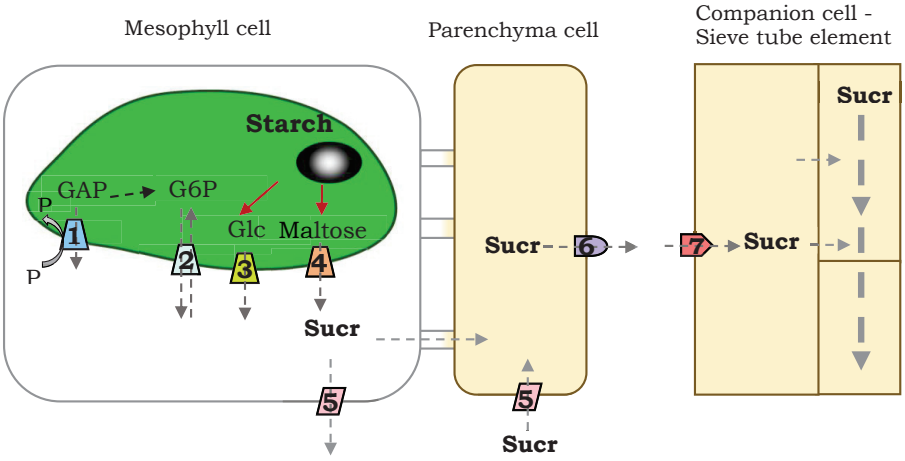


Figure 4. Sugar and sugar phosphate transporters in source leaves and transport of sucrose to phloem. 1, triosephosphate/phosphate translocator (TPT); 2, glucose-6-phosphate transporter (GPT); 3, plastidic glucose transporter pGlcT; 4, maltose exporter MEX1; 5, sucrose/H+ symporter SUC2/SUT1; 6, SWEET transporter; 7, sucrose/H+ symporter SUC2/SUT1. GAP, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; Glc, glucose; Sucr, sucrose.

Next, the apoplastic sucrose has to be actively taken up by the phloem companion cells to be loaded in the phloem. This active transport across the plasma membrane is accomplished by the sucrose/H^+ symporter SUC2/SUT1. Similar to the *sweet11 sweet12* double mutant, the *suc2* mutant is dwarfed and accumulates sugars in the leaves (Srivastava *et al*, 2008; Truernit & Sauer, 1995). In Arabidopsis, there are nine putative sucrose transporters (SUC1-9) located on the plasma membrane, vacuole or chloroplast membranes, but *SUC6* and *SUC7* were suggested to be non-functional pseudogenes (Kuhn & Grof, 2010; Sauer *et al*, 2004).

Finally, sucrose has to be transported from the phloem to enter the sink cells. How this transport is facilitated remains unknown, but both a role of the active SUC/H^+ symporters and complete symplastic transport have been suggested (Srivastava *et al*, 2008). Thus, when arriving to the sink tissue, sucrose enters the sink cell via plasmodesmata or it is first relieved in the apoplast through sucrose transporters. In the apoplast, sucrose is converted to glucose and fructose by cell wall invertases and these hexoses are subsequently imported via hexose transporters in the sink cells. When arriving in the sink cell, sucrose or its hexose products, glucose and fructose, can influence transcription, translation, cell division and expansion to coordinate growth and development with the environment.

Sugar-mediated transcriptional responses

Besides their role as carbon building blocks of primary and secondary metabolism in both source and sink cells, sugars have been widely accepted to act as signaling molecules (Lastdrager *et al*, 2014; Rolland *et al*, 2006; Smeekens *et al*, 2010). Plants have to adjust their growth with a changing environment and sugars can act as endogenous signals to link plant development with carbon and energy availability. Extensive efforts have been done in unravelling the sugar-mediated regulation of gene expression depending on environmental and developmental influences.

Generally, it is well-established that low sugar levels induce the transcription of photosynthesis-related and sugar metabolizing genes, whereas sugar accumulation triggers sugar storage and utilization, through increased expression of genes

encoding key enzymes in starch biosynthesis and sucrose metabolism (Koch, 1996; Pego *et al*, 2000; Sheen, 1990). Furthermore, it is well-known that high sugar levels repress photosynthesis genes encoded by the nuclear genome as well as by the cpDNA (Krapp *et al*, 1993; Paul & Foyer, 2001; Sheen, 1994). However, it remains challenging to decipher whether the transcriptional effects are a consequence of the changing cellular metabolite levels or are due to sugars as signaling molecules. In the last decade, numerous studies have been analyzing the short-term transcriptional responses in Arabidopsis with different sugar starvation protocols or short-term sugar treatments. As illustrated in Table 2, a wide variety of sugars, sugar concentrations, plant material, growth conditions, duration of the treatment were tested. The sugar-mediated transcriptional responses are generally involved in the same cellular processes. Sugar treatment commonly results in the repression of genes involved in the light-dependent reactions and the Calvin cycle of photosynthesis, of starch degradation, plastid protein synthesis, autophagy, and sucrose metabolism, such as invertases. Oppositely, sugar treatment generally induces expression of genes involved in respiration and major synthetic pathways (such as nucleotide, amino acid, protein, starch and cell wall synthesis), sucrose breakdown and cell cycle. Additionally, also chloroplast specific sugar-responsive effects were demonstrated. Usadel and co-workers reported a decreased expression of genes involved in chloroplast biogenesis, chlorophyll biosynthesis and photosynthesis in the light when sucrose is produced (Usadel *et al*, 2008). Other studies demonstrated specific repression of plastid gene expression upon different sucrose and glucose treatments (Gonzali *et al*, 2006; Osuna *et al*, 2007; Price *et al*, 2004).

In conclusion, irrespective of the used tissues, sugars and concentrations, sugar treatment always elicits major transcriptional responses in which repression of photosynthesis-related gene is commonly found, highlighting the importance of sugar-mediated feedback regulation of photosynthesis.

Table 2. Studies reporting sugar-mediated transcriptional responses using different tissues, sugars, sugar concentrations and treatment conditions.

Plant material	Sugar treatment	Harvesting time point	Ref.
Whole seedlings grown in liquid culture for 7 d	2 days C-starvation, + 15 mM sucr	30 min, 3 h	Osuna *et al* (2007)
5-weeks old rosettes	Dark	End of night, 4-8-24-48 h extended night	Usadel *et al* (2008)
Whole seedlings grown in liquid culture for 7 d	2 days C-starvation, + 100 mM glc	3 h	Blasing *et al* (2005)
Whole seedlings grown in liquid culture for 7 d – continuous light	1 d C-starved, + 167 mM glc	2, 4, 6 h	Li *et al* (2006)
Whole seedlings grown in liquid culture for 5 d	1 d C-starved in dark, + 167 mM glc	3 h	Price *et al* (2004)
Leaf segments of 6-weeks old plants	100 mM sucr	16 h	Muller *et al* (2007)
Stem-cell-like cell suspension culture	1 mM glc/sucr/fru	1 h	Kunz *et al* (2014)
Whole seedlings grown in liquid culture for 4 d - dark	90 mM sucr	6 h	Gonzali *et al* (2006)
Cell suspension cultures	Sucr-starvation	48 h	Contento *et al* (2004)
Whole 14 d-old seedlings grown on plates	Dark for 48 h, + 30 mM sucr	8 h	Thum *et al* (2004)

Sugar-mediated translational control

Besides carbon sources, cells also need to synthesize novel proteins to be able to grow. Several links between the cellular sugar status and mRNA translation or protein synthesis have been reported. Price and colleagues found that treatment with cycloheximide, a protein synthesis inhibitor, impaired part of glucose-mediated upregulation of gene expression (Price *et al*, 2004).

One of the best characterized translational control systems by sugars is the sucrose-induced repression of translation of S1-type basic region-leucine zipper (bZIP) transcription factors (Rook et al, 1998). This repression is facilitated by the presence of a conserved upstream open reading frame (uORF) in the 5' untranslated region of the S1-class of bZIP genes (bZIP1/2/11/44 and 53) (Wiese *et al*, 2004). The uORF encodes an attenuator sucrose-control peptide that probably arrests the ribosome and stops further translation when sucrose levels accumulate (Hummel *et al*, 2009; Rahmani *et al*, 2009).

Besides translation initiation, also control of translation termination has been suggested through glucose-induced expression of the eukaryotic RELEASE FACTOR1-2 (eRF1-2). Seedlings overexpressing eRF1-2 are hypersensitive to high glucose concentrations, resulting in altered germination and seedling development compared to wild-type plants (Zhou *et al*, 2010).

Another interesting link between sugars and translation comes from studies where polysomal mRNA and total mRNA were extracted and compared to distinguish between translational and transcriptional control. In a first study, sucrose-starved cell suspension cultures were used and polysomal and total mRNA were hybridized to oligonucleotide microarrays (Nicolai *et al*, 2006). This analysis revealed a general translational repression after sucrose starvation. Furthermore, it was shown that cytosolic polysome loading correlates well with the sucrose content of Arabidopsis rosettes (Pal *et al*, 2013). A more recent study demonstrated that sucrose treatment of Arabidopsis seedlings in the light increased the polysomal occupancy of ribosomal protein mRNAs (Gamm *et al*, 2014).

Taken together, although an obvious interconnection between sugar status and protein synthesis is suspected, still little is known about which proteins are affected by sugars and how sugars exactly control *de novo* protein synthesis. At least part of the sugar-induced transcriptional responses might indirectly result from prior sugar-induced regulation at the mRNA translational level.

Sugar regulation of cell division and expansion

When sucrose enters the sink cell, it can also be metabolized to its hexose products by cytosolic invertases or sucrose synthases, or it is stored in the vacuole, where it can also be converted into its hexose products via vacuolar invertases. These metabolic steps are essential in providing the carbon and energy sources for primary and secondary metabolism. When enough carbon is available, cells can divide and molecular mechanisms signal the carbon status to the cell cycle machinery. Sugar-mediated regulation of the cell cycle was reported both during the primary cell cycle control point, the G1 phase as well as during the G2-to-M phase. An induction of the expression of *CYCD2s* and its partner *CDKA;1*, and of *CYCD3s* was seen 2 h and 6 h, respectively, after addition of sucrose or glucose to sugar-depleted Arabidopsis

seedlings grown in liquid growth medium (Riou-Khamlichi *et al*, 2000). Expression of *CYCD3;1* is associated primarily with proliferating tissues and its repression might be an important factor in mitotic cell cycle exit and onset of cellular expansion and differentiation (Dewitte & Murray, 2003). Furthermore, sucrose was found to induce *CYCP2;1* expression (Peng *et al*, 2014). P-type cyclins are mainly active in proliferating tissues mediating the G2-to-M transition (Torres Acosta *et al*, 2004).
Next to its role in cell cycle control, sugars are main metabolic building blocks of the cell wall as well as storage compounds in the vacuole and therefore important in regulating cell expansion (Wang & Ruan, 2013). In the vacuole, sucrose is converted to its hexose products which are osmotically active components that lowers the osmotic potential. When the osmotic potential in the vacuole is lower than the osmotic potential outside the vacuole, water is imported, increasing cell turgor pressure, which leads to cell expansion by cell wall extension (Sturm & Tang, 1999). Also during the latter process, sucrose plays an important role by delivering UDP-glucose for cellulose biosynthesis via sucrose synthase enzymes (Baroja-Fernandez *et al*, 2012).

In summary, sugars are important determinants of cell division and expansion. However, the exact mechanisms how sugars are signaled are still poorly understood but in the last decades several master regulators have been characterized in more detail.

Key sugar signaling regulators and plant growth

To elicit the sugar-mediated transcriptional and translational responses, sugars have to be sensed by specific proteins that can link the environmental conditions with plant development. Several conserved regulatory networks have been described that can couple the cellular sugar status with growth, to adjust organ development depending on the fluctuations in the environment resulting in changing nutrient and energy sources. To date, three conserved sugar signaling mechanisms with partially linked and independent signaling pathways are considered to be central in this cellular sugar responsive network: i) the Sucrose Non-Fermenting1 (SNF1) - Related Kinase (SnRK1), which is inhibited when sugars accumulate, and its inhibitor trehalose-6-phosphate (T6P), ii) the Target Of Rapamycin (TOR) protein kinase and iii) the

glucose sensor Hexokinase1 (HXK1). SnRK1, TOR and HXK1 are independently regulated by glucose.

i) *SnRK1/T6P-mediated signaling and growth*

Arabidopsis SnRK1 and its orthologues, existing in all eukaryotes, exhibit a conserved heterotrimeric structure consisting of one catalytic α-subunit and two regulatory ɣ- and β-subunits. In yeast and animals these orthologues are called SNF1 and AMPK, respectively. AMPK is allosterically activated by binding of AMP to the energy-sensing ɣ-subunit under stress conditions (Xiao *et al*, 2011). Also in higher plants, SnRK1 is known to be activated by different stresses such as darkness, hypoxia, sugar depletion or energy deficiency, but like yeast SNF1, SnRK1 is not responsive to AMP (Baena-Gonzalez *et al*, 2007; Ramon *et al*, 2013; Emanuelle *et al*, 2015). Upon activation, SnRK1-mediated signaling triggers an enormous amount of transcriptional and post-transcriptional responses, such as phosphorylation of transcription factors, to maintain cellular homeostasis. SnRK1 will enhance energy-producing catabolic processes and prevent energy-consuming biosynthetic pathways, resulting in a change in the metabolism to monitor plant development. For example, SnRK1 signaling regulates sucrose and starch biosynthesis through phosphorylation, and thereby inactivation, of sucrose phosphate synthase *in vitro* (Sugden *et al* 1999) and through redox activation of ADG via trehalose-6-phosphate (T6P) (Kolbe *et al*, 2005).

In Arabidopsis, different genes encode the α-, ɣ- and β-subunits of the SnRK1 complex (Polge & Thomas, 2007; Emanuelle *et al*, 2016). Two homologous genes encode the catalytic subunits, AKIN10 and AKIN11, which contain a conserved serine/threonine kinase and a C-terminal domain for interaction with its regulatory subunits (Fig. 5) (Baena-Gonzalez *et al*, 2007). Three subgroups of ɣ-like proteins are described in Arabidopsis based on the structure similarity with ɣ-subunits of yeast SNF1 and mammalian AMPK: AKINβɣ, AKINɣ and PV42-type proteins. These proteins all contain four highly conserved tandem repeated cystathionine β-synthase (CBS) domains (Fig. 5). Howver, only the AKINβɣ was shown to function in the heterotrimeric SnRK1 complex, whereas the AKINɣ regulatory subunit cannot interact with the β-subunits and does not affect expression of typical SnRK1 target genes, such as the dark-induced *SEN1/DIN1*, *DIN6* and *DIN10* (Emanuelle *et al* 2015;

Ramon *et al*, 2013). AKINβγ has a conserved CBS tandem repeat domain but also a glycogen-binding domain (GBD), which is one of the three domains that specifies β-subunits. The other two domains of the β-subunits are an internal kinase-interacting domain (KIS) and an ASC (association with SNF1 complex) domain to interact with ɣ-subunits (Fig. 5). Plants have two groups of β-subunits, AKINβ1 and 2 proteins that contain all three GBD, KIS and ASC domains and an atypical AKINβ3 protein with a truncated structure, i.e. no GBD domain (Fig. 5).

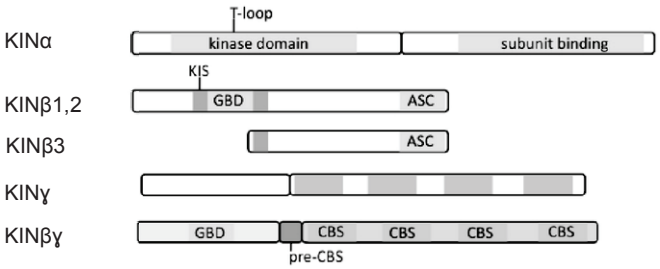


Figure 5. Structure and domain composition of the plant SnRK1 subunits. The GBD (light grey) in the β subunits (KINβ1 and KINβ2) overlaps with the kinase-interacting sequence (KIS) domain (dark grey). Taken from Ramon *et al*, 2013.

Interestingly, the subunits of SnRK1 have distinctive roles in growth regulation. Overexpression of the catalytic α-subunit KIN10 confers enhanced tolerance to nutrient starvation, and delayed flowering and onset of senescence, whereas *kin10 kin11* double mutants exhibit severe growth defects (Baena-González *et al*, 2007). The homologous proteins PV42a and PV42b have been implicated in reproductive development during male gametogenesis and pollen tube formation (Fang *et al*, 2011). Expression of both PV42-members is reduced in a mutant for HISTONE ACETYLTRANSFERASE 1 (HAC1). *hac1* mutants are insensitive to high sugar concentration, show delayed flowering and produce less siliques and seeds (Heisel *et al*, 2013).

Trehalose-6-phosphate (T6P) is synthesized from UDP-glucose and G6P by the T6P synthase1 (TPS1) enzyme in the first step of trehalose biosynthesis. Subsequently, T6P can be further converted to trehalose by T6P phosphatase (TPP). Trehalose is a glucose disaccharide present in low micromolar concentrations in the cell. In

Arabidopsis, 11 genes are encoding for proteins with TPS or TPS-like structures and 10 genes are encoding TPPs (Delorge *et al*, 2015; Vandesteene *et al*, 2012). It has long been suggested that only TPS1 is enzymatically active, but this has recently also been demonstrated for TPS2 and 4 (Delorge *et al*, 2015). High concentrations of trehalose inhibit growth and this effect is attributed to an accumulation of T6P and a decrease in cellular G6P levels (Schluepmann *et al*, 2012; Schluepmann *et al*, 2004). Contrastingly, T6P is also indispensable for growth as *tps1* mutants show a developmental arrest during embryo maturation, i.e. impairment in the transition of cell division-to-expansion (Eastmond *et al*, 2002). Plants that overexpress TPS1, have increased T6P levels (and decreased G6P levels) and can make better use of the exogenously supplied sugars resulting in increased shoot branching, small dark green leaves and early flowering (O'Hara *et al*, 2013; Schluepmann *et al*, 2003). In accordance, reduced T6P levels (and increased G6P levels) through TPP overexpression, result in growth repression *in vitro* because the exogenously supplied sugars are not efficiently used. So, T6P acts as a sugar signal that monitors the supply and utilization of sugars during plant growth and development (Paul, 2008). A T6P-sucrose nexus model has been proposed, postulating that T6P acts both as a signal of sucrose availability and as a negative feedback regulator of sucrose levels. By this, optimal sucrose concentrations for the cell and in function of the developmental stage of the plant are maintained (Yadav *et al*, 2014; Lunn *et al*, 2014). T6P levels increase when seedlings are treated with sucrose (Lunn *et al*, 2006), whereas high T6P levels transiently decreases sucrose levels (Figueroa *et al*, 2016).

To date, it is known that T6P and SnRK1 signaling closely interact to regulate sugar metabolism and plant growth (Baena-Gonzalez, 2010; Emanuelle *et al*; Lastdrager *et al*, 2014; O'Hara *et al*, 2013). T6P, and also G6P, appear to inhibit SnRK1 activity, although, the exact mechanism is still not known (Nunes *et al*, 2013; Toroser *et al*, 2000; Zhang *et al*, 2009). In potato tubers, T6P activates ADP-glucose pyrophosphorylase (ADG), involved in the rate-limiting step of starch biosynthesis, in a SnRK1-dependent manner (Kolbe *et al*, 2005). Furthermore, Arabidopsis double *kin10 kin11* mutants are unable to mobilize their starch at the end of the night (Baena-Gonzalez *et al*, 2007). These findings suggest a role of both SnRK1 and T6P during starch biosynthesis and remobilization. However, recently it was shown that increased T6P levels in plants expressing T6P synthase gene (*otsA*) under control of

an ethanol-inducible promoter did not influence the redox state of ADG, whereas strach degradation was repressed (Martins *et al,* 2013).
Interestingly, also a connection between SnRK1 and the S1-class of bZIP transcription factors has been demonstrated in growth regulation. Expression of both KIN10 and the bZIP transcription factors synergistically activated the expression of overlapping target genes indicating that they partially mediate SnRK1-signaling (Baena-Gonzalez *et al*, 2007; Tome *et al*, 2014). In addition, both KIN10 and bZIP11 overexpression lines are resistant to high growth-inhibitory trehalose concentrations (Delatte *et al*, 2011). Furthermore, SnRK1 is also able to phosphorylate bZIP63, a member of the C-class bZIPs which forms dimers with S1-class bZIP members (Mair *et al*, 2015).

T6P has been reported to also directly influence growth. A direct link between T6P and cell growth was demonstrated by the transcriptional analysis of transgenic potato plants with reduced T6P levels, showing down-regulation of genes involved in cell proliferation and growth as well as up-regulation of a transcript corresponding to a CDK inhibitor (p27KIP1; Debast *et al*, 2011). Contrastingly, SnRK1-dependent phosphorylation of two other cell-cycle inhibitors and p27KIP1 homologs, KIP-RELATED PROTEIN6 (KRP6) and KRP7, resulting in inactivation and maintained cell proliferation, has been described (Guerinier *et al*, 2013).

In conclusion, SnRK1 and T6P play essential and contrasting roles in regulating plant growth and development. Increased sugar levels result in higher T6P levels which inactivate SnRK1s to promote growth, whereas low sugar levels induces SnRK1-signaling to mediate growth inhibition and energy conservation.

ii) *Glucose-activated TOR signaling*

In animals, AMPK interacts with another conserved regulator TOR that is activated by hormones, nutrients and energy to coordinate growth with environmental signals. The direct connection between SnRK1 and TOR is not yet elucidated in plants, although similar target genes are oppositely affected. Unlike animals that contain two TOR complexes, mTORC1 and mTORC2, only one gene in Arabidopsis encodes for a TOR protein which forms the TOR complex with additional proteins that are however still not fully characterized (Xiong & Sheen, 2014; Xiong & Sheen, 2015). In

Arabidopsis, TOR contains a conserved C-terminal serine/threonine kinase and can associate with REGULATORY ASSOCIATE PROTEIN OF TOR1/2 (RAPTOR 1/2) and LETHAL WITH SEC-13 PROTEIN8 1/2 (LST8 1/2) (Xiong & Sheen, 2014). The importance of TOR in plant growth has first been demonstrated by the embryo lethality of T-DNA insertion mutants disrupting TOR expression (Moreau *et al*, 2012). TOR is mainly expressed in proliferating tissues, such as meristems, but not in differentiated cells. Knock-down mutants of TOR via RNA interference also show reduced growth, whereas TOR overexpression increases growth and seed production (Deprost *et al*, 2007). Downregulation of TOR also resulted in accumulation of starch, amino acids and lipids, indicating a central role of this protein in primary metabolism. Also disruption of one of the putative *LST8* genes, *LST8-1*, leads to arrested growth with increased levels of starch and amino acid (Moreau *et al*, 2012). In root meristems, TOR is activated by photosynthesis-derived glucose to mediate root growth (Xiong *et al*, 2013).

Glucose-induced TOR activity mediates many different transcriptional responses to stimulate biosynthetic pathways and inhibit catabolic processes to sustain growth. The TOR-activated processes are protein synthesis, DNA synthesis, cell cycle, primary and secondary metabolism, whereas TOR-inactivated processes are autophagy and protein degradation (Xiong & Sheen, 2014). TOR affects different aspects of protein synthesis, such as rRNA expression, the expression of ribosomal proteins and polysome loading of the mRNAs (Deprost *et al*, 2007; Rexin *et al*, 2015). TOR signaling thus controls cell proliferation both at the transcriptional as well as translational level. In animals, TOR regulates protein synthesis by phosphorylating the ribosomal protein S6 kinase1 (S6K1) that, on its turn, phosphorylates the ribosomal protein S6 (RPS6) to promote mRNA translation (Magnuson *et al*, 2012). Also in Arabidopsis, RAPTOR1 interacts with TOR as well as with S6K1 to activate the latter protein (Mahfouz *et al*, 2006). Furthermore, upon activation by auxin, TOR phosphorylates S6K1, which phosphorylates an eukaryotic initiation factor (eIF3h) to promote initiation of translation and polysome loading (Schepetilnikov *et al*, 2013). At the transcriptional level, glucose-induced TOR and E2Fa target genes largely overlap. E2Fa is a conserved transcription factor that, upon release of the RETINOBLASTOMA-RELATED1 (RBR1) protein induces the expression of S-phase specific genes involved in cell cycle progression and DNA replication (Polyn *et al*,

2015). Moreover, TOR phosphorylates E2Fa directly and *e2fa* mutants are compromised in the glucose-induced activation of root meristems (Xiong *et al*, 2013). In conclusion, TOR is a master regulator of cell proliferation, growth and metabolism regulating transcription and translation in response to environmental signals.

iii) Glucose-mediated signaling through HXK1

In Arabidopsis, the HXK1 protein belongs to a family of six members with three HXKs (HXK1-3) that have the capacity to phosphorylate glucose, and three HXK-Like proteins (HXL1-3) that lack this catalytic function and show around 50% amino acid sequence similarity with HXK1 (Karve *et al*, 2008). All these members are ubiquitously expressed in different plant organs except for HXL3, which only shows expression in flowers. Five of the six proteins, HXK1-2 and HXL1-3, have a predicted N-terminal mitochondria anchor domain, and this localization was confirmed with C-terminal GFP fusion proteins in different plant species, such as in Arabidopsis, tobacco, pea and tomato (Balasubramanian *et al*, 2007; Granot, 2008; Karve *et al*, 2008). HXK3 is predicted to have a chloroplast transit peptide and chloroplast stromal localization of other HXK orthologues has also been verified in moss (Olsson *et al*, 2003), tobacco (Giese *et al*, 2005), tomato (Kandel-Kfir *et al*, 2006), rice (Cho *et al*, 2006a) and Arabidopsis (Granot, 2008).

HXK1 is a conserved enzyme responsible for the phosphorylation of glucose in the first step of glycolysis but it is already known for a long time to also have glucose-sensing capacity (Jang & Sheen, 1994). Interestingly, this dual functionality of HXK1 has recently been structurally explained (Feng *et al*, 2015). The glucose-sensor role was first suggested by the study of *HXK1* antisense and overexpression lines showing hyposensitivity and hypersensitive growth responses to high glucose concentrations, respectively (Jang *et al*. 1997). Later, the *glucose insensitive2* (*gin2*) mutant, which can grow on high concentrations of glucose while wild type plants are developmentally arrested, display repression of cotyledon expansion and chlorophyll accumulation as well as anthocyanin production, was identified in the Landsberg *erecta* (L*er*) background (Fig. 4; Moore *et al*, 2003). Furthermore, in the *gin2* mutant, glucose does not repress the expression of nuclear-encoded photosynthesis-related genes, such as chlorophyll a/b binding proteins (*CAB)*, the small subunit of Rubisco

(*RBCS*), carbonic anhydrase (*CAA*) and sedoheptulose-biphosphatase (*SBP*) (Moore *et al,* 2003). To exclude that these effects were metabolic, inactive HXK1 alleles, which lack the catalytic phosphorylation domain, were expressed in the *gin2* mutant background (Moore *et al*, 2003). When grown under high light, the *gin2* mutant normally displays general growth defects such as small and dark green leaves with delayed senescence, a shorter root system and inflorescences with fewer flowers. The leaf expansion as well as the glucose-mediated repression of gene expression was restored by the HXK1 mutant alleles not affecting glucose phosphorylation activity and, thus, G6P levels, suggesting a HXK1-mediated signaling effect on growth (Moore *et al*, 2003). Contrastingly, over-expression of HXK1 in Arabidopsis and its orthologues in tomato and rice (Cho *et al*, 2009; Dai *et al*, 1999; Kelly *et al*, 2012; Kelly *et al*, 2014) led to growth inhibition with reduced chlorophyll accumulation and accelerated senescence, supporting a role of HXK1 in growth promotion as well as growth inhibition depending on the environmental conditions.

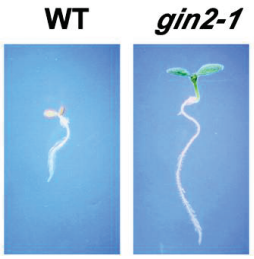


Figure 4. *Arabidopsis* HXK1 has a predominant role in glucose signaling. *Arabidopsis gin2-1* mutants and wild-type (WT) in the *Ler* background. Plants were grown on 6% glucose. Picture taken from Moore *et al*, 2003.

The essential role of HXK1 in mediating the repression of photosynthesis genes suggests that at least part of the HXK1 proteins have to be present in the nucleus. A nuclear HXK1 complex has been isolated by proteomics based on nuclear fractions (Cho *et al*, 2006b). Two unconventional HXK1-interacting proteins, the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B), were found in the nucleus in a complex together with HXK1 to regulate CAB expression. Similar to the *gin2* mutant, the *vha-b1* and *rpt5b* mutants are insensitive to the repression of cotyledon expansion, chlorophyll accumulation, true-leaf development and root elongation by high glucose concentrations and showed the same growth defects as *gin2* when grown in soil (Cho *et al*, 2006b).

Glucose-mediated HXK1 signaling is closely linked with hormonal signaling, highlighted by the glucose hyper- and hyposensitive responses of many different hormone biosynthesis and signaling mutants (Leon & Sheen, 2003; Rolland *et al*, 2006). Glucose and ethylene signaling act antagonistically as shown by the glucose hypersensitivity of the ethylene signaling mutants, *etr1* and *ein2* (Zhou et al, 1998) and by the glucose-mediated and HXK1-dependent degradation of ETHYLENE-INSENSITIVE3 (EIN3), a key transcriptional regulator in ethylene signaling (Yanagisawa *et al*, 2003). Also abscisic acid (ABA) synthesis and signaling mutants (*abi*) are insensitive to glucose, and could not be restored by HXK1 expression, indicating that ABA signaling works downstream of the HXK1-dependent signaling (Arenas-Huertero *et al*, 2000). Furthermore, clear interaction with auxin and cytokinin during shoot induction has been shown (Moore *et al*, 2003) and, recently, HXK1-signaling was reported to depend on the presence of brasissinosteroids during lateral root development and hypocotyl elongation (Zhang & He, 2015).

This close interconnection of the HXK1-mediated signaling pathway with hormones and the HXK1-dependent regulation of photosynthesis gene expression emphasizes a pivotal but yet unclear role of HXK1 in monitoring plant organ development.

Conclusions

Sugars are essential and central players during plant growth and development. They not only act as structural components of the cell wall, of DNA, RNA and proteins but are also the major energy storage molecules of the cell. Sugar metabolism has been an important target in metabolic engineering to enhance yield. Several efforts have been made in improving source-to-sink regulation, the photosynthetic carbon fixation by Rubisco, simplifying the photorespiration pathway and increasing photosynthesis to boost crop productivity and yield (Raines, 2011). However, sugars also elicit cellular signaling pathways integrating environmental conditions with plant development. Sugar signaling pathways closely interconnect with hormonal signaling and plant growth-regulatory networks in controlling and fine-tuning growth. Substantial progress has been made to understand the molecular mechanisms but it still remains challenging to uncouple their metabolic and signaling functions. This is

because the sugar status is tightly linked with the environment continuously changing the photosynthetic capacity of source leaves which also alters the sugar allocation to sink tissues. Different master regulators involved in sugar signal transduction have been identified involved in reprogramming gene expression and protein synthesis to maintain growth. However, the sugar-responsive underlying molecular mechanims are still poorly understood, especially those integrating development such as early leaf growth and the sink-to-source transition. Obviously, a better understanding of these pathways and how they exactly affect development is necessary to further unravel the complexity of plant growth.

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CHAPTER 2

Plants Grow with a Little Help of their Organelle Friends

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ABSTRACT

Chloroplasts and mitochondria are indispensable for plant development. They not only provide carbon sources and energy to cells, but have evolved to become major players in a variety of processes such as amino acid metabolism, hormone biosynthesis and cellular signalling. As semi-autonomous organelles, they contain a small genome that relies mostly on nuclear factors for its maintenance and expression. An intensive cross-talk between the nucleus and the organelles is therefore essential to ensure proper functioning and the nuclear genes encoding organelle proteins involved in photosynthesis and energy production are obviously crucial for plant growth. Organ growth is determined by two main cellular processes, i.e. cell proliferation and subsequent cell expansion. To date, the role of the nuclear-encoded organelle proteins in plant growth has not been investigated in much detail. Here, we review how Arabidopsis plant growth is affected in mutants involved in organelle biogenesis and physiology. Our findings indicate a clear role for organellar proteins in plant organ growth, primarily during cell proliferation. We therefore encourage researchers to extend their phenotypic characterisation beyond macroscopic features in order to get a better view on how chloroplasts and mitochondria regulate the basic processes of cell proliferation and expansion, essential to drive growth.

Introduction

Plant growth and development are complex and multifactorial traits, intensively studied from the molecular to the whole plant level. Plant organ growth relies on two main processes, cell proliferation and cell expansion, which are influenced by specific and interconnected regulatory networks. Many regulators of root, leaf, flower or seed growth have been functionally characterized (for reviews, see Gonzalez & Inze, 2015; O'Maoileidigh *et al*, 2014; Satbhai *et al*, 2015). In particular genes have been studied that, when mutated or ectopically expressed, positively affect plant growth.

During plant growth, cellular energy is provided as sugars and ATP by two key organelles in plant cells, chloroplasts and mitochondria. Both organelles are surrounded by an external and internal envelope membrane and also contain characteristic internal membranes, called thylakoids in chloroplasts and cristae in mitochondria. The latter membranes harbour the protein complexes necessary for photosynthesis and oxidative phosphorylation to produce sugars and energy in chloroplasts and mitochondria, respectively. Next to these indispensable cellular

functions, both organelles are involved in many other essential metabolic functions, such as amino acid and hormone biosynthesis (Berkowitz *et al*, 2016; de la Torre *et al*, 2014). Both chloroplasts and mitochondria contain an organelle genome that is largely dependent on nuclear factors for their organisation and expression. It is clear that a correct communication between nucleus and organelles is necessary to ensure proper organelle functioning. The proteins encoded by the nucleus but having a function in the organelles have strict transport mechanisms, and nucleus and organelles interact with each other by nucleus-to-organelle (anterograde) and organelle-to-nucleus (retrograde) signalling.

Organ growth is a tightly controlled process and a correct number, size, distribution and differentiation of chloroplasts and mitochondria is necessary at any developmental stage to support growth. The effect of disturbed organelle biosynthesis, division, function or physiology on cell proliferation and expansion during organ growth has not been studied intensively. A major hurdle is that generating mutations in organelle genomes is challenging, although feasible (Chuah *et al*, 2015; Daniell *et al*, 2002), and inducing these mutations is likely to be lethal. On the other hand, many nuclear-encoded organelle proteins have been characterised for their roles in organelle functioning and further knowledge on how the mis-expression of these proteins affects plant development is instrumental to understand the role of mitochondria and chloroplasts in organ growth.

In this review, we highlight how dysfunctional organelles affect growth at the organ and cellular level. First, we evaluate the differential expression of nuclear genes encoding organellar proteins during leaf and root development to illustrate their potential role during cell proliferation or expansion. We then compile available mutant phenotypic data on genes involved in organelle biogenesis and gene expression as well as in organellar carbon or amino acid biosynthesis, hormonal regulation of organelles and organelle-nucleus signalling. Since the functions of mitochondria and chloroplasts are broad, we restricted this review to studies addressing growth phenotypes at the cellular level, with a focus on leaf and root development in Arabidopsis. Finally, we plead for a more thorough evaluation and characterisation of growth phenotypes in order to get a better grasp on the involvement of organelle function on growth.

Differential Expression during Organ Development

Plant growth can be improved or compromised when cell proliferation and/or cell expansion is affected during development due to mutation in genes expressed specifically during these developmental phases (Gonzalez *et al*, 2012). The expression pattern of nuclear genes encoding organelle proteins has been analysed to some extent over different plant developmental phases or upon treatments (Andriankaja *et al*, 2012; Berkowitz *et al*, 2016). During development the expression pattern varies and subsets of these genes appear to be specifically more expressed in leaf, root or flower or at specific time points. For example, it is well established that during germination most nuclear genes encoding mitochondrial proteins are upregulated (Howell *et al*, 2007). On the other hand, transcript levels of nuclear genes encoding photosynthesis-related proteins are generally upregulated during early leaf development (Andriankaja *et al*, 2012). The establishment of the photosynthetic machinery and chloroplast differentiation happens just before the onset of cell expansion during early leaf development (Andriankaja *et al*, 2012), suggesting that a plastid-derived retrograde signal is necessary for triggering cells to stop dividing and start to expand.

In order to get a better view on how genes encoding organellar proteins are expressed during organ development, we investigated how their transcript levels change in publicly available developmental datasets. We used two datasets, one during leaf and one during root development, allowing to follow the expression profile of these genes in proliferative, transitioning and differentiating or expanding tissue (Andriankaja *et al*, 2012; Birnbaum *et al*, 2003). The expression profiles were obtained from samples harvested over six consecutive days during early leaf development and three stages of root development, and differentially expressed genes (DEGs) were identified (Fig. 1, Supplemental Table S1). The nuclear genes encoding organellar proteins chosen for this analysis were selected with SUBA3 software and were either experimentally confirmed by GFP fusions or correspond to an organellar protein identified by MS/MS analysis of organelles (Tanz *et al*, 2013). Although these selection criteria are stringent, we are aware that some false positives, not specifically located in chloroplast or mitochondria, might be included in this list since the organelle localisation of some of the genes is based on a prediction.

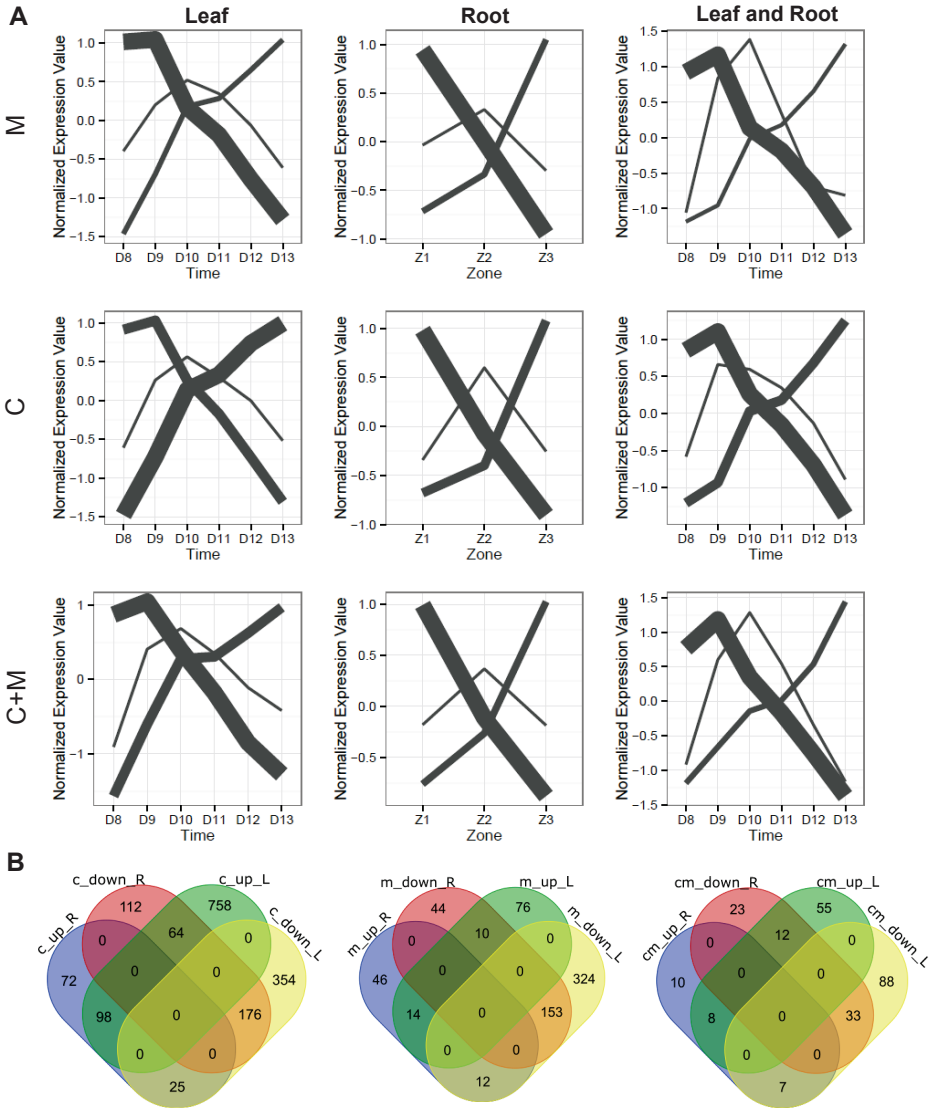

Figure 1. Expression profiles of nuclear genes encoding organellar proteins during Arabidopsis leaf and root development. A, Relative expression of genes encoding proteins localized to mitochondria (M), chloroplasts (C) or both (C+M). For leaf development, we selected a microarray analysis performed over six consecutive days during early development of the third true leaf, i.e. 8 to 13 days after stratification (DAS) (Andriankaja *et al*, 2012). This dataset encompasses the developmental phases during which the third leaf exclusively grows through cell proliferation (8-9 DAS) and a transitioning phase (10-11 DAS) to a cell-expansion based growth (12-13 DAS). For the expression patterns during root development, a microarray analysis of a total of 15 different zones of the root corresponding to different tissues and developmental stages was used (Birnbaum *et al*, 2003). The expression profile in the different root tissue types were averaged for each gene and correspond to three zones (Z) of root development: Z1 corresponding to the root tip where cells are proliferating, Z2 in which cells are transitioning to expansion and Z3 consists of fully expanded and differentiated cells. The expression profile of DEGs was normalised using MeV software (www.tm4.org) and subsequently CAST clustered (Cluster Affinity Search Technique, using Pearson correlation at a threshold of 0,8) according to their specific profile over the developmental zones. Each line represents the expression pattern of a group of DEGs and the thickness of each line correlates with the number of DEGs. B, Venn diagrams illustrating the amount of DEGs presented in (A) and their overlap over leaf (L) and root (R) development of genes encoding proteins localized to mitochondria (M), chloroplasts (C) or both (C+M).

Currently, in Arabidopsis, a total of 1095 proteins are annotated to localize in mitochondria and 2720 proteins in chloroplasts, of which 381 proteins are assigned to both organelles.

54% (596 out of 1095) of the genes encoding mitochondrial proteins are differentially expressed in the leaf dataset compared to 28% (307 out of 1095) in the root dataset. For genes encoding plastid proteins, 59% (1475 out of 2511) are differentially expressed in the leaf dataset and 25% (615 out of 2511) in the root dataset. The genes encoding proteins targeted to both organelles are included in these percentages, but taken separately 56% (203 out of 364) are differentially expressed in the leaf dataset and 27% (99 out of 364) in the root dataset (Fig. 1).

In both the leaf and root developmental dataset a general decrease in expression of nuclear genes encoding mitochondrial proteins was found when cells transition from cell proliferation to cell expansion (83% and 68% of DEGs, respectively; Fig. 1). Conversely, the major trend for genes encoding plastid proteins was an increase in expression when cells enter the expansion phase in leaves (63% of DEGs; Fig. 1), but most DEGs in roots had a decrease in expression during development (57% of DEGs; Fig. 1). Furthermore, several genes encoding proteins targeted to both organelles show a decrease in expression over development both in the leaf and root dataset (63% and 69% of DEGs). A considerable amount of DEGs shows a similar expression profile during leaf and root development. GO enrichment of the differentially expressed genes in both leaves and roots reveals an important role of mitochondrial gene expression regulation during organ development (Supplemental Table S1). For the gene sets encoding chloroplast proteins that are upregulated during development a clear enrichment is seen for GO categories related to photosynthesis and water transport. Water transport through chloroplast membranes is necessary for differentiation of proplastids in meristematic tissue to have fully functional chloroplasts in expanding and maturing tissue. For the genes encoding chloroplast proteins downregulated during development several categories related to enzymes involved in amino acid metabolism, such as glutamine, aspartate and lysine, are enriched (Supplemental Table S1). Finally, also genes encoding proteins involved in redox reactions were repressed or upregulated during development.

In total, 449 genes showed similar expression profiles during root and leaf development, being up-or downregulated in the same direction when proliferating cells gradually start to expand. A literature survey revealed that for 80 of these genes a mutant growth phenotype has been reported. For example, the *GENOMES UNCOUPLED5* (*GUN5*) gene, is upregulated during leaf development, and encodes a subunit of the Mg-chelatase involved in the first steps of chlorophyll biosynthesis. *gun5* mutants are small, pale green and impaired in chloroplast-to-nucleus signalling (Mochizuki *et al*, 2001). The increase in expression during development is indicative of the onset of photosynthesis in leaves and underlines the importance of retrograde signalling during plant development. In contrast, the expression level of several genes is downregulated when transitioning from cell proliferation to cell expansion. For example, the gene encoding the PENTATRICOPEPTIDE REPEAT (PPR) protein known as RNA-EDITING INTERACTING PROTEIN1 (RIP1), involved in mitochondrial and chloroplast RNA editing, is down-regulated. *rip1* plants are dwarfed and sterile (Bentolila *et al*, 2012). A second example of a gene exhibiting downregulation over development encodes the DNA gyrase protein A (GyrA), homologous to the bacterial topoisomerase that induces topological changes to DNA during replication and transcription (Wall *et al*, 2004). *gyrA* plants are embryo lethal illustrating the importance of this protein in plant development.

In summary, a large amount of nuclear encoded genes encoding organellar proteins are differentially expressed over development. Except for genes encoding photosynthesis-related and chloroplast proteins in leaves, the majority of genes are downregulated when cells transition from cell proliferation to cell expansion. This trend points towards an important role of organellar proteins in the support of cell proliferation during early organ development. At the same time, the onset of photosynthesis when cells start expanding and differentiating in leaves highlights the active role of chloroplasts in cell expansion (Andriankaja *et al*, 2012).

Organelle Biogenesis, Division and Gene Expression

When organ primordia develop, chloroplasts differentiate from proplastids in the shoot meristems in the light (Fig. 2). Light triggers in the proplastid the formation of a

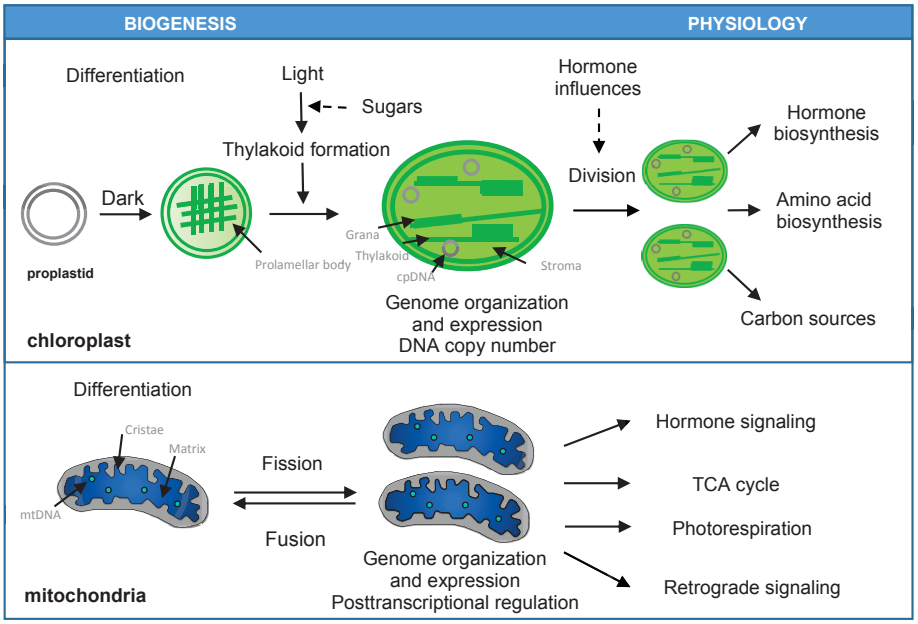

Figure 2. Overview of the mitochondria and chloroplast characteristics. Schematic representation of chloroplast differentiation from proplastid stage and the mitochondrial fission process. Different aspects of the organelle biogenesis and physiology that will be discussed in this review are highlighted.

preliminary thylakoid structure, the prolamellar body, which further develops in the typical thylakoid membranes of a differentiated chloroplast.

The protein complexes necessary to perform the light reactions of photosynthesis, photosystem I and II (PSI and PSII) with their respective light-harvesting complexes I and II (LHCI and LHCII), are localized in specific regions on these thylakoid structures (Eberhard *et al*, 2008). To absorb efficiently light energy, a coordinated formation of the photosynthetic pigments, chlorophyll and carotenoids, with the photosynthetic complexes is needed. In contrast, mitochondria are formed independent from light through binary fission of pre-existing ones (Millar *et al*, 2008) (Fig. 2) and do not exhibit drastic morphological and functional changes, except in shoot apical meristem (SAM) and leaf primordial meristematic cells. In these cells, a large and complex mitochondrial mass exists around the nucleus that undergoes structural changes during the cell cycle (Seguí-Simarro *et al*, 2008). Still, their differentiation is strictly regulated. The inner mitochondrial membrane forms structures, termed cristae, holding the electron transport chain (ETC) complexes and necessary for generating a proton gradient to drive ATP synthesis. To exert their

cellular functions chloroplasts and mitochondria depend on the correct expression of their organelle genome encoding the core components of photosynthesis and respiration. Mutations in genes involved in organelle biogenesis, expression or physiology results in diverse growth defects and an overview of all mutants that will be discussed in this review can be found in Supplemental Table S2, with their corresponding plant, organelle and cellular phenotype.

Biogenesis

Disturbing differentiation into photosynthetic active chloroplasts through incorrect biosynthesis of the thylakoid structures, chlorophyll or carotenoids has major effects on the photosynthetic capacity of the leaves, and thus affects leaf development. The majority of mutants with improper chloroplast development are typically pale green, variegated or albino (Aluru *et al*, 2006; Yu *et al*, 2007). Variegated mutants produce green leaves with white sectors that contain abnormally formed or less chloroplasts. In all these mutants disrupted chloroplast development results in an impaired chloroplast-to-nucleus signalling which affects cell differentiation. Therefore, most of these mutants have pleiotropic plant growth defects (Aluru *et al*, 2006; Sakamoto, 2003; Yu *et al*, 2007). In the *immutans* (*im*) mutant, for example, the white sectors of the leaf consist of heteroplastidic cells containing both normal and abnormal chloroplasts without internal thylakoid structures (Wetzel *et al*, 1994). *IM* encodes a protein associated with the thylakoid membranes in chloroplasts and acts as a cofactor in phytoene desaturation in the first steps of the carotenoid biosynthesis during early chloroplast development (Carol *et al*, 1999; Foudree *et al*, 2012) Plants lacking a functional IM have a delay in shoot and root growth compared to wild type plants and their leaves are thicker due to larger cells and more intercellular spaces in their mesophyll layers (Aluru *et al*, 2001). Another example of variegated mutants with characterized cellular effects are the *var2* and *var3* mutants (Chen *et al*, 2000; Kato *et al*, 2009; Naested *et al*, 2004; Sakamoto *et al*, 2002; Takechi *et al*, 2000). *VAR2* encodes the ATP-dependent chloroplast metalloprotease known as FILAMENTATION TEMPERATURE SENSITIVE2 (FtsH2) (Chen *et al*, 2000; Sakamoto *et al*, 2002; Yu *et al*, 2005). *var2* mutants have more and smaller cells in both white and green sectors compared to wild type leaves (Sakamoto *et al*, 2009). *VAR3* encodes a zinc-finger protein that interacts with NINE-CIS-

EPOXYCAROTENOID DIOXYGENASE4 (NCED4), a protein similar to carotenoid deoxygenase and consequently *var3* mutant has reduced chlorophyll and carotenoid levels (Naested *et al*, 2004). *var3* mutant produces yellow instead of white sectors in the green leaves and have a severe reduction in palisade mesophyll cells in these yellow sectors (Naested *et al*, 2004). Finally, *anu10* mutants are also impaired in thylakoid biogenesis and grana formation but these exhibit small, pale green leaves due to less but larger cells with small chloroplasts (Casanova-Saez *et al*, 2014). *ANU10* or *ANGULATA10* encodes a protein of unknown function localized to chloroplasts and is required for chloroplast and mesophyll cell development.

In contrast to the chloroplast differentiation mutants, the phenotypes of mutants in mitochondrial differentiation related genes have not been described in much detail. In general, the mitochondria of these mutants are characterised by a reduction in cristae development and are swollen and less electron-dense (Teardo *et al*, 2015). The *RETARDED ROOT GROWTH* (*RRG*) gene encodes a mitochondrial membrane protein of unknown function and root growth is disturbed when *RRG* is mutated (Zhou *et al*, 2011). In the *rrg* mutant, the size of the root meristem is much smaller due to a decrease in cell number. The cells in the meristem are however larger, and mature root cells are similar in size to wild-type, suggesting that cells enter expansion and differentiation earlier. The difference in root size arises after embryogenesis and is probably due to aberrant mitochondrial structure. Two mitochondrial proteins belonging to the Type I PROHIBITIN (PHB) class, PHB3 and PHB4, are also involved in the biogenesis of mitochondria (Van Aken *et al*, 2007). Growth of *phb3* plants is markedly reduced and a developmental delay was observed both in roots and shoots. *phb3* root meristem contains fewer cells, which are smaller initially but attain wild-type size later in development, similar to the *rrg* mutant. *phb3* mutant plants exhibit swollen, rounder mitochondria compared to the wild-type. The double *phb3 phb4* mutant is lethal, suggesting that both genes are essential for meristematic cell production.

Although a correct differentiation of organelles seems crucial for cell growth, the cellular phenotypes of the important players of organelle differentiation is not reported. Usually, the effect on organelle morphology is generally described and linked to macroscopic phenotypes (Teardo *et al*, 2015; Yua *et al*, 2014). However, from the examples presented here, disruption of chloroplast biogenesis-related

proteins generally results in less but larger mesophyll cells, indicating a primary effect on cell proliferation, which likely is compensated by an increase in cell size and/or an early onset of differentiation. Similarly, the few examples of mutants involved in mitochondrial biogenesis show clear defects in cell proliferation.

Division and Fission

When cells divide, not only the nuclear but also the organellar genetic information needs to be distributed equally among the daughter cells. Therefore, organelles also undergo a division cycle, comparable to the binary fission in bacteria. In the centre of the bacterial cell, a contractile ring, made by a polymer of the tubulin homolog FILAMENTING TEMPERATURE-SENSITIVE MUTANT Z (FtsZ) anchored to the membrane, is formed and the correct positioning of this division midpoint is monitored by the intracellular localisation of three MIN proteins (MinC, MinD and MinE). Chloroplasts are surrounded by two membranes and need the formation of both a stromal FtsZ ring and an external/cytosolic ring composed of a dynamin-like protein (DRP) for their division. Mitochondria of several eukaryotes including higher plants lack FtsZ, and fission is governed solely by an external DRP ring (Margolin, 2005). Several nuclear-encoded homologs of the prokaryotic division-genes work together with eukaryote-specific proteins to form the organellar dividing machinery.

In Arabidopsis, several mutants with defects in chloroplast division have been described (Osteryoung *et al*, 1998; Yoder *et al*, 2007). Both loss- and/or gain-of function of *MinD1*, *MULTIPLE CHLOROPLAST DIVISION SITE1* (*MCD1*) or *ACCUMULATION AND REPLICATION OF CHLOROPLASTS3* (*ARC3*) result in asymmetric chloroplast divisions forming fewer but enlarged chloroplasts with different sizes and shapes (Colletti *et al*, 2000; Maple *et al*, 2007; Nakanishi *et al*, 2009; Shimada *et al*, 2004). Interestingly, transgenic lines with perturbed plastid division do not show clear plant growth defects, since the total ratio chloroplast area/mesophyll area is always maintained since the reduction in chloroplast number is compensated by an increased chloroplast area (Pyke & Leech, 1994). In the SAM, proplastid division positively correlates with the cell cycle, similarly to other organelles (Segui-Simarro *et al*, 2008; Segui-Simarro & Staehelin, 2009), whereas plastids generally divide nonsynchronously (Miyagishima, 2011). In addition,

expression of FtsZ is cell-cycle dependent (El-Shami *et al*, 2002) and some proteins seem to be involved in both plastid and cell division processes. CDC TARGET1a (CDT1a) facilitates, together with other proteins, the assembly of the pre-replication complex to the DNA in order to initiate DNA replication. At the transition from G1 to S phase, CDT1 is degraded after phosphorylation by cyclin-dependent kinases to permit DNA replication (Blow & Dutta, 2005). The CDT1a protein is localised both in the nucleus and plastids, where it interacts with the chloroplast division ACCUMULATION AND REPLICATION OF CHLOROPLASTS6 (ARC6) protein. The simultaneous down-regulation of *CDT1a* and *CDT1b* in RNAi plants leads to the formation of small pale green leaves. At cellular level, these leaves consist of less and smaller cells that contain less but enlarged chloroplasts. Disturbing the function of another nuclear-encoded protein, CRUMPLED LEAF (CRL), located on the outer membrane of chloroplasts, results in severe growth inhibition in shoot and root. All the organs of the *crl* mutant contain cells with discontinuous and unorganized cell division planes. Leaves of *crl* mutant plants have less and smaller cells and also root growth is negatively affected. Additionally, several important cell cycle genes such as the cyclin *CYCD3;1* are downregulated in the *crl* mutant, whereas the cell-cycle inhibitor *SIAMESE-RELATED5* is upregulated. As a consequence, *crl* cells differentiate earlier and endoreduplication is enhanced in both shoot and root (Asano *et al*, 2004; Hudik *et al*, 2014).

In contrast to plastids, mitochondria undergo massive fusion-fission events during the cell cycle (Mitra, 2013). The occurrence of large sheet or cage-like structures of fused mitochondria around the nucleus during the cell cycle has been observed in certain cell types of plants (Segui-Simarro *et al*, 2008; Segui-Simarro & Staehelin, 2009). During G1/S, this fusion of mitochondria is hypothesized to occur in response to the high demand for energy needed for nuclear DNA replication and cell division as well as to ensure proper mixing and recombining of the mitochondrial DNA (mtDNA). During mitosis, two important proteins involved in mitochondrial fission, DRP3A and DRP3B, are activated through phosphorylation and, when cells are treated with an inhibitor of CDKB/CYCB and related kinases, fission is compromised (Wang *et al*, 2012). From these evidences, it is clear that the regulation of mitochondrial and cell division must involve common players. When mitochondrial fission is perturbed due to the mutation of one or more of the most important

members of the division apparatus (*DRP3A*, *DRP3B*, *DRP5B* and *ELONGATED MITOCHONDRIA1* (*ELM1*)), a reduction in plant growth is observed (Arimura *et al*, 2008; Aung & Hu, 2012; Fujimoto *et al*, 2009; Mano *et al*, 2004). To our knowledge, only the *friendly* mutant, which displays clusters of mitochondria, has been characterised at the cellular level. *FRIENDLY* is member of the CLUSTERED MITOCHONDRIA (CLU) superfamily and is involved in mitochondrial fussion (Zawily et al 2014). *friendly* produces smaller shoots, hypocotyls and roots. In *friendly* roots, an increase in cell number was observed, accompanied by an important reduction in cell size (El Zawily *et al*, 2014).

In conclusion, the division of mitochondria and chloroplasts needs to be coordinated with cell division during organ development. For both chloroplast and mitochondria, clear molecular and/or morphological links between factors involved in organelle division machinery and the cell cycle have been revealed.

Genome Organization and Expression

Although the genomes of chloroplasts and mitochondria contain relatively few genes, they encode key proteins involved in organelle gene expression, photosynthesis or electron transport and the correct expression of these organelle genes is crucial for plant development. In Arabidopsis, the mitochondrial genome (mtDNA, 366 kb) encodes three rRNAs and 57 proteins, mainly subunits of the ETC and ribosomal proteins (Unseld *et al*, 1997). The 154 kb chloroplast genome (cpDNA) contains 45 RNA- and 87 protein-coding genes (Sakamoto *et al*, 2008; Sato *et al*, 1999). The proteins encoded by the cpDNA are mainly involved in transcription, translation and photosynthesis. Organelle DNA copies are organised in protein complexes called nucleoids. Arabidopsis rosette leaves harbour hundreds to thousands cpDNA copies per cell and less than hundred mtDNA copies (Draper & Hays, 2000; Preuten *et al*, 2010). Curiously, some mitochondria only contain a fraction of the mtDNA or no genome at all (Preuten *et al*, 2010). Generally, the number of organelle DNA copies per cell increases with cell size but there is some controversy regarding the decline of the amount of cpDNA copies during proplastid-to-chloroplast conversion and during leaf development (Oldenburg & Bendich, 2015; Rowan & Bendich, 2009; Zoschke *et al*, 2007). Mitochondrial DNA copy number remains low and constant

during development and the highest copy number is found in root tips containing several hundred copies per cell.

Two homologs of the bacterial DNA polymerase I (PolI), *PolIA* and *PolIB*, are encoded by the Arabidopsis nuclear genome and responsible for organelle DNA replication. Mutation in *PolIA* does not affect plant growth, but roots and shoots of *polIb* mutant lines develop slowly compared to the wild-type probably due to a defect in cell elongation (Cupp & Nielsen, 2013; Parent *et al*, 2011). At the subcellular level, the *polIB* mutant has an increase in the number of mitochondria compensated by a decrease in mitochondrial area density, but chloroplast ultrastructure is not affected. WHIRLY2 (WHY2), a nucleoid-binding protein localized in mitochondria and belonging to the plant-specific Whirly protein family, also plays an important role in maintaining mtDNA copy number (Cai *et al*, 2015). Overexpression of *WHY2* in pollen vegetative cells, normally harboring extremely low mtDNA copy numbers, results in decreased mitochondrial division, increased mtDNA levels and reduced pollen tip growth. DNA replication depends on a sufficient supply of deoxyribose nucleotide triphosphates (dNTPs). Ribonucleotide reductase (RNR) determines the last step in de novo formation of dNTPs in the cell. In Arabidopsis, RNR is formed by two large subunits (R1) and two small subunits (R2). A mutation in *CRINKLED LEAVES8* (*CLS8*), encoding RNR1, leads to the production of rough and uneven green leaves with white regions as well as reduced root growth (Garton *et al*, 2007). Mesophyll cell size is not affected but both green and white regions of *cls8* leaves contain less chloroplasts, which are enlarged in green areas and smaller in white areas. The reduction in dNTP level also causes lower plastome copy numbers. Single or double mutants of the three redundant R2 genes (*TSO2*, *RNR2A*, *RNR2B*) display similar effects on plant growth (Wang & Liu, 2006) and mesophyll cells are small and form large intercellular spaces, whereas epidermal cells are clustered and enlarged on the surface of the leaf causing the rough leaf phenotype. A reduced and imbalanced dNTP pool probably underlies these developmental defects, which result in a block of organelle DNA replication and chloroplast-deficient cells in the white sectors.

Mutants involved in organelle genome maintenance generally also exhibit plant growth defects. MUTS HOMOLOG1 (MSH1) is a plant-specific mitochondrial- and plastid-targeted protein homologous to the bacterial mismatch repair protein MutS,

involved in organelle genome stability. Suppression of *MSH1* results in numerous phenotypes, such as leaf variegation, enhanced branching, dwarfism and delayed maturity of the vegetative and reproductive structures (Xu *et al*, 2011; Xu *et al*, 2012). Also, partial transition to perennial growth on short days is observed and the stems of *msh1* are thicker due to an increase in secondary xylem and accumulate much more lignin compared to the wild-type (Xu *et al*, 2012). Interestingly, several genes encoding important regulators of cell division and expansion, such as *CDKB2;1*, *CYCB1;4* and *CYCD3;1, EXPA8* and *EXPA11*, are downregulated in *msh1* mutants, indicating defects in both processes (Xu *et al*, 2012).

Organelle gene expression also is under nuclear control. In Arabidopsis chloroplasts, two types of RNA polymerases are responsible for the transcription of specific plastid-encoded genes, the phage-type nuclear-encoded RNA polymerase (NEP) and a multi-subunit plastid-encoded polymerase (PEP). NEP is responsible for the transcription of the different core subunits of RNA polymerase (rpoA, rpoB, rpoC1 and rpoC2) of the PEP, which is responsible for the transcription of photosynthesis-related genes. Arabidopsis encodes three NEPs, RPOTp, RPOTm and RPOTmp targeted to plastids, mitochondria and both organelles, respectively (Borner *et al*, 2015). Knock-out of the mitochondria-localized RPOTm is lethal and heterozygous plants have a reduction in male and female gamete fitness (Kuhn et al, 2009). Roots and rosettes of the single *rpoTmp* mutants grow slower compared to the wild-type (Baba et al, 2004; Courtois et al, 2007; Kuhn et al, 2009). Mutations in *SCABRA3* (*SCA3*), encoding RPOTp, lead to the production of reticulate leaves that are round, reduced in size and have deep serrations (Hricova *et al*, 2006). Reticulate mutants show reticulation pattern of dark green veins on a green or pale green lamina. The *sca3* leaf surface is wrinkled to crumpled but does not exhibit changes in epidermal cell size or morphology. However, mesophyll cells are less dense and more irregularly shaped making it impossible to distinguish between palisade and spongy layers. The growth defects in *sca3* mutant, as in most reticulate mutants, are not solely attributed to defects in cell proliferation or expansion, but also result from an incorrect development of the distinctive tissue layers. Since the mesophyll layer in leaves is the most important site of photosynthesis, a link between plastid gene expression and mesophyll differentiation can be proposed. However, most known reticulated mutants have roles in primary plastid metabolism, and some hypotheses

have been put forward to explain the defects in mesophyll development in light of impaired metabolism (see below and reviewed in (Lundquist *et al*, 2014)).

In chloroplasts and mitochondria, primary transcripts undergo posttranscriptional processing, which has been proposed as an important determinant of organelle gene expression (Colas des Francs-Small & Small, 2014; Sakamoto *et al*, 2008). For all mitochondrial protein complexes of the electron transport chain (ETC), except the nuclear-encoded Complex II, so-called surrogate mutants have been characterized (reviewed in (Colas des Francs-Small & Small, 2014); (Colas des Francs-Small *et al*, 2014; Hsieh *et al*, 2015; Hsu *et al*, 2014)). These mutants are disturbed in nuclear-encoded genes encoding proteins involved in various steps of posttranscriptional regulation in the mitochondria: RNA processing, stabilization, splicing, editing or translation. A large number of these surrogate mutants displays pleiotropic phenotypes due to a reduction in accumulation and/or activity of ETC complexes, such as a reduced germination capacity, slow development of roots and shoots and fertility problems. However, many of these surrogate mutants have no apparent macroscopic phenotype under normal conditions, indicating that a threshold level of the organelle protein complex abundance or activity exists above which plants can develop and grow normally (Colas des Francs-Small & Small, 2014). With the exception of mutants affecting posttranscriptional processing of *nad* genes, encoding subunits of complex I of the ETC, only few surrogate mutants have been described at cellular level. The *slo3* mutant, for example, defective in splicing of *nad7*, germinates late and displays delayed root and shoot development due to a reduction of cell proliferation (Hsieh *et al*, 2015). Cell proliferation is a process requiring high amounts of energy; in *slo3* mutants a decline in ATP production caused by a reduction in the major subunits of the ETC leads to growth defects. Also, the *slg1* mutant, defective in editing of *nad3*, displays slower development of roots and leaves (Yuan & Liu, 2012). This mutant has a reduction in root meristem cell number due to a lower cell division activity as well as a reduction in epidermal cell size in the maturation zone. *slg1* mutant shows reduced complex I activity and ATP content, and activation of the alternative respiratory pathway. A third surrogate mutant, *abo8* (*aba overly sensitive8*), is defective in splicing of *nad4* and also has reduced complex I abundance and activity, resulting in decreased ATP production and increased ROS production (Yang *et al*, 2014). *abo8* plants produce smaller roots, due to a reduction

in root meristem cell number which is partially compensated by an increase in cell size.

Finally, also organelle translation is important for normal organ development. In the *regulator of fatty acid composition 3* (*rfc3*) mutant, a nuclear-encoded plastid-localized S6-like ribosomal protein is mutated, resulting in low levels of unsaturated fatty acids and impaired lateral root development (Horiguchi *et al*, 2003; Horiguchi *et al*, 2011). The lateral roots of the mutant do not develop in a distal direction when grown on high sucrose levels and do not have a quiescent center.

In conclusion, strict regulation of organelle genome copy number, maintenance, expression and translation is important for sustaining normal organ growth, at the cellular and macroscopic level. This plethora of phenotypic consequences can be explained since the organellar genome encodes solely proteins implicated in its core functions, photosynthesis in chloroplasts and energy production in mitochondria. Mis-expression of these proteins, if not lethal, has pleiotropic consequences on plant growth.

Physiology

The key function of the organelles is to generate enough carbon sources and energy during the growth of plant organs. Additionally, organelles are important hubs for various interconnected metabolic pathways such as amino acid metabolism, fatty acid and secondary metabolite production, vitamin, hormone biosynthesis and signalling.

Carbon metabolism

During the day, chloroplasts of leaves actively performing photosynthesis (source leaves) convert light energy into chemical energy stored in carbohydrates. The primary end products of photosynthesis are triose phosphates which are subsequently used to form starch, as storage compound, in the chloroplast, or sucrose in the cytosol (Stitt & Zeeman, 2012). Sucrose is then used as a long-distance transport molecule to other parts of the plants that act as sink tissues, such

as roots and young growing leaves that do not yet photosynthesize. The inner membrane of the chloroplast contains different phosphate translocators (PT), necessary for the exchange of important metabolites between the cytosol and plastid stroma (Flugge, 1999; Flugge *et al*, 2011). One of the major translocators is the antiporter Triose Phosphate/PT (TPT), involved in exporting triose phosphates into the cytosol in exchange for Pi. *tpt* mutant does not display a growth phenotype, probably because of the presence of redundant phosphate translocators, but has larger chloroplasts (Hausler *et al*, 2009; Schneider *et al*, 2002). The starch biosynthesis mutant *adg1-1,* impaired in ADP-glucose phosphorylase, only displays severe growth retardation when grown under short-days or high light and has smaller chloroplasts (Kunz *et al* 2010; Lin *et al*, 1988). The double mutant *adg1-1/tpt* exhibits severe growth arrest under high light and produces thick pale green leaves due to less but larger mesophyll cells (Hausler *et al*, 2009; Heinrichs *et al*, 2012; Schmitz *et al*, 2012). When TPT exports triose phosphates into the cytosol for sucrose biosynthesis, Pi is imported in the chloroplast stroma. As Pi is an important regulator of chloroplast enzymes and Pi homeostasis has to be maintained in the cell, it has to be exported into the cytosol through specific Pi transporters. In Arabidopsis, two sink-specific plastid-localized Pi translocator family proteins have been identified, PHT2 and PHT4. One of these proteins, PHT4;2, is mainly localized in roots and *pht4;2* mutant rosettes produce more biomass compared to the wild type under short-day conditions. This increase in size is due to an increase in epidermal cell number but the exact mechanism is unclear (Irigoyen *et al*, 2011).

Following transport to the sink tissues, sugars are used for glycolysis in the cytosol and in the citric acid cycle (TCA) in mitochondria to generate ATP. Glycolysis and the TCA have to work together efficiently and perturbing this interaction has phenotypic consequences. An example is related to the functionality of a multi-enzyme pyruvate dehydrogenase complex, responsible in the mitochondria for converting pyruvate imported from the cytosol into acetyl-CoA and NADH. When one subunit, the E2 dihydrolipoyl acetyltransferase, is downregulated, reduced influx in the TCA cycle leads to an increase in TCA intermediate metabolic products and amino acids. The reduced metabolic flux reduces ATP production, leading to severe growth penalties in the roots and the shoot. The extreme reduction in primary root length and mature

leaves size is due to a reduction in cell proliferation but also cell differentiation is impaired in this mutant (Yu *et al*, 2012).

Mitochondria and chloroplasts play in C3 plants an important role during the photorespiration pathway, which scavenges toxic waste products that are made when RUBISCO interacts with oxygen instead of carbon dioxide. The pathway involves a network of enzymes exchanging metabolites between three subcellular compartments: chloroplasts, peroxisomes and mitochondria. The main mitochondrial step involves the conversion of glycine into serine, catalyzed by the glycine decarboxylase complex (GDC) and a serine hydroxymethyltansferase (SHMT) (Eisenhut *et al*, 2013; Kuhn *et al*, 2013; Lawand *et al*, 2002). The mitochondrial carrier A BOUT DE SOUFFLE (BOU) plays a role in this pathway although its substrate has not been identified yet. *bou* mutant plants have a reduced GDC activity and accumulate glycine. The mutant of SHMT, *shm1-1*, displays a similar metabolic phenotype. As a consequence, both *bou* and *shm* exhibit a photorespiratory phenotype as they grow normally at high CO_2 but fail to develop photoautotrophically under ambient CO_2 levels due to a sucrose and CO_2-dependent reduction in cell-cycle activity in the root and shoot apical meristem.

Many proteins involved in respiration and photosynthesis contain Fe-S clusters as important cofactors in redox reactions. Fe-S cluster assembly mainly occurs in the cytosol, but also in mitochondria and plastids (Balk & Pilon, 2011). The ATP-BINDING CASETTE TRANSPORTER OF MITOCHONDRIA3 (ATM3) transports mitochondrial glutathione persulfide (GS-S^0-SG) to the cytosol where the sulfur is used in Fe-S cluster assembly (Chen *et al*, 2007; Kim *et al*, 2006; Schaedler *et al*, 2014; Teschner *et al*, 2010). Mutants of *ATM3* germinate less efficiently on medium without sucrose and produce small roots and shoots (Bernard *et al*, 2009; Kushnir *et al*, 2001). *atm3* leaves are chlorotic and thicker due to less but enlarged cells, and produce a poorly developed mesophyll layer (Kushnir *et al*, 2001).

In summary, photosynthesis and mitochondrial respiration are tightly coupled and need different transporters for shuttling metabolites from one organelle to another to maintain cellular homeostasis. When this transport is impaired cell proliferation is mainly affected.

Amino acid biosynthesis

Chloroplasts and mitochondria have a central position in the biosynthesis of amino acids. The aromatic amino acids (AAAs) tryptophan, tyrosine and phenylalanine are synthesized via the shikimate pathway that starts with the import of phosphoenolpyruvate in the chloroplasts via a phosphate transporter, the phosphoenolpyruvate translocator (PPT) (Maeda & Dudareva, 2012). Interestingly, the correct biosynthesis of AAAs and functional PPT proteins are crucial for plant growth since all mutants in chloroplast amino acid biosynthesis produce reticulated leaves (Lundquist *et al*, 2014; Knappe *et al*, 2003; Li *et al*, 1995; Streatfield *et al*, 1999). *cue* or *chlorophyll a/b binding protein* (*CAB) underexpressed* mutants, impaired in *PPT* expression, produce small reticulate leaves when grown on medium with an external carbon source (Li *et al*, 1995). The palisade mesophyll cell layer in the pale green sections of the leaf is underdeveloped with less cells and larger intercellular spaces, whereas the number and size of epidermal and spongy mesophyll cells are unaffected (Streatfield *et al*, 1999). Furthermore, chloroplasts are smaller in *cue1*, while their number is not changed. Treatment of the *cue1* mutants with a cocktail of AAA, rescues the reticulated leaf phenotype but not the reduced shoot growth (Staehr *et al*, 2014; Streatfield *et al*, 1999). These treatments restore the reduced mesophyll cell density, but further reduce *cue1* root growth. A distinct role for PPT1 was suggested in shoot and root, working as an importer in chloroplasts in leaves and as an exporter in the root (Staehr *et al*, 2014). Most reticulate mutants described have clear effects on the number of cells, but in the *small organ1* (*smo1/trp2*) reticulate mutant, mutated in the gene encoding the β-subunit of tryptophan synthase (TBS1) smaller leaves are produced due to a decrease in the average size of the palisade mesophyll cells. In addition, chloroplasts are under-developed and contain less starch granules and thylakoid structures (Jing *et al*, 2009). Also mutants with a defect in the biosynthesis of other amino acids have been described. *venosa3* (*ven3*) and *ven6* reticulate mutants are impaired in arginine biosynthesis due to a loss-of-function of the large and small subunit of the chloroplast-localized carbamoyl phosphate synthase, required for the conversion of ornithine into citrulline in the arginine pathway. Both mutants produce small leaves that contain less and smaller palisade mesophyll cells as well as large intercellular spaces (Molla-Morales *et al*, 2011).

In summary, the reticulate phenotype highlights the importance of proper amino acid biosynthesis in chloroplasts for correct establishment of the palisade mesophyll cell layer and, thus, for normal leaf development. Although it is not sure how this reticulated phenotype arises, it represents a good model to study mesophyll development.

Hormone biosynthesis and regulation

Since chloroplasts and mitochondria are involved in plant development, their function is connected to several hormones which steer plant growth. The plant hormones auxin, gibberellin (GA), cytokinin (CK) and brassinosteroids (BR) are hormones that are predominately defined as regulating growth and development, while abscisic acid (ABA), salicylic acid (SA), jasmonates and ethylene (ET) are traditionally involved in the responses to abiotic and biotic stresses. Chloroplasts play an important role in the biosynthesis of several plant hormones. The first biosynthetic steps of ABA, CK, GA, BR and other phytohormones take place in (pro)plastids (Pfannschmidt & Munné-Bosch, 2013). Mitochondria are not directly involved in hormone biosynthesis, but recent studies indicate that mitochondrial function and the hormonal regulation of growth are coupled (Berkowitz *et al*, 2016).

ABA is an important hormone in plant physiology having roles in the response to a variety of stress conditions. One of the consequences of plant stress is the increase in reactive oxygen species (ROS), generated by mitochondria and chloroplasts, and ABA promoted ROS in mitochondria are involved in retrograde signaling. Two PENTATRICOPEPTIDE REPEAT (PPR) proteins, SLG1 and ABO8, with roles in posttranscriptional regulation of *nad* genes, encoding subunits of mitochondrial complex I of the ETC, and plant growth have been implicated in ABA responses (Yuan & Liu, 2012; Yang *et al*, 2014). The *slg1* mutant is more responsive to ABA and displays an increased tolerance to drought stress. Cell-cycle activity is diminished in *abo8* roots, which can be further enhanced by ABA treatment and rescued by addition of the reducing agent glutathione (GSH). The mutant contains lower transcript levels of the auxin-inducible *PLETHORA* (*PLT*) genes, illustrating a connection between ABA-mediated ROS production in mitochondria and auxin homeostasis in developing organs (Yang *et al*, 2014).

Auxin is involved in both cell proliferation and cell expansion and plays a role in all different stages during plant development, such as apical dominance, root initiation and growth tropisms. A mitochondrial tetratricopeptide protein, SHORT AND SWOLLEN ROOT1 (SSR1), is involved in auxin polar transport during root development (Zhang *et al*, 2015). When SSR1 is mutated, the expression of several PIN proteins is downregulated and auxin levels are reduced in roots. As a consequence, the roots of the *ssr1* mutant have a compromised stem cell niche, which causes a reduction in both the number and the size of cells in the primary root (Zhang *et al*, 2015). In addition to the root phenotype, the vegetative rosettes and inflorescences of *ssr1* are smaller compared to the wild-type.

The phenotype of BR biosynthesis and signalling mutants corresponds typically to dwarfed plants with dark-green round leaves (Zhu *et al*, 2013). Most of these mutants also exhibit de-etiolation, i.e. short hypocotyl and expanded cotyledons in the dark, because they express light- and photosynthesis-related genes in the dark (Azpiroz *et al*, 1998; Chory *et al*, 1991; Chory & Peto, 1990). The first BR mutants isolated are the so-called *det* (*de-etiolated*) mutants, *det1* and the less severe *det2.* Both *DET* genes encode reductases involved in the first steps of BR biosynthesis (Fujioka *et al*, 1997). *det1* root cells are small and contain a large number of green chloroplasts, instead of amyloplasts, whereas *det2* does not show major differences in chloroplast programming (Chory *et al*, 1991; Chory & Peto, 1990). All these characteristics clearly demonstrate an important role of BR in chloroplast differentiation and in photosynthesis. A role for mitochondria in BR signalling has been reported as well, although, no steps of BR signalling occur inside this organelle. The *Brz-insensitive-long hypocotyls 2-1D* (*bil2-1D*) mutant, insensitive to brassinazole (Brz) an inhibitor of BR biosynthesis, is defective in a mitochondrial DnaJ/Heat shock protein 40 (DnaJ/Hsp40) (Bekh-Ochir *et al*, 2013). This mutant produces outward-curled leaves with long petioles similar to plants overexpressing the BR receptor BRI1 (Gonzalez *et al*, 2010). When *BIL2* is overexpressed, an increase in biomass is observed. The roots of this gain-of-function mutant are longer and have more lateral roots. Although the cellular composition of these organs was not investigated, it is assumed that *BIL2* plays a role in the induction of cell elongation during BR signalling (Bekh-Ochir *et al*, 2013).

Also CK are involved in a variety of developmental processes such as cell division, shoot development, leaf senescence and photomorphogenic development. CK play a role in regulating cell proliferation in shoots but also promote cell differentiation in the roots (Schaller *et al*, 2014). Several genetic studies with mutants of the different components of the CK signalling revealed a clear link between CK and chloroplast development (reviewed by (Cortleven & Schmulling, 2015)). Loss-of-function of both the ARABIDOPSIS HISTIDINE KINASES (AHKs) and ARABIDOPSIS RESPONSE REGULATORS (ARRs) results in reduced chlorophyll content in the shoot as well as reduced shoot growth (Argyros *et al*, 2008; Riefler *et al*, 2006). Leaves are smaller due to less but enlarged epidermal cells in the *ahk* mutants, and less but similar epidermal cell sizes in *arr* mutants. Furthermore, disturbing organelle-localized ATP/ADP and isopentenyltransferases (IPTs) involved in CK biosynthesis, results in decreased levels of the CK isopentenyladenine (iP) and trans-zeatin. These reduced CK levels cause several plant developmental phenotypes such as reduced shoot growth, reduction in leaf number, SAM and stem size but also an increase in primary and lateral root length have been observed (Miyawaki *et al*, 2006). Moreover, when plants are treated with CK, chloroplast transcripts are commonly upregulated and chloroplast division is promoted (Brenner *et al*, 2005). Also endogenous increase in CK levels through overexpression of *CYTOKININ RESPONSE FACTOR2* (*CRF2*) induces PLASTID DIVISION PROTEIN2 (PDV2) which stimulates chloroplast division (Okazaki *et al*, 2009). Finally, overexpression of the transcription factor GROWTH REGULATING FACTOR5 (GRF5) also stimulates chloroplast division and acts synergistically with CK to promote the cell proliferation phase during leaf development (Vercruyssen *et al*, 2015). Transgenic plants overexpressing *GRF5* produce larger and dark green leaves due to more and smaller cells (Gonzalez *et al*, 2010).

The last phytohormone linked to organelles is GA, involved in many different processes during plant growth, such as germination, vegetative growth, and floral induction (Gupta & Chakrabarty, 2013). GA responses are mediated through the degradation of DELLA proteins, transcriptional regulators that negatively influence these responses in absence of GA. In Arabidopsis DELLA proteins are encoded by five genes: *RGA*, *GAI*, *RGL1*, *RGL2* and *RGL3*. GA deficient mutants are known for their semi-dwarf phenotypes (Claeys *et al*, 2014a) and are affected in both cell

proliferation and cell expansion. In addition, these GA deficient mutants are darker green compared to wild type plants. The GA-deficient mutant *ga1-3*, for example produces small leaves containing less and smaller mesophyll cells that contain more chloroplasts compared to the wild type (Jiang *et al*, 2012). Furthermore, GA treatment increases chloroplast number per cell, chloroplast division frequency, cell division rate and mesophyll cell area in the *ga1-3* mutant (Jiang *et al*, 2012). Also the GA-response mutant *rga-24 gai-t6* leaves contain more chloroplasts, whereas chloroplast number of the *della* pentuple mutant is similar to wild-type, suggesting additional factors responsible for chloroplast division exist next to the DELLA regulators.

In conclusion, organelle functioning and hormone biosynthesis and signalling are closely connected. This link has important consequences on plant growth, depending on the organelle that is involved and the regulated hormone. Strong interconnections between hormonal regulation, organelle function and plant growth have only recently been uncovered.

Signaling –adaptations to changing conditions

Many metabolic signals and proteins have been proposed to be involved in retrograde signaling, generating a complex and intertwined network of signals that are sensed by the nucleus to control gene expression and organelle development. This crosstalk between the nucleus and organelles is crucial for cell functioning under normal but also stress conditions. Indeed, when chloroplasts or mitochondria function is affected through application of chemicals or due to mutations in genes encoding organellar proteins, changes in the nuclear gene expression is observed, indicating a general response to organelle dysfunction. Furthermore, mitochondria and chloroplasts are also responsible for specific responses to stress conditions (Schwarzlander *et al*, 2012; Van Aken & Whelan, 2012).

Chloroplasts signal their developmental status to the nucleus to adapt to environmental conditions and maintain the necessary carbon and energy for the plant to grow. Efforts has been made to identify these signals and the responses they elicit, but for most of these signaling pathways the effect on cell proliferation and cell expansion during organ growth is not characterized (Reviewed by (Chi *et al*, 2015;

Chi *et al*, 2013; Kleine & Leister, 2013; Pogson *et al*, 2008)). The best known retrograde signals are derivatives of the tetrapyrrole, carotenoid and isoprenoid biosynthetic pathways. These factors have essential roles in the regulation of photosynthesis in coordination with the nucleus. For example, the *chloroplast biogenesis5* (*clb5*) mutant is impaired in ζ-CAROTENE DESATURASE (ZDS), involved in the biosynthesis of an apocarotenoid of yet unknown nature. *clb5* mutant exhibits severe defects in leaf development, with no clear leaf differentiation, translucent and irregular leaf surfaces. Most of the mesophyll tissue is absent in *clb5* and the expression of nucleus- and chloroplast-encoded genes is impaired, resulting in a lack of chloroplast development (Avendano-Vazquez *et al*, 2014).

In mitochondria, several nuclear-encoded proteins are targets of retrograde signaling for which expression is induced upon mitochondrial dysfunction. Most of the genes encoding these proteins share a CTTGNNNNNCA[AC]G cis-regulatory motif and are termed the Mitochondrial Dysfunction Stimulon (MDS) (De Clercq *et al*, 2013). Several NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION FACTOR ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) transcription factors have been shown to bind this motif in the promoter of MDS genes, and for ANAC13 and ANAC17 a role in oxidative stress tolerance has been shown through the activation of the MDS genes (De Clercq *et al*, 2013; Ng *et al*, 2013). When plants are subjected to stress conditions, they actively reprogram their growth (Claeys *et al*, 2014b). Typically, reports on stress response score the phenotype on growth media containing ABA or stress-inducing chemicals such as mannitol, sorbitol, NaCl or H_2O_2. However, the severity of the stresses applied is often extreme and only measurements of germination efficiency or survival are tested. In contrast, the developmental response to mild stress is more subtle and allows deciphering the molecular networks involved in the active regulation of the growth arrest (Skirycz *et al*, 2011). As such, a role of mitochondria in adaptation to mild stress conditions has been elucidated. When grown on mild osmotic stress conditions (25mM mannitol), a clear induction of MDS gene expression, including *ALTERNATIVE OXIDASE1a* (*AOX1a*), is observed in young leaf primordia which grow exclusively through cell proliferation (Skirycz *et al*, 2010). The proposed role of these MDS genes is to preserve mitochondrial morphology and function during adverse conditions (De Clercq *et al*, 2013). AOX1A mediates the plant-specific alternative oxidase pathway,

transferring electrons directly to O_2 as a shortcut for the ETC (Berthold *et al*, 2000). Upregulation of this protein leads to a reduction in the amount of harmful ROS, but also ATP production. As a result, plants have a growth penalty but are able to support the energetically demanding process of cell proliferation in a normal way under mild stress conditions. Plants overexpressing AOX1A are more tolerant to mild drought stress from early developmental time points onwards, indicating that AOX1A is necessary to maintain a normal cell proliferation phase under stress conditions. Contrastingly, *aox1a* mutant plants exhibit an increase in leaf area under normal conditions indicating that the mitochondrial ETC is the preferably energy source under control conditions, whereas alternative oxidase is preferred under stress conditions (Skirycz *et al*, 2010).

In summary, the interaction between nucleus and organelles ensures that plants are able to grow and develop even under adverse conditions. Organelles generate both general and specific retrograde signals which feed information to the nucleus, upon which the nucleus can anticipate through the induction of, for example, stress response genes. When this signaling is impaired, plants are unable to respond to environmental cues and display growth penalties.

Conclusion and Future Perspectives

In this review, we present a resource of mutant phenotypes for nuclear genes encoding proteins localised in chloroplasts, mitochondria, or both organelles. Based on the genetic evidences we find that most of these mutants are affected in cell proliferation (Fig. 3). Since chloroplasts mainly deliver carbon sources and mitochondria cellular energy, it is not surprising that compromising their function leads to a reduction in cellular energy needed for cell division. Furthermore, most mutants analysed at cellular level either exhibit a specific defect on cell proliferation or reduction in cell number compensated by an increase in cell expansion. This compensation effect is often insufficient to completely rescue the decrease in cell numbers and therefore these mutants still display growth phenotypes (Horiguchi *et al*, 2006). This phenomenon is commonly observed in mutants of cell-cycle proteins (Blomme *et al*, 2014). Furthermore, the number of chloroplasts per mesophyll cell normally correlates positively with the cell area during cell growth (Pyke & Leech, 1992) and chloroplast proliferation is enhanced in compensation mutants (Andriankaja *et al*, 2012; Kawade *et al*, 2013). So, although the genetic evidence presented in this review proposes a strong involvement of organellar proteins mainly in cell proliferation, their role in the regulation of cell expansion and differentiation cannot be ignored but largely remains to be elucidated.

The involvement of chloroplasts and mitochondria in the cellular processes driving organ growth is undisputable. Many nuclear genes encoding organellar proteins are differentially expressed during organ development, and growth phenotypes have been described for several gain- or loss-of function mutants of these genes. Although still many genes have not been characterised at the phenotypical level, they seem interesting candidates to further elucidate the role of chloroplast and mitochondrial proteins in cell proliferation, the transition to cell expansion and/or differentiation. Moreover, based on the expression profiles, some of these genes could encode proteins with organ-specific roles.

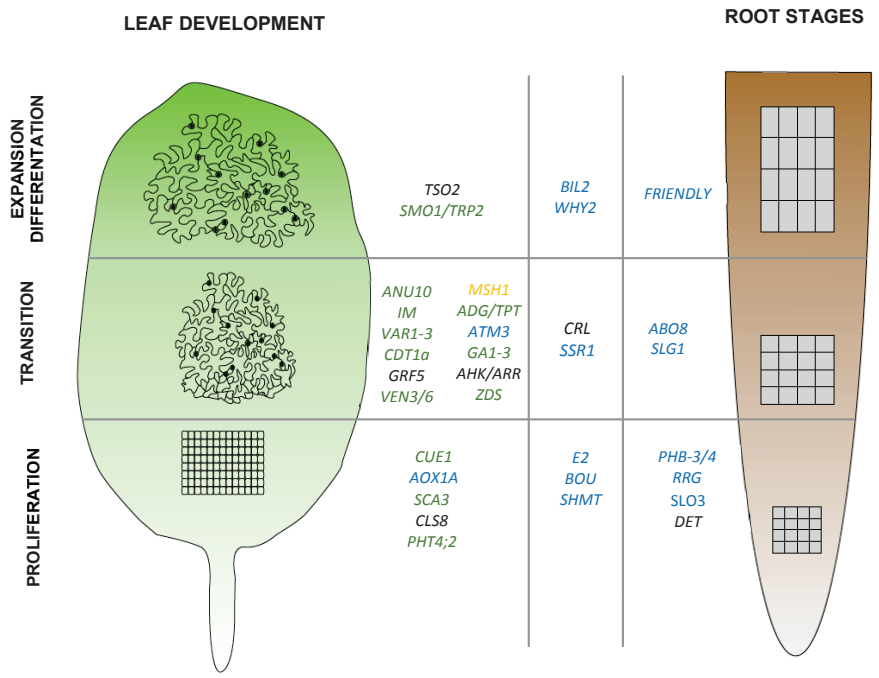

Figure 3. Summary of the genes reported to have a plant, cellular and organelle phenotype when disturbed or overexpressed. Genes are subdivided according to the effect on the cellular level when mutated or overexpressed. Three developmental zones can be distinguished. Bottom of the leaf or the root tip mainly contains proliferating cells. In the middle of the root or leaf, cells are transitioning from dividing to elongation or from cell proliferation to cell expansion, respectively. At the tip of the leaf cell expansion starts and in the upper part of the root most cells are elongated and differentiated. Genes are ordered next to the organ in which a phenotype has been described upon loss- or gain-of-function; if both a root and shoot phenotype was reported genes are central. Genes are color-coded according to the subcellular localization of the encoded protein: green for chloroplasts, blue for mitochondria, yellow for both and black for any other localization.

Looking beyond the number or size of cells composing a leaf or root, several mutants display a more complex reticulated or variegated phenotype where the development of palisade mesophyll tissue is hampered. It is still not understood how these complex phenotypes arise, although some hypotheses have been put forward (Lundquist et al, 2014). These mutants represent excellent genetic tools to better understand how chloroplasts regulate the development of specific cell layers such as mesophyll and how this can be connected to other tissues such as the epidermal layer.

Unfortunately, the majority of studies reporting growth phenotypes of organelle proteins only discuss the macroscopic defects in shoot and root growth. Organellar proteins exert crucial functions during plant development, but for a lot of proteins the direct or indirect regulation of plant growth remains uncharacterised or has not been

characterised at the cellular level. The additional investigation of the cellular effects of a mutant of interest would allow to better understand the growth phenotype (Gonzalez *et al*, 2012). Therefore, we plead for a more thorough cellular characterisation of mutants affected in growth.

SUPPLEMENTAL DATA

Supplemental tables are listed below and can be found at the end of this chapter.

Supplemental Table S1. Gene Ontology (GO) categories of nuclear genes encoding organellar proteins differentially expressed over development.

Supplemental Table S2. Genes involved in organelle function and plant growth.

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Supplemental Table S1. Gene Ontology (GO) categories of nuclear genes encoding organellar proteins differentially expressed over development. GO enrichment analysis using PLAZA (Proost *et al*, 2009) of differentially expressed genes during development in roots and leaves combined. Mito_Down and Mito_Up represents GOs of genes encoding mitochondrial proteins downregulated and upregulated during development, Chloro_Down and Chloro_Up represents GOs of genes encoding chloroplast proteins downregulated and upregulated during development.

Mito_Down		Mito_Up	
p-value	**Description**	**p-value**	**Description**
1.31E-20	structural constituent of ribosome	1.31E-20	structural constituent of ribosome
3.13E-11	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	3.13E-11	P-P-bond-hydrolysis-driven protein transmembrane transporter activity
3.46E-5	protein heterodimerization activity	7.77E-6	copper ion binding
7.77E-6	copper ion binding	9.36E-5	RNA binding transcription antitermination factor activity
6.73E-4	RNA binding	9.36E-5	RNA binding transcription factor activity
6.84E-4	cobalt ion binding	3.46E-5	protein heterodimerization activity
9.36E-5	RNA binding transcription antitermination factor activity	6.73E-4	RNA binding
9.36E-5	RNA binding transcription factor activity	6.84E-4	cobalt ion binding
0.01	carbon-nitrogen ligase activity, with glutamine as amido-N-donor	0.01	carbon-nitrogen ligase activity, with glutamine as amido-N-donor
0.01	chaperone binding	0.01	chaperone binding
0.01	single-stranded DNA binding	0.01	single-stranded DNA binding
0.01	rRNA binding	0.01	rRNA binding
0.01	ATPase activity	0.01	ATPase activity
0.01	ATP binding	0.01	ATP binding
0.01	nucleoside phosphate binding	0.01	nucleoside phosphate binding
0.01	nucleotide binding	0.01	nucleotide binding
0.01	phosphotransferase activity, alcohol group as acceptor	0.01	phosphotransferase activity, alcohol group as acceptor
Chloro_Down		**Chloro_Up**	
5.91E-19	structural constituent of ribosome	1.79E-12	chlorophyll binding
3.92E-7	small molecule binding	1.20E-8	water transmembrane transporter activity
8.62E-6	protein heterodimerization activity	1.20E-8	water channel activity
1.91E-6	nucleoside phosphate binding	2.36E-4	metal ion binding
1.39E-6	anion binding	4.29E-4	oxidoreductase activity
1.34E-5	lyase activity	5.44E-4	copper ion binding
1.05E-4	rRNA binding	5.73E-4	urea transmembrane transporter activity
1.24E-4	carbamoyl-phosphate synthase (glutamine-hydrolyzing) activity	0.01	formate dehydrogenase (NAD+) activity
1.24E-4	Ran GTPase activator activity	0.01	nitrite reductase (NO-forming) activity
1.26E-4	carbon-carbon lyase activity	0.01	magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase activity
1.33E-4	cofactor binding	0.01	zinc transporting ATPase activity
1.60E-4	copper ion binding	0.01	phosphatidylinositol-3,4,5-trisphosphate binding
2.01E-4	pyridoxal phosphate binding	0.01	oxidoreductase activity, acting on other nitrogenous compounds as donors, cytochrome as acceptor
3.70E-4	amidophosphoribosyltransferase activity	0.01	oxidoreductase activity, acting on other nitrogenous compounds as donors,

			iron-sulfur protein as acceptor
3.70E-4	L,L-diaminopimelate aminotransferase activity	0.01	phytoene synthase activity
5.44E-4	purine NTP-dependent helicase activity	0.01	ribulose-1,5-bisphosphate carboxylase/oxygenase activator activity
5.44E-4	ATP-dependent helicase activity	0.01	geranylgeranyl reductase activity
7.36E-4	cyclo-ligase activity	0.01	neoxanthin synthase activity
7.36E-4	ATP-dependent 5'-3' DNA helicase activity	0.01	geranylgeranyl-diphosphate geranylgeranyltransferase activity
7.36E-4	carboxyl- or carbamoyltransferase activity	0.01	9,9'-dicis-carotene:quinone oxidoreductase activity
0.01	glycogen debranching enzyme activity	0.01	7,9,9'-tricis-neurosporene:quinone oxidoreductase activity
0.01	uracil phosphoribosyltransferase activity	0.01	FMN reductase activity
0.01	cyclohydrolase activity	0.01	ferredoxin-nitrite reductase activity
0.01	oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	0.01	carotene 7,8-desaturase activity
0.01	fatty acid synthase activity	0.01	ferredoxin-nitrate reductase activity
0.01	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	0.01	poly-pyrimidine tract binding
0.01	NAD binding	0.01	poly(U) RNA binding
0.01	cobalt ion binding	0.01	oxidoreductase activity, acting on the CH-CH group of donors
0.01	carbon-oxygen lyase activity	0.01	transferase activity, transferring alkyl or aryl (other than methyl) groups
0.01	nucleic acid binding transcription factor activity	0.01	calcium ion binding

Supplemental Table S2. Genes involved in organelle function and plant growth. For each gene, the published effects on plant development, cellular and organelle phenotype of its mutation are given. The expression profile over development is indicated for differential expression. Abbreviations used: M: Mitochondria; C: Chloroplast, N: Nucleus; Mes: Mesophyll, Mer: Meristem, Epi: Epidermis; D: downregulated during development, U: upregulated during development; L: leaf; R: root.

AGI	NAME	LOF/GOF	Expression	Organelle	Plant effect	Cellular effect	Organelle effect
Biogenesis							
AT1G28530	*ANGULATA10*	LOF	C_D_L	C	Small, pale green	Less and larger Mes cells	Impaired thylakoid biogenesis, small C
AT4G22260	*IMMUTANS*	LOF	-	C	Variegated, small	Larger Mes cells	No thylakoid membranes
AT2G30950	*VARIEGATED2*	LOF	C_U_L	C	Variegated, small	More and smaller cells	Impaired thylakoid biogenesis
AT5G17790	*VARIEGATED3*	LOF	-	C	Variegated, yellow sectors, small	Few Mes cells	Impaired thylakoid biogenesis
AT1G69380	*RETARDED ROOT GROWTH*	LOF	-	M	Reduced root growth	Less and larger Mer cells	Aberrant M structure
AT5G40770 / AT3G27280	*PROHIBITIN-3/-4*	LOF	M_U_L / CM_D_LR	M	Reduced shoot and root growth	Less and smaller	Swollen, rounder M
Division and fission							
AT2G31270 / AT3G54710	*CDC TARGET1-a/-b*	LOF	- / -	C + N	Small + pale green	Less and smaller Mes cells	Less but larger C
AT5G51020	*CRUMPLED LEAF*	LOF	CM_U_L	C	Reduced shoot and root growth	Unorganized cell division planes	Less but larger C
AT3G52140	*FRIENDLY*	LOF	C_D_LR	M	Reduced shoot and root growth	More and smaller cells	M clusters
Genome Organization and expression							
AT3G20540	*DNA POLYMERASE IB*	LOF	-	C + M	Reduced shoot and root growth	Defects in cell elongation	Low M area density
AT1G71260	*WHIRLY2*	LOF	M_D_LR	M	Normal growth	Reduced pollen growth	Increased mtDNA and reduced M division
AT2G21790	*CRINKLED LEAF 8*	LOF	-	C	Variegated leaves, reduced root growth	Normal Mes cell size	Less and smaller C
AT3G27060	*TSO2*	LOF	-	C	Rough leaves with white spots	Small Mes cells, clustered and large Epi cells	No C in white sectors
AT3G23580	*RNR2A*	LOF	-	C	Rough leaves with white spots	Small Mes cells, clustered and large Epi cells	No C in white sectors
AT5G40942	*RNR2B*	LOF	-	C	Rough leaves with white spots	Small Mes cells, clustered and large Epi cells	No C in white sectors
AT3G24320	*MUTS PROTEIN HOMOLOG 1*	LOF	-	C + M	Reduced growth, variegation	Less and smaller cells	Increase in recombination
AT2G24120	*SCABRA3*	LOF	-	C	Reticulate - reduced shoot growth	Less and irregular Mes cells	Less and smaller C

AT3G61360	*SLOW GROWTH3*	LOF	-	M	Reduced shoot and root growth	Less root cells	Reduced complex I activity
AT5G08490	*SLOW GROWTH1*	LOF	-	M	Reduced root growth	Less root Mer cells and smaller Epi cells	Reduced complex I activity
AT4G11690	*ABA OVERLY SENSITIVE8*	LOF	-	M	Reduced root growth	Less and larger root Mer cells	Reduced complex I activity
AT1G77470	*REGULATOR OF FATTY ACID COMPOSITION3*	LOF	-	C	Disorganized lateral root	No quiescent center in lateral roots	Not determined
Carbon metabolism							
AT5G48300 / AT5G46110	*ADP GLUCOSE PYROPHOSPHORYLASE / TRIOSE PHOSPHATE TRANSOLACTOR*	Double Mutant	C_U_R / C_U_LR	C	Reduced growth, pale green	Less and larger Mes cells	Smaller chloroplast, grana hyperstacking
AT2G38060	*PHOSPHATE TRANSLOCATOR4;2*	LOF	-	C	Increased shoot growth	More Epi cells	Not determined
AT1G61800	*GLUCOSE-6-PHOSPHATE TRANSPORTER2*	LOF	-	C	Increased shoot growth	More Epi cells	Less differentiated C
AT3G25860	*E2 DIHYDROLIPOYL ACETYLTRANSFERASE*	LOF	C_D_LR	M	Reduced shoot and root growth	Less cells	Reduced TCA cycle influx and ATP
AT4G32520	*SERINE HYDROXY METHYLTRANSFERASE*	LOF	C_D_LR	M	Reduced shoot and root growth	Less Mer cells	Not determined
AT5G46800	*A BOUT DE SOUFFLE*	LOF	CM_U_L	M	Reduced shoot and root growth	Less Mer cells	Not determined
AT2G15570	*ATP-BINDING CASETTE TRANSPORTER OF MITOCHONDRIA3*	LOF	-	M	Reduced shoot and root growth, chlorotic	Less and larger cells	Not determined
Amino acid metabolism							
AT5G33320	*CHLOROPHYLL A/B BINDING PROTEIN UNDEREXPRESSED*	LOF	C_D_L	C	Reticulate, reduced shoot and root growth	Less Mes cells	Smaller C
AT5G54810	*SMALL ORGAN 1/TRP SYNTHASE β SUBUNIT2*	LOF	C_D_L	C	Reticulate, reduced shoot growth	Smaller Mes cells	Impaired C development
AT1G29900 / AT3G27740	*VENOSA3/6*	LOF	C_D_LR / CM_D_LR	C	Reduced shoot growth	less and smaller Mes cells	Not determined
Hormone biosynthesis and regulation							
AT5G02130	*SHORT AND SWOLLEN ROOT1*	LOF	-	M	Reduced shoot and root growth	Less and smaller cells	Not determined
AT4G10180 / AT2G38050	*DE-ETIOLATED1/2*	LOF	-	C	Reduced root growth	Small root cells	Green chloroplasts in roots
AT2G42080	*Brz INSENSITIVE LONG HYPOCOTYL*	GOF	-	M	Increased root growth	Cell elongation	Not determined

	ARABIDOPSIS HISTIDINE KINASES	LOF	-	C	Reduced shoot growth	Less and larger Epi cells	Less chlorophyll
	ARABIDOPSIS RESPONSE REGULATORS	LOF	-	C	Reduced shoot growth	Less Epi cells	Less chlorophyll
AT3G13960	*GROWTH REGULATING FACTOR 5*	GOF	-	N	Increased growth, dark green	More and smaller Epi cells	More C
AT4G02780	*GA-DEFICIENT MUTANT1-3*	LOF	-	C	Reduced growth + dark green	Less and smaller Mes cells	More C
AT2G01570 / AT1G14920	*REPRESSOR OF ga1-3 GA INSENSITIVE T6*	Double Mutant	-	C	Reduced growth + dark green	Not determined	More C
Signaling - adaptations to changing conditions							
AT3G04870	*CHLOROPLAST BIOGENESIS 5*	clb5	C_U_LR	C	Translucent and irregular leaves	No Mes cell layer	Impaired C development
AT3G22370	*ALTERNATIVE OXIDASE1a*	aox1a	-	M	Increased leaf area	Involved in cell proliferation	Not determined

CHAPTER 3

The dual effects of Strobilurins on Crops: Disease Control and Yield-Enhancement

The Origin of the Strobilurin Fungicides

Strobilurins were originally discovered as naturally occurring fungicidal compounds produced by the *Basidiomycetes* fungi species (Balba, 2007; Bartlett *et al*, 2002). These fungi mainly live on decaying wood material and produce fungicidal products to eliminate other fungi and yeasts from competing for nutrient resources. The name strobilurins comes from the first identified natural derivative Strobilurin-A, isolated from liquid cultures of *Strobilurus tenacellus* (Anke *et al*, 1977), which grows on rotting pine cones (Fig. 1). The identification of these natural strobilurins, which are very unstable in light, volatile and have rather weak activity, initiated the development of synthetic strobilurins (Beautement *et al*, 1991). Different companies started research programs to chemically modify the natural strobilurins to improve their photostability and activity (Balba, 2007; Bartlett *et al*, 2002). Most attempts have been focusing on the modification of the toxophoric group or (*E*)-methyl β-methoxyacrylate structural group, which is the fungicidal active part of the molecule (Fig. 1). The first strobilurin fungicides were simultaneously released by Syngenta, which discovered azoxystrobin, and BASF, which generated kresoxim-methyl (KM; Fig. 1). Nowadays, many different synthetic strobilurin formulations are on the worldwide market for use in agriculture and all exhibit diverse physical and biological properties due to their structural differences.

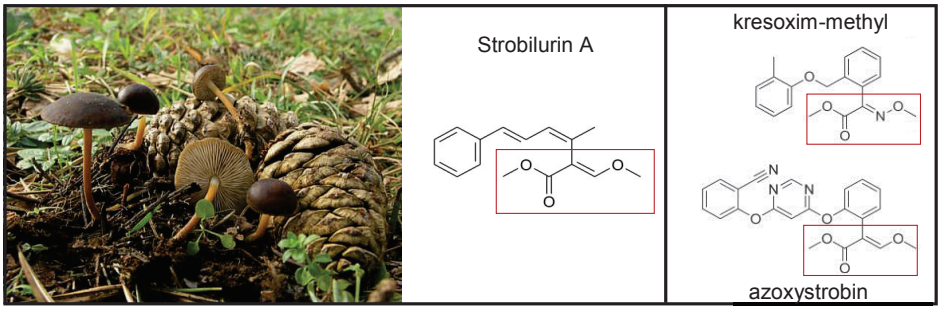

Figure 1. Strobilurins originate from the natural fungicide Strobilurin A produced by *Strobilurus tenacellus.* Image of *S. tenacellus* commonly found to grow on pine cones. Structures of the natural occurring strobilurin A and two synthetic strobilurin fungicides used in agriculture: azoxystrobin (Syngenta) and kresoxim-methyl (BASF). Red square highlights the toxophoric group. Modified from Liu *et al,* 2013.

Fungicidal Mode-Of-Action

Strobilurins are nowadays one of the most widely used fungicides in the world because of their broad-spectrum disease control against all four major groups of plant pathogenic fungi, i.e. *Ascomycetes*, *Basidiomycetes*, *Deuteromycetes* and *Oomycetes* as well as their use on a wide variety of plant species. Based on field trials, KM has been shown to facilitate protection against scab in apples and pears, downy and powdery mildew in apples, grapevines, cucurbits, sugar beet, and several fungal diseases in cereals and rice (Ammermann, 1992). Strobilurins are particularly efficient as preventive fungicide to eliminate spore germination and mycelium formation of the fungi.

Strobilurins exert their fungicidal effect by inhibiting the fungal mitochondrial respiration through their binding on the Quinone (Q_o) site of the electron transport chain (Fig. 2). By this binding, electron transfer between cytochrome b and c (Complex III) is blocked, the fungus is not able to oxidize nicotinamide adenine dinucleotide (NADH) and to produce ATP and eventually dies (reviewed by Leroux, 1996). Hence, strobilurins belong to a general fungicide class named Quinone outside inhibitors or Q_oI. Unfortunately, this highly specific single-site inhibition made Q_o inhibitor fungicides easy targets to acquire resistance and different examples of resistant fungi have been reported (Fernandez-Ortuno *et al*, 2008).

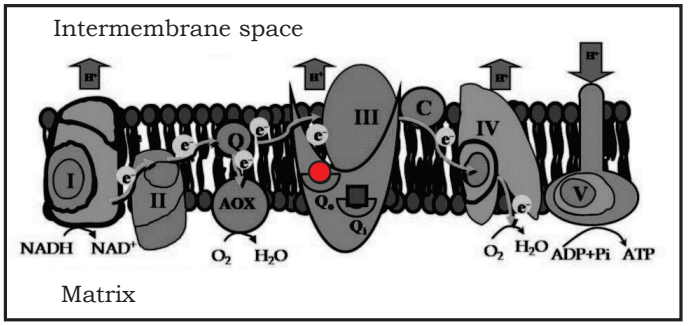

Figure 2. Schematic representation of the mitochondrial electron transport system. Different complexes of the electron transport chain are indicated with I, II, III, IV and V. Arrows indicate direction of the electron flow. Q is the ubiquinone pool and the Quinone-binding sites on Complex III are indicated with Qo, the Quinol oxidation site, and Qi, the Quinone reduction site. Red circle represents binding site of Q_O inhibitor fungicides. Taken from Fernández-ortuño *et al,* 2010.

Since the proteins of the mitochondrial respiration are highly conserved in eukaryotes, it could be expected that strobilurins also target mitochondria of plants or other eukaryotic species. Target activity of KM was evaluated in maize, rat and fly, and it was shown that KM is also active on mitochondria of these species (Roehl & Sauter, 1994). However, the toxicity effect depends on the biokinetic properties of this compound, such as uptake, transport and metabolism in these species, and is most effective against yeasts and fungi. In addition, also the developmental stage of the organism might affect the toxicity effect. In other words, whether the mitochondrial respiration is of major importance for the organism at the moment of application.

Strobilurins are commonly used as foliar fungicides. However, leaf uptake characteristics differ between the strobilurin structures and depend on the treated plant, the environmental conditions and the formulation since surfactants or other compounds can be added. Generally, strobilurins are not easily absorbed by the leaf and only few of them are systemically transported via the xylem. Azoxystrobin and picoxystrobin are systemic since they control powdery mildow in wheat leaves at regions distant from the application site (Bartlett *et al*, 2002). KM, trifloxystrobin and pyraclostrobin, on the other hand, are not systemic since application of these compounds did not result in a systemic disease control in wheat leaves. Strobilurins, with the exception of azoxystrobin and pyraclostrobin, are also transferred via air, in a process called vapor phase molecular distribution. In addition, all strobilurins are translaminar compounds since they are capable to diffuse across the leaf, leaking into the underlying plant cells (Reddy, 2012). Generally, strobilurin fungicides are rapidly metabolized in soil or are degraded by soil-grown bacteria (Howell *et al*, 2014). However, several studies have been reporting the toxicity for non-target water organisms demonstrating a risk in the use of strobilurins for the aquatic ecosystem (Liu *et al*, 2013; Liu *et al*, 2015).

Plant Physiological Benefits of the Strobilurins

Interestingly, in the absence of diseases, strobilurins were also found to be growth-promoting compounds by affecting multiple physiological pathways of the plant (Fig. 3), which sometimes, but not always, result in yield improvement. It has been hypothesized that the observed positive effects result from a general inhibition of all micro-organisms in the soil, by which the plant does not have to put energy in its defense response. On the other hand, it has also been suggested that strobilurins might transiently inhibit plant mitochondrial respiration by which photosynthetic carbon fixation might be favored (Bartlett *et al*, 2002; Grossmann & Retzlaff, 1997).

The first positive effects of strobilurins were reported in wheat (Ammermann *et al*, 1992; Grossmann & Retzlaff, 1997; Noon, 1997) but other studies demonstrated a general strobilurin-induced yield-enhancing effect in diverse plant species (Bartlett *et al*, 2002; Reddy, 2012). Multiple field trials independently reported an increase in wheat yield and grain size when treated with different strobilurins and strobilurin combinations (Beck *et al*, 2002; Bertelsen *et al*, 2001; Bryson *et al*, 1999; Gerhard, 1999). One of these positive effects was termed the ‘greening’ effect of strobilurins. Treatment with strobilurins led to a prolongation of the leaf greenness and, thus, to a delay in leaf senescence, which results in a longer grain-filling period and, consequently, more yield. This visible ‘greening’ effect has been confirmed by measurements of the photosynthetic activity by gas exchange and of the chlorophyll content of wheat plants grown under field conditions and treated with different strobilurin mixtures (Beck *et al*, 2002).

Azoxystrobin and pyraclostrobin treatment of wheat and barley plants also resulted in higher antioxidative enzyme activities, such as superoxide dismutase, catalase and peroxidase, in elevated hydrogen peroxide and lower superoxide levels accompanied with an increase in yield (Wu & von Tiedemann, 2001; Wu & von Tiedemann, 2002; Zhang *et al*, 2010). Recently, pre-treatment of seeds with KM was also found to protect *Medicago truncatula* plants against abiotic stresses, such as drought and salt stress (Filippou *et al*, 2015).

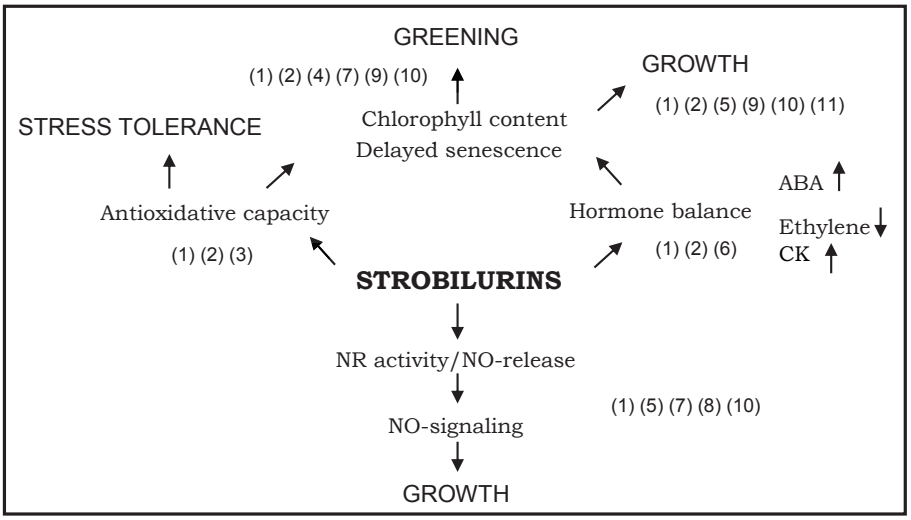

Figure 3. Physiological effects of strobilurins in plants. Summary of the reported physiological effects of strobilurin treatment in different plant species: (1) wheat, (2) barley, (3) Medicago, (4) duckweed, (5) tobacco, (6) grapevine, (7) spinach, (8) soybean, (9) rapeseed, (10) lettuce, (11) potato, (12) corn. NO, nitric oxide; NR, nitrate reductase; CK, cytokinin; ABA, abscisic acid.

However, bioassays with KM demonstrated contrasting growth effects depending on the plant species studied and the concentrations used (Grossmann & Retzlaff, 1997). Treatment with KM at a concentration of 10^{-4} M, inhibited growth of maize suspension cells, of *Lemna* (or duckweed) and germination of cress seeds but these inhibitory effects decreased with lower KM concentrations (Grossmann & Retzlaff, 1997). The inhibitory effects on *Lemna* were found to be accompanied with intensive greening. Remarkably, KM at a concentration of 10^{-7} M was able to induce shoot and root regeneration of tobacco explants, similarly as the auxin indol-3-acetic acid (IAA) at a concentration of 10^{-8} M (Grossmann & Retzlaff, 1997). This observation led to the hypothesis that KM could mimic the effect of auxin in plants. Additional studies with wheat leaf discs demonstrated that treatment with higher concentrations of KM (between 10^{-4} and 10^{-5} M) also affects ethylene and cytokinin levels, similarly as auxin signaling. Reduced aminocyclopropane-1-carboxylic acid (ACC) levels as well as a reduction in ACC synthase activity, involved in ethylene biosynthesis in converting S-adenosyl-methionine to ACC was found together with increased cytokinin levels. Chlorophyll content was also increased (Grossmann *et al*, 1999; Grossmann & Retzlaff, 1997). Similar results were obtained after pyraclostrobin foliar treatment of both barley and wheat which resulted in decreased ACC synthase activity and ACC levels (Köhle, 2002; Wu & von Tiedemann, 2002). Furthermore, IAA

and ABA levels were increased in wheat leaf discs and less chlorophyll degradation was observed (Köhle, 2002). Also in grapevine, pyraclostrobin resulted in increased ABA levels (Diaz-Espejo *et al*, 2012). Together with the increased antioxidative effects, these shifts in hormone levels, could explain the delayed leaf senescence phenotype and the improved stress tolerance after strobilurin treatment.

Several studies reported an interesting link between strobilurins and nitric oxide (NO) signaling which can regulate diverse physiological functions (Yu et al, 2014). KM treatment was found to induce nitrate reductase activity in spinach leaf discs (Glaab & Kaiser, 1999). The same effect was shown for treatment of wheat plants with pyraclostrobin, in its commercial formulation F500 (BASF), which increased nitrate reduction and uptake as well as fresh weight (Köhle, 2002). The positive effects on wheat plant physiology and growth was proposed to be a consequence of a possible increase in NO levels and subsequent NO signaling. Later, it was confirmed that pyraclostrobin treatment at a concentration of around 10^{-6} M induces NO-release in soybean cells and tobacco leaves (Conrath *et al*, 2004). Furthermore, field experiments with different strobilurins, including azoxystrobin, in wheat demonstrated improved grain yield, biomass and harvesting index due to increased nitrogen in the above-ground biomass and in the grains (Ruske *et al*, 2003).

More recent reports on azoxystrobin-induced advantages in other crops include an increase in chlorophyll content without an effect on yield of baby spinach (Conversa *et al*, 2014), an increase in grain number in barley (Bingham *et al*, 2012), higher seed yield and greening of field grown-rapeseed (Ijaz & Honermeier, 2013), and higher yield, reduced nitrate content and less chlorophyll degradation during storage in lettuce (Bonasia *et al*, 2013). Moreover, field trials with both azoxystrobin and pyraclostrobin resulted in increased potato tuber yield (MacDonald *et al*, 2007).

Whilst many advantages on plant growth have been reported, several studies also demonstrated no effect or even negative effects on plant physiology after treatment with strobilurins as well as location-dependent positive effects. Growth of unicellular algae *Chlorella vulgaris* was reduced by azoxystrobin application. Treatment with azoxystrobin resulted in a reduction in photosynthesis as measured by chlorophyll content and photosynthesis-related gene expression as well as an increase in reactive oxygen species (ROS) production (Liu *et al*, 2015). Field experiments with four different soybean varieties treated with pyraclostrobin in the absence of diseases

did not result in changes in yield measured by seed mass, seed number, pod number and harvest index (Swoboda & Pedersen, 2009). Also in dry bean, no difference in seed weight or yield was observed after spraying with azoxystrobin or pyraclostrobin (Mahoney & Gillard, 2014). Foliar treatment of corn with pyraclostrobin increased grain yield on two of three research locations tested, probably because of the differences in environmental conditions during time of application (Shetley *et al*, 2015). Also spraying of orchards on two different locations (South Carolina and California) with pyraclostrobin did not result in increased fruit quality, size or yield of peach (Schnabel & Crisosto, 2008).

Taken together, these findings show that strobilurins are important class of fungicides used throughout the world on a wide variety of crops, vegetables and fruits, against a wide variety of fungal diseases. They are extremely popular not only for their broad-spectrum disease control, but also because they often contribute to higher yield and biomass. However, these positive effects on growth are not always present and do depend on the plant species, the treatment and environmental conditions. Several paths and hypotheses have been reported (Fig. 3) to explain these positive effects, but it remains largely unclear how strobilurins work. Hence, further research is necessary and will be of major interest to unravel the underlying effects of strobilurin treatment on plant growth and development.

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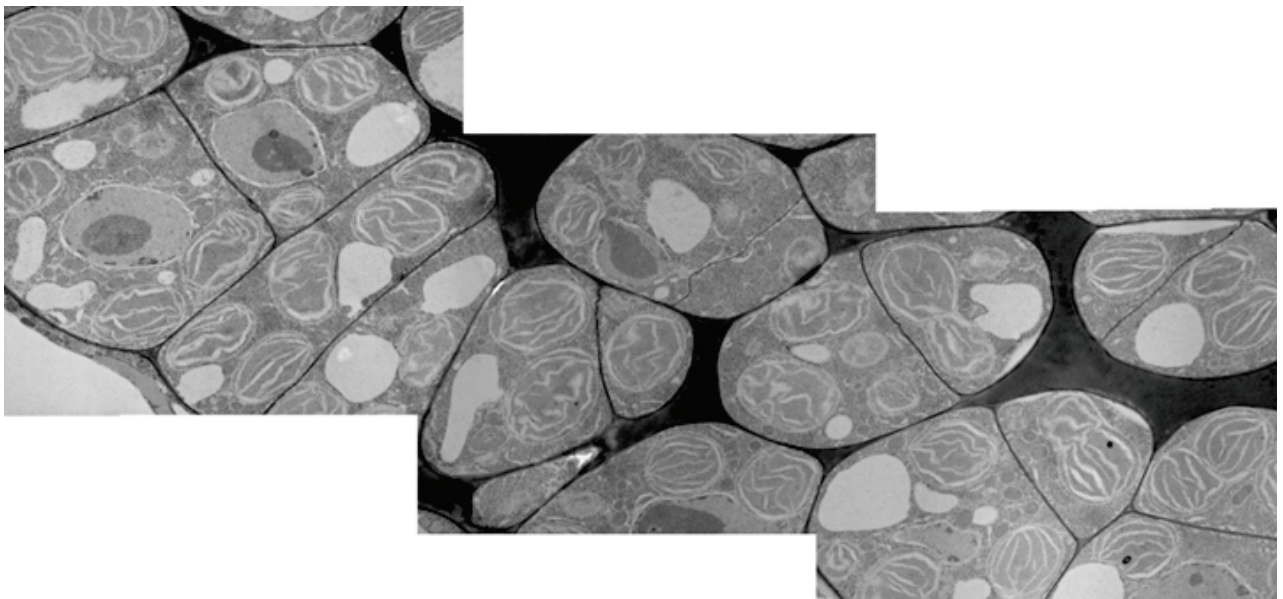
Part 2:

Results



CHAPTER 4

Chloroplasts Are Central Players in Sugar-Induced Leaf Growth

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ABSTRACT

Leaves are the plant's powerhouses, providing energy for all organs through sugar production during photosynthesis. However, sugars do not only serve as metabolic energy source for sink tissues, but also as signaling molecules, affecting gene expression through conserved signaling pathways to regulate plant growth and development. Here, we describe an *in vitro* experimental assay, allowing to alter the sucrose availability during early Arabidopsis (*Arabidopsis thaliana*) leaf development, with the aim to identify the affected cellular and molecular processes. The transfer of seedlings to sucrose-containing medium showed a profound effect on leaf growth by stimulating cell proliferation and postponing the transition to cell expansion. Furthermore, rapidly after transfer to sucrose, mesophyll cells contained less and smaller plastids which are irregular in shape and contain less starch granules compared with control mesophyll cells. Short-term transcriptional responses after transfer to sucrose revealed the repression of well-known sugar-responsive genes and multiple genes encoded by the plastid on the one hand, and up-regulation of a *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER*, *GPT2*, on the other hand. Mutant *gpt2* seedlings showed no stimulation of cell proliferation and no repression of chloroplast-encoded transcripts when transferred to sucrose, suggesting that GPT2 plays a critical role in the sucrose-mediated effects on early leaf growth. Our findings therefore suggest that induction of *GPT2* expression by sucrose increases the import of glucose-6-phosphate into the plastids that would repress chloroplast-encoded transcripts, restricting chloroplast differentiation. Retrograde signaling from the plastids would then delay the transition to cell expansion and stimulate cell proliferation.

INTRODUCTION

The energy needed for plant growth and development is produced by photosynthesis in leaves, capturing and converting light into chemical energy, which is stored into sugars and transported to all other plant organs to meet their energy demands.

Arabidopsis leaves arise from the shoot apical meristem as leaf primordia, which initially grow exclusively by cell proliferation. Subsequently, cell proliferation ceases at the tip of the leaf and gradually the cells start to expand in a tip-to-base direction (Andriankaja *et al*, 2012; Donnelly *et al*, 1999). After a few days, this cell cycle arrest front abruptly disappears at the base of the leaf and further leaf growth is driven by cell expansion (Andriankaja *et al*, 2012; Kazama *et al*, 2010) and the asymmetric division of meristemoids, i.e. precursors of stomata in the epidermis (Geisler *et al*, 2000; Gonzalez *et al*, 2012; Kazama *et al*, 2010).

Leaves, which actively perform photosynthesis, so-called source leaves, produce their own energy and carbon sources for growth and development. Contrastingly, plant tissues that are unable to photosynthesize, such as roots, flowers and young growing leaves, depend on these source leaves for carbon supply to grow (Turgeon, 1989). The primary end-products of photosynthesis are triose phosphates, which are rearranged into glucose-6-phophate (G6P) and used for the formation of starch as storage molecules or transported into the cytosol to form sucrose. In the source leaves, sucrose can be metabolized to its hexose products, i.e. glucose and fructose, it can be stored in the vacuole, or it can be transported through the phloem to the sink tissues (Lemoine *et al*, 2013). Sucrose is either imported directly in the sink cells via active sucrose transporters located at the plasma membrane (Kühn & Grof, 2010) or via plasmodesmata, or it is first cleaved to its hexose products by cell wall invertases in the apoplast (Ruan *et al*, 2010; Sturm, 1999). In the sink cells, hexoses are imported in the plastids for starch biosynthesis or for the oxidative pentose phosphate pathway. The main glucose transporters in photosynthetically inactive sink cells are the plastid-located G6P transporters, i.e. GPT1 and GPT2, which import G6P in exchange for phosphates (Kammerer *et al*, 1998). *GPT1* is expressed throughout plant development (Niewiadomski *et al*, 2005). However, *GPT2* expression is limited to certain tissues, such as senescing leaves, and is induced under different conditions, such during relief of seed dormancy (Finch-Savage *et al*, 2007), during acclimatization to high light (Dyson *et al*, 2015), during glucose-induced senescence (Pourtau *et al*, 2006), in starch-free mutants (Heinrichs *et al*, 2012; Kunz *et al*, 2010) and when sugar levels increase (Gonzali *et al*, 2006; Müller *et al*, 2007; Osuna *et al*, 2007; Price *et al*, 2004). Chloroplasts are the central organelles performing photosynthesis and producing sugars. Photosynthetic active chloroplasts are derived from proplastids present in the meristematic cells (Charuvi *et al*, 2012; Sakamoto *et al*, 2009). Functional chloroplasts contain about 3,000 different proteins mainly involved in photosynthesis, transcription and translation, of which most are encoded by the nuclear genome. However, plastids also have their own DNA, the so-called plastome, consisting of 133 genes in Arabidopsis, of which 87 encode proteins with different functions, such as photosynthetic and ribosomal proteins (Sato *et al*, 1999; Wicke *et al*, 2011). Genes of the plastome are transcribed by two different RNA polymerases, i.e. a nucleus-encoded polymerase (NEP) and a plastid-encoded

polymerase (PEP) (Liere *et al*, 2011; Shiina *et al*, 2005). PEP consists of the plastome-encoded core subunits rpoA, rpoB, rpoC1 and rpoC2, and one of the six nucleus-encoded sigma-factors which define promoter specificity (Lerbs-Mache, 2011). Besides this core PEP complex, some non-core subunits have been identified to exhibit additional transcriptional functions, the so-called polymerase-associated proteins (PAPs; Steiner *et al*, 2011). During leaf development, both the NEP and PEP actively transcribe their specific target genes (Zoschke *et al*, 2007), of which most are organized in operons and transcribed into polycistronic mRNA from one single promoter, such as their bacterial ancestors (Wicke *et al*, 2011).

Obviously, the nucleus and chloroplasts have to exchange information to regulate photosynthesis in function of environmental conditions. To date, different signals have been described to be involved in this chloroplast-to-nucleus or 'retrograde' signaling (reviewed by Kleine & Leister, 2013). The best-known retrograde signals are the intermediates of tetrapyrrole synthesis, i.e. the precursors of chlorophyll, which have been identified through the analysis of *genomes uncoupled* (*gun*) mutants, in which nuclear photosynthesis-related gene expression is maintained when chloroplast differentiation is perturbed by norflurazon (NF) treatment (Susek *et al*, 1993; Terry & Smith, 2013). Furthermore, reactive oxygen species, the redox state of the plastoquinone pool of the chloroplasts and of redox components such as glutathione and ascorbate (Oelze *et al*, 2012; Pfalz *et al*, 2012; Shapiguzov *et al*, 2012), as well as different hormone signals and the plastid gene expression itself (Tiller & Bock, 2014) have been reported to exert signals from chloroplasts to regulate nuclear gene expression. Additionally, sugars can act as signals in retrograde and other signaling pathways, integrating environmental and developmental changes during plant growth (Häusler *et al*, 2014; Smeekens & Hellmann, 2014). For example, sugars can modulate nuclear gene expression, especially the repression of nucleus-encoded photosynthesis genes, such as *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) and the small subunit of RUBISCO (*RBCS*), to control feedback regulation of photosynthesis (Krapp *et al*, 1993). However, our knowledge of sugar-regulated transcripts comes from studies using a wide variety of plant organs and tissues, developmental stages, treatments with different sugars and growth conditions. Furthermore, in most studies, sugars are applied to cell suspension cultures, detached leaves or liquid cultures (Kunz *et al*,

2014; Li *et al*, 2006; Müller *et al*, 2007; Osuna *et al*, 2007; Price *et al*, 2004; Usadel *et al*, 2008), highlighting the need for more targeted experimental designs to study the effect of sucrose on organ growth such as leaves. In addition, most sugar-feeding experiments make use of high, non-physiological glucose or sucrose concentrations (Gonzali *et al*, 2006; Heinrichs *et al*, 2012; Li *et al*, 2006; Müller *et al*, 2007; Price *et al*, 2004).

During leaf development, it has been observed that the transition from cell proliferation to cell expansion occurs simultaneously with the onset of photosynthesis (Andriankaja *et al*, 2012). Furthermore, an up-regulation of transcripts encoding proteins involved in tetrapyrrole synthesis has been observed in leaves just before the start of the transition to cell expansion. These findings suggest a role for differentiation of the photosynthetic machinery and associated retrograde signaling in the transition to cell expansion. Contrastingly, in a recent study using the *crumpled leaf* mutant deficient in chloroplast development, it has been demonstrated that impaired chloroplast differentiation affects cell proliferation and induces an early onset of cell differentiation (Hudik *et al*, 2014). Young proliferating leaves first depend on the supply of sugars, produced by photosynthetically active source leaves, to grow. Reduced photosynthetic activity of source leaves or reduced sugar availability triggers young, proliferating leaves to produce their own sugars and energy for further growth (Li *et al*, 2006). To do so, chloroplasts need to differentiate to start photosynthesis, producing sugars and other retrograde signals, which could trigger the transition to cell expansion. Hence, it is obvious that a cross talk exists between sugars, chloroplasts and leaf growth, but which process, i.e. cell proliferation or expansion, is affected by sugars and how sugars are sensed during leaf growth still need to be investigated in detail.

Here, we exogenously supplied sucrose during growth of Arabidopsis seedlings and found that sucrose increases final leaf size by promoting cell proliferation and postponing the transition to cell expansion. Furthermore, transcriptome and microscopic analyses of the growing leaves revealed a central role for chloroplast differentiation and GPT2 during the sucrose-induced promotion of leaf growth. Transfer of seedlings to sucrose resulted in reduced plastome transcription and smaller chloroplasts per mesophyll cell, which were irregular in shape and less differentiated compared with control seedlings. Also, in *gpt2* mutant seedlings, cell

proliferation was not stimulated and chloroplast transcription was not repressed upon transfer to sucrose.

RESULTS

Development of an Experimental Setup to Analyze the Influence of Exogenously Supplied Sucrose on Final Leaf Size

To investigate in detail how sugars regulate early leaf growth, we designed an experimental assay in which the sugar status is changed at a specific developmental stage during growth of Arabidopsis seedlings, and in which the impact of this change on leaf growth can then easily be monitored.

We first tested the effect of three different sucrose and glucose concentrations (6 mM, 15 mM and 30 mM) and found none of the glucose concentrations to reproducibly increase the third leaf size at 21 days after stratification (DAS) (Supplemental Fig. S1). On the other hand, when plants were germinated and grown under a 16-h day/8-h night cycle on sucrose-containing medium, a clear effect could be measured at 21 DAS both on rosette and individual leaf areas (Fig.1, A and B). The three concentrations tested, i.e. 6 mM, 15 mM, and 30 mM, resulted in a significant average increase in rosette area (40%, 56% and 44%, respectively; $P < 0.05$) compared with plants grown on medium without sucrose (Fig. 1A). The measurements of individual leaf area showed that all leaves (with the exception of the cotyledons and the two first leaves) of the plants grown on sucrose-containing medium were larger ($P < 0.05$) compared with control plants (Fig. 1B). Because a concentration of 15 mM sucrose resulted in the largest significant increase in size both of the whole rosette and of the third true leaf ($P < 0.0001$), it was retained for further characterization at the cellular level.

We also tested two different light intensities to optimize the effect of the supplemented sucrose on the final leaf size, because different light intensities may change the photosynthetic capacity and, consequently, the endogenous sugar production in the leaf. At a light intensity of 65±5 µmol m^{-2} s^{-1}, germination on medium containing 15 mM sucrose resulted at 21 DAS in an increase in the third leaf size of on average 16% as compared with control plants germinated on medium without sucrose (Fig. 1C).

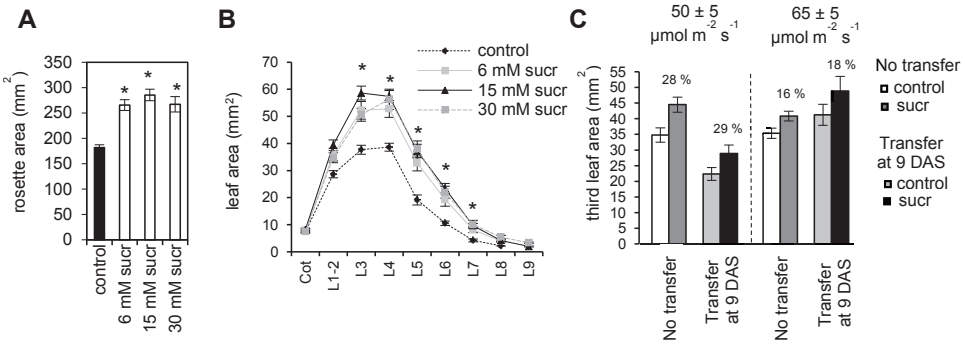

Figure 1. Rosette and individual leaf area increase upon sucrose treatment. A-B, Plants were germinated on different sucrose (sucr) concentrations (0 (control), 6, 15 or 30 mM), and the average rosette area (A) and average individual leaf area (B) were measured at 21 DAS. Cot = cotyledons; Lx = leaf position x in the order of appearance on the rosette. Values are the means of three biological repeats with their SE. Rosette and leaf area was measured for 6 to 10 plants in each repeat. C, Third leaf area of plants germinated on 15 mM sucrose or germinated on sucrose-free (control) medium (no transfer) and transferred at 9 DAS to 15 mM sucrose or control medium, measured at 21 DAS at a light intensity of approximately 65 µmol m-2 s-1 or at a lower light intensity of 50 µmol m-2 s-1. Values are the means of three biological repeats with their SE. Leaf area was measured for 4 to 33 leaves in each repeat. *, adjusted $P < 0.05$ for log-transformed values in (A), mixed models (see Supplemental Methods).

However, when plants were grown at a lower light intensity (50±5 µmol m^{-2} s^{-1}), 15 mM sucrose resulted in an on average 28% increase of the third leaf size (Fig. 1C). Additionally, to study the short-term effects of sucrose during early leaf growth, seedlings were first grown on a mesh (see Material and Methods) covering a sugar-free medium, and subsequently transferred to medium supplemented with 15 mM sucrose. Transfer was done at 9 DAS, the time point at which the third leaf is fully proliferating (Andriankaja *et al*, 2012), demonstrating a similar increase of the third leaf area at 21 DAS of on average 18% and 29% at both light intensities (65±5 µmol m^{-2} s^{-1} and 50±5 µmol m^{-2} s^{-1}, respectively), compared with the control plants transferred to medium without sucrose (Fig. 1C). Statistical analysis revealed a significant average increase in the third leaf area upon sucrose supplementation independent of the use of meshes or not as well as the different light intensities ($P < 0.05$).

In conclusion, we developed an experimental assay, in which transfer of plants grown at a light intensity of 50±5 µmol m^{-2} s^{-1} at 9 DAS to a growth medium with 15 mM sucrose, reproducibly increases the size of the third leaf at 21 DAS. This setup, using the meshes, was used in all following experiments to study the underlying cellular and molecular mechanisms by which sucrose affects plant growth.

Sucrose Positively Affects Leaf Growth by Promoting Cell Proliferation

To identify the cellular process involved in the sucrose-induced enlarged leaf size, the pavement cell number, cell size and stomatal index were measured at 21 DAS, twelve days after transfer of seedlings to medium with or without 15 mM sucrose (at 50±5 µmol m^{-2} s^{-1}). Transfer of plants to sucrose resulted in a significant average increase of the third leaf area of 47% ($P < 0.05$) (Fig. 2A) due to a significantly higher total pavement cell number (37%; $P < 0.05$), whereas the cell size remained unchanged ($P = 0.11$) (Fig. 2B). Also the stomatal index, i.e. the fraction of guard cells in the total population of epidermal cells, was slightly but significantly increased compared with the control (7%; $P < 0.05$) (Fig. 2B). Thus, sucrose increases the final third leaf size mainly by promoting cell proliferation.

To analyse the effect of sucrose on cell proliferation in more detail, a time-course experiment was performed by harvesting the third leaf daily after transfer, from 10 DAS until 21 DAS, and measuring its leaf area. At 12 DAS (3 days after transfer), the third leaf size of sucrose-transferred plants was significantly larger than that of control plants with an average increase of 39% (Fig. 2C; $P < 0.05$). Additionally, the relative leaf growth rate of sucrose-grown plants was slightly, but not significantly, higher, whereas later during development, the growth rates remained unchanged compared with control plants (Supplemental Fig. S2). Because sucrose significantly increased the third leaf area within three days after transfer, cellular measurements were performed on early time points during leaf growth (10-16 DAS; Fig. 2D-F). The total pavement cell number was significantly increased by 35% ($P = 0.02$; Fig. 2E), already 24 h (10 DAS) after transfer (Fig. 2D), whereas the cell area did not change ($P = 0.32$; Fig. 2F). The total pavement cell number remained higher until 16 DAS (Fig. 2D). No consistent changes in the average cell size were found between control and sucrose-transferred plants at early time points during leaf growth (10-16 DAS; Fig. 2F).

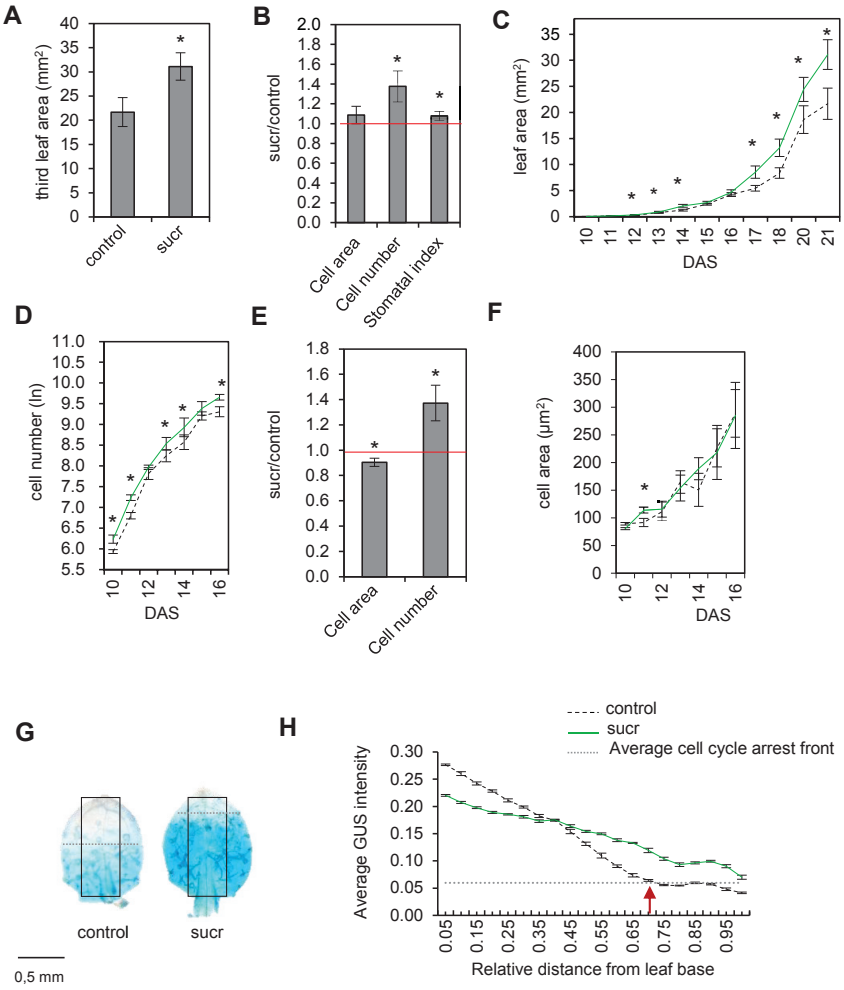

Figure 2. Cellular changes upon transfer to sucrose. Seedlings were first grown on medium without sucrose (sucr) and, at 9 DAS, transferred to medium supplemented with or without 15 mM sucrose. A, Third leaf area at 21 DAS. B, Ratio of the pavement cell number, cell area and stomatal index of the third leaf of plants transferred to 15 mM sucrose relative to the control (0 mM sucr), at 21 DAS. C, Leaf area from 10 DAS until 21 DAS. The inset is a close-up of 10 DAS until 14 DAS. D, Pavement cell number from 10 DAS until 16 DAS. E, Ratio of the pavement cell area and number of the third leaf of seedlings, 24 hours after transfer to 15 mM sucrose (sucrose) relative to the control (0 mM sucr). F, Cell area from 10 DAS until 16 DAS. G and H, GUS-stained third leaves at 13 DAS of *pCYCB1;1::CYCB1;1-D-box:GUS* seedlings transferred to control or sucrose-containing medium (G) and the GUS intensity plot of these leaves (H). GUS staining was in a defined region from the base to the tip of each leaf as indicated by the black rectangle in G. The dotted lines indicate the cell cycle arrest front. Above this front is the division zone, below is the expansion zone. Red arrow indicate the position of the average cell cycle arrest front of control leaves. Values in (A), (B) and (C) are the means of three biological repeats with their SE. Leaf area was measured for 5 to 20 leaves in each repeat. Cellular data are from five leaves in each repeat. Values in (D), (E) and (F) are the means of four to five leaves with their SE. Values in (H) are the means of two biological repeats with their SE. GUS intensity was measured for 8 to 10 leaves in each repeat. *, adjusted $P < 0.05$ for log-transformed values in (A) to (F), mixed models (see Supplemental Methods).

To further analyze the effect of the transfer to sucrose on cell proliferation and the transition to cell expansion, the *pCYCB1;1::CYCB1;1-D-box:GUS* (Eloy *et al*, 2012) reporter line, which allows visualizing actively dividing cells, was used. After 9 days of growth without sucrose, *pCYCB1;1::CYCB1;1-D-box:GUS* seedlings were transferred to control and sucrose-supplemented medium and grown for four additional days until 13 DAS. Subsequently, the third leaf was harvested, and stained with 5-bromo-4-chloro-3-indolyl-β-glucuronide. The GUS intensity was measured in a defined region from the base to the tip of each leaf, as indicated in Figure 2G. At 13 DAS, control leaves showed a cell cycle arrest front positioned closer to the leaf base (Fig. 2H, dotted line) compared with sucrose-transferred leaves. Hence, a large number of third leaf cells of plants grown for four days on sucrose were still proliferating, whereas cell expansion was initiated in most cells of the control leaves at 13 DAS.

Taken together, above results indicate that addition of sucrose to the medium promotes early leaf growth by stimulating cell proliferation. Although the positive effect of sucrose on leaf size was only clear after three days of growth on sucrose, a significant underlying effect on the cell number was observed already after 24 h.

Short-Term Effects of Sucrose on the Transcriptome

To gain more insight into the molecular mechanisms driving leaf growth upon exogenous sucrose application, short-term transcriptional responses were analyzed using RNA-sequencing (RNA-seq). Because sucrose solely affects cell proliferation, we micro-dissected the third leaf very early during development (at an average size of 0.04 mm^2) and extracted RNA to be used for RNA-seq. Nine-day-old plants were transferred to medium with or without sucrose for 3 and 24 h. Only 19 genes and 69 genes were found to be differentially expressed 3 and 24 h after transfer, respectively (Log_2FC of > 0.58 and a FDR < 0.05) (Fig. 3A, Supplemental Table S1).

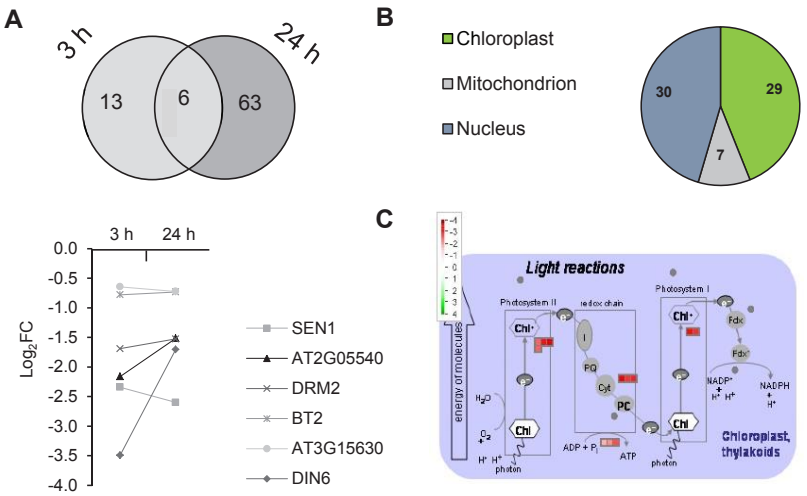

Figure 3. Sucrose-induced transcriptional responses in growing leaves. A, Overlap between differentially expressed genes in the third leaf, micro-dissected at 3 h and 24 h of seedlings transferred to 15 mM sucrose or control medium, and $\log_2$ fold-changes (Log_2FC) at 3 h and 24 h of the six common genes. Data are from a RNA-sequencing analysis. B, Pie chart of differentially expressed transcripts 24 h after transfer to 15 mM sucrose. C, MAPMAN representations of enriched genes differentially repressed 24 h after transfer to 15 mM sucrose in the third leaf.

At three hours, only three of the 19 differentially expressed genes were found to be induced: AT3G49110 and AT5G58390, encoding peroxidase proteins, and *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER2* (*GPT2*), which was the highest up-regulated gene with a Log_2FC of 2.04. Six of the 16 repressed genes at 3 h (*DIN6, SEN1*, AT2G05540, *DRM2*, *BT2*, AT3G15630) remained repressed 24 h after transfer (Fig. 3A). Two of these genes, i.e. *SEN1* and *DIN6*, belong to the so-called *DARK INDUCED* class of genes (Fujiki *et al*, 2000), both well-known as sugar starvation markers, and showed highly reduced transcript levels at 3 h with a Log_2FC of -2.34 and -3.49, respectively (Fig. 3A). The remaining four genes, which were repressed both at 3 h and 24 h, encode a protein with telomerase activity (BT2), a glycine-rich protein (AT2G05540), a dormancy/auxin associated protein (DRM2) and a protein with unknown function (AT3G15630). The other ten genes, which were down-regulated at 3 h but regained normal expression levels at 24 h, encode three hydrolase superfamily proteins (AT2G32150, AT2G39400 and AT1G04280), an unknown protein (AT1G68440), an aluminium-induced protein (AT3G15450), an oxidative stress protein (OXS3; AT5G56550), another glycine-rich protein (AT2G05380) as well as another dormancy-associated protein (DRM1), a

chloroplast-targeted DnaJ protein J8 (AT1G80920) and PV42a (AT1G15330). *PV42a* was the second highest repressed gene at 3 h and encodes a protein belonging to the class of γ-subunits of the plant-specific Sucrose Non-Fermenting1 (SNF1)-related Protein Kinase 1 (SnRK1) complexes, which are central metabolic sensors activated when environmental stress conditions deplete carbon and energy supply and which are known to link the sugar status with organ growth (Baena-González *et al*, 2007; Fang *et al*, 2011; Gissot *et al*, 2006).

The transcript levels of 69 genes were significantly changed 24 h after transfer to sucrose, of which the vast majority (66) showed a decrease in expression compared with the control. The three up-regulated genes encode a cytochrome P450 protein, i.e. CYP710A2 (AT2G34490) with C22-sterol desaturase activity, a pentatricopeptide repeat (PPR) superfamily protein (AT5G06400) and a stearoyl-acyl-carrier-protein desaturase family protein (AT1G43800). Surprisingly, of the 66 (6 also at 3 h and 60 only at 24 h) repressed genes, 30 were encoded by the nuclear genome, while 29 were located on the chloroplast DNA and 7 on mitochondrial DNA (Fig. 3B). A list of the sucrose-repressed, chloroplast-encoded transcripts at 3 h and 24 h is shown in Table 1. From the genes encoded by the nuclear genome, several have been reported to be induced by sugar starvation and were here found to be repressed by short-term sucrose treatment, e.g. genes encoding anhydrases, oxygenases and hydrolases, as well as *DIN* genes (Fujiki *et al*, 2000; Lee *et al*, 2007). Sucrose-repressed genes located on mitochondrial DNA encode subunits of the electron transport chain complexes and mitochondrial ribosomal proteins. The repressed genes located on chloroplast DNA represented a mixture of genes belonging to different operons and coding for photosynthesis-related proteins involved in the light reactions, such as photosystem I and II proteins (*psa* and *psb*), proteins part of the cytochrome b_6f complex (*pet*), and subunits of NADPH dehydrogenase and ATP synthase (*atp*) (Fig. 3C). Furthermore, genes encoding proteins involved in photosystem I and II assembly and stability (*ycf*), chloroplast ribosomal proteins (*rps*), a maturase involved in intron splicing (*matK*), an acetyl-CoA carboxylase subunit (*accD*), the large subunit of ribulose-1,5-bifosfate carboxylase oxygenase (Rubisco, *rbcL*) and one gene encoding the β-subunit of the plastid-encoded RNA polymerase (PEP, *rpoC2*), were found to be down-regulated. These transcriptional changes were

confirmed by quantitative reverse transcription (qRT)-PCR analysis for a set of selected chloroplast genes (Supplemental
Fig. S3).

Table 1. Log_2 fold changes and corresponding *P*-values for the 29 sucrose-repressed chloroplast-encoded transcripts 3 h and 24 h after transfer to sucrose.

Gene ID	Name	Description	3 h		24 h	
			Log_2FC	*P*-value	Log_2FC	*P*-value
ATCG01090	***ndhI***	Subunit of the chloroplast NAD(P)H dehydrogenase complex	-0.54	0.47	-3.57	2.42E-04
ATCG00360	***ycf3***	Protein required for photosystem I assembly and stability	0.10	0.72	-3.38	3.42E-05
ATCG00020	***psbA***	photosystem II reaction center protein A	-0.14	0.93	-3.37	2.28E-09
ATCG00730	***petD***	Subunit IV of the cytochrome b6/f complex	-0.18	0.94	-3.27	3.06E-07
ATCG00340	***psaB***	D1 subunit of photosystem I and II reaction centers	-0.09	0.81	-3.14	1.85E-08
ATCG00280	***psbC***	CP43 subunit of the photosystem II reaction center	-0.16	0.96	-3.12	2.67E-08
ATCG00520	***ycf4***	Protein required for photosystem I assembly and stability	-0.23	1.00	-3.07	8.37E-06
ATCG00720	***petB***	Cytochrome b(6) subunit of the cytochrome b6f complex	-0.23	0.89	-3.03	1.38E-07
ATCG01100	***ndhA***	NADH dehydrogenase ND1	-0.09	0.89	-3.02	4.18E-05
ATCG00490	***rbcL***	Large subunit of RUBISCO	-0.18	0.95	-2.99	5.49E-08
ATCG00350	**psaA**	Protein comprising the reaction center for photosystem I along with psaB protein	-0.13	0.92	-2.93	1.09E-07
ATCG00540	***petA***	Cytochrome f apoprotein	-0.31	0.65	-2.89	9.87E-07
ATCG00650	***rps18***	Chloroplast ribosomal protein S18	-0.50	0.38	-2.76	1.89E-04
ATCG01040	***ycf5***	Hypothetical protein	-0.49	0.31	-2.76	3.72E-04
ATCG00140	***atpH***	ATPase III subunit	0.10	0.77	-2.68	2.23E-04
ATCG00040	***matK***	Maturase located in the trnK intron in the chloroplast genome	-0.32	0.68	-2.55	8.28E-08
ATCG00160	***rps2***	Chloroplast ribosomal protein S2	-0.39	0.65	-2.55	2.03E-04
ATCG00270	***psbD***	Photosystem II reaction center protein D	-0.05	0.74	-2.54	4.03E-06
ATCG00680	***psbB***	Photosystem II reaction center protein B	-0.11	0.87	-2.52	5.41E-07
ATCG00330	***rps14***	Chloroplast ribosomal protein S14	0.20	0.55	-2.48	5.54E-05
ATCG00420	***ndhJ***	NADH dehydrogenase subunit J	-0.31	0.63	-2.46	6.86E-05
ATCG00500	***accD***	Carboxytransferase βsubunit of the Acetyl-CoA carboxylase (ACCase) complex	-0.70	0.18	-2.32	3.90E-04
ATCG01050	***ndhD***	Subunit of a NAD(P)H dehydrogenase complex	-0.11	0.90	-2.26	8.12E-05
ATCG00380	***rps4***	Chloroplast ribosomal protein S4	-0.07	1.00	-2.19	2.25E-04
ATCG00120	***atpA***	ATP synthase subunit alpha	-0.20	0.92	-2.13	5.70E-06
ATCG01110	***ndhH***	49KDa plastid NAD(P)H dehydrogenase subunit H protein	-0.52	0.28	-2.08	3.45E-04
ATCG00170	***rpoC2***	DNA-directed RNA polymerase β' subunit-2	-0.16	0.98	-1.87	8.94E-05
ATCG01130	***ycf1.2***	Hypothetical protein	-0.35	0.60	-1.81	2.03E-05
ATCG00150	***atpI***	Subunit of ATPase complex CF0	-0.36	0.59	-1.78	3.43E-04

Furthermore, because not all genes encoded by the plastome were found to be significantly differentially expressed by sucrose at 24 h, the effect of sucrose on the complete plastome was studied by a gene set enrichment analysis, in which all chloroplast-encoded transcripts were analyzed together as a single gene set. We found that the chloroplast gene set was significantly down-regulated compared with all other genes (21,481 genes, P = 0), 24 h after transfer to sucrose. The repression of plastome expression upon transfer to sucrose can result from either a reduced

plastome copy number or less chloroplasts per cell. However, quantitative PCR (qPCR) on total cellular DNA demonstrated no differences in chloroplast DNA copy number in the third leaf, 3 h and 24 h after transfer to sucrose-supplemented or control medium (Supplemental Fig. S4).

In conclusion, transfer to sucrose resulted in repression of plastome transcription, while plastome copy number was not affected, which suggests reprogramming of chloroplasts upon transfer to sucrose.

Blocking Chloroplast Differentiation Also Induces Cell Proliferation

To investigate if changes in chloroplast differentiation affect the sucrose-induced cellular processes, seedlings were treated with norflurazon (NF), a herbicide that inhibits phytoene desaturase by competition with the cofactors. Phytoene desaturase is involved in carotenoid biosynthesis and treatment of plants with NF inhibits chloroplast development (Koussevitzky *et al*, 2007).

Seedlings were transferred at 9 DAS to Murashige and Skoog (MS) medium with or without sucrose (MS±S), and medium with or without sucrose supplemented with 5 µM NF (MS±S+NF). Three days after transfer to NF with or without sucrose (12 DAS), smaller seedlings with bleached leaves could be observed, indicating a clear effect on chloroplast differentiation (Fig. 4A). At 10 DAS, or 24 h after transfer, the third leaf area was significantly increased by sucrose with 42% ($P < 0.05$; Fig. 4B), and with 16%, although not significantly, when transferred to sucrose-containing medium supplemented with NF ($P = 0.65$; Fig. 4B). To study the underlying cellular processes induced by sucrose, the relative increases in cell size and pavement cell number on sucrose-containing medium supplemented with NF or not were determined. Sucrose did not result in an altered cell size, and addition of NF did not change this, whereas the pavement cell number was significantly increased by sucrose with 32% ($P < 0.05$) and with 34% by addition of NF, albeit not significantly ($P = 0.10$) compared to control seedlings (Fig. 4C). Furthermore, cell size was significantly decreased when NF was added to the medium, and this reduction was equal between leaves of seedlings grown on sucrose and without sucrose in the medium ($P < 0.05$; Fig. 4D, left). Remarkably, third leaves of seedlings grown without sucrose, but with NF, had a similar average increase in total pavement cell number

(27%; P = 0.10) as seedlings grown with sucrose and without NF (34%; P < 0.05) compared with control seedlings on MS medium (Fig. 4D, right). Moreover, combination of both sucrose and NF resulted in significant increase in pavement cell number of 62% (P < 0.05) compared with control leaves, which was equal to the sum of the effects of NF and sucrose separately (61%; Fig. 4D, right). We therefore hypothesize that NF and sucrose act additively on cell number.

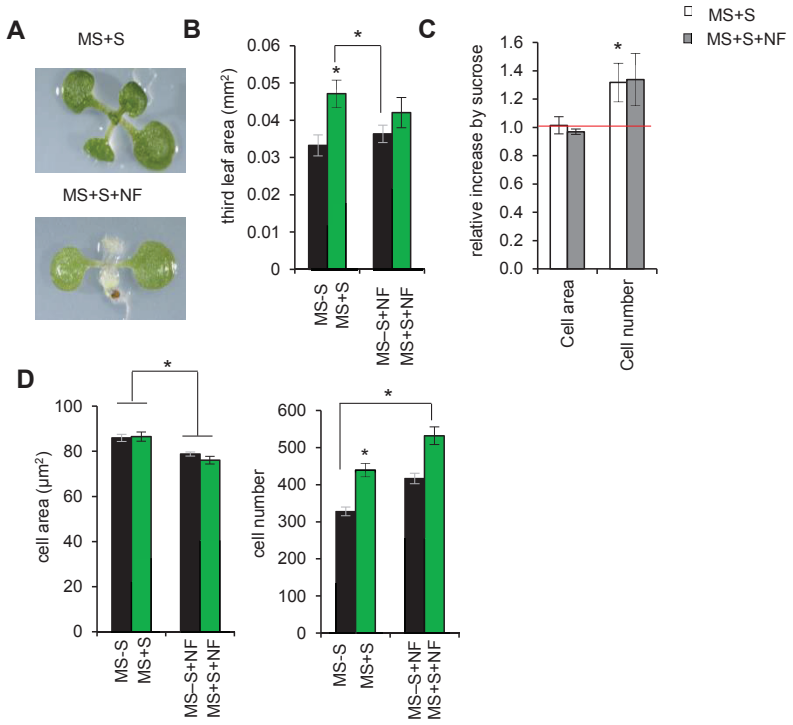

Figure 4. Cellular effects of sucrose with or without NF. Seedlings were transferred at 9 DAS to normal MS medium with sucrose (MS+S), MS medium without sucrose (MS-S), and MS±S supplemented with 5 µM norflurazon (MS±S+NF). A, Image of seedlings, 3 days after transfer to MS+S or MS+S+NF. B, Third leaf area, 24 hours after transfer to MS±S or MS±S+NF. C, Relative increase of cell area and pavement cell number of the third leaf, 24 h after transfer to MS±S or MS±S+NF. D, Cell area and pavement cell number of the third leaf of seedlings transferred to MS±S or MS±S+NF. Values are the means of three biological repeats with their SE. Leaf area was measured for 4 to 15 leaves in each repeat. Cellular data are from three to five leaves in each repeat.*, adjusted P < 0.05 for log-transformed values in (B), mixed models (see Supplemental Methods).

Transfer to Sucrose Results in Fewer, Smaller and Less Differentiated Chloroplasts

Because sucrose rapidly represses chloroplast-encoded transcripts, on the one hand, and cell proliferation can be stimulated by blocking chloroplast differentiation by NF, on the other hand, we set out to study the effect of sucrose on chloroplast number and morphology by transmission electron microscopy.

Leaves were harvested 24 h (10 DAS) after transfer to sucrose-containing or control medium and, subsequently, transverse sections were made to examine differences in chloroplast thylakoid structure, chloroplast size and number. For each leaf, 17 to 87 mesophyll cells of the tip and the base of the leaf, representing the cell expansion and proliferation region, respectively, were analysed. A clear difference in thylakoid structure and organization, as well as chloroplast shape, could be observed between leaves of control and sucrose-transferred seedlings (Fig. 5). Generally, in control leaves, chloroplasts seem to be more differentiated compared with leaves of seedlings transferred to sucrose. Some chloroplasts already start to form starch granules, they generally have more thylakoid membranes and start to form typical lens-shapes of mature chloroplasts (Fig. 5A). In contrast, the chloroplasts of sucrose-transferred seedlings are generally more irregular in shape and less starch formation could be observed. To quantify the difference in starch formation, starch grains were counted in transverse sections of mesophyll cells of the control and sucrose-transferred leaves. In general, leaves of sucrose-transferred seedlings contained on average 0.14 starch granules per mesophyll cell, whereas control leaves had 0.29 starch granules (Fig. 5B). Similarly, increased starch accumulation was seen by Lugol's staining at 12 DAS in control leaves compared with leaves of sucrose-treated seedlings (Supplemental Fig. S5). Mesophyll cell area, chloroplast number and chloroplast size were measured and statistically analyzed, taking into account the differences between the tip and base of the leaf. The average mesophyll cell area did not differ significantly between leaves of control and sucrose-transferred seedlings (Fig. 5C). Chloroplasts were significantly larger at the tip compared with the base of the leaves in control seedlings (44%, $P < 0.05$; Fig. 5D). In sucrose-transferred leaves, the chloroplast areas were not significantly different between the tip and the base ($P = 0.45$). Additionally, chloroplasts were significantly larger in the tip of control leaves compared with the tip of sucrose-transferred leaves (50%, $P < 0.05$; Fig. 5D).

Furthermore, leaves of sucrose-transferred seedlings had less chloroplasts in transverse sections, although not significantly (P = 0.12; Fig. 5E).

In conclusion, transfer of seedlings to sucrose resulted in fewer, smaller and less differentiated chloroplasts with limited formation of thylakoid membranes and less starch granules.

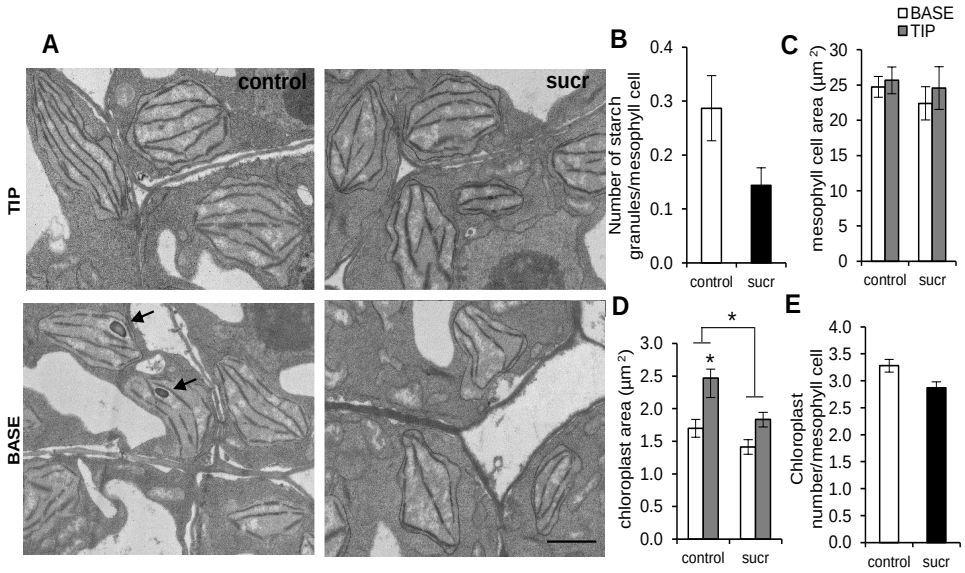

Figure 5. Differences in chloroplast morphology, number and size in the tip and base of sucrose-treated and control leaves. A, Transmission electron micrographs of tip and base of the 10-d-old third leaf, 24 hours after transfer to control or 15 mM sucrose (sucr) supplemented medium. Arrows point to starch granules. The bar represents 1 µm. B, Average number of starch granules per mesophyll cell counted in approximately 60 cells of two leaves of control and sucrose-transferred seedlings. C and D, Average mesophyll cell area (C) and average chloroplast size (D) in the tip and base of the third leaf of control and sucrose-treated seedlings. E, Chloroplast number of the third leaf of control and sucrose-treated seedlings. Values are the means of two independent leaves with their SE. Chloroplast data are form 17 to 87 mesophyll cells in the tip and the base of each leaf. *, adjusted P < 0.05 for log-transformed values in (D), mixed models (see Supplemental Methods).

Role of GPT2 in Sucrose-Induced Stimulation of Cell Proliferation

The above-described results demonstrate a clear negative effect of sucrose on plastome transcription as well as chloroplast development, resulting in stimulation of cell proliferation. Remarkably, one of the three nucleus-encoded genes that was induced by sucrose three hours after transfer, encodes the glucose-6-phosphate/phosphate transporter, GPT2. Recently, a central role of GPT2 in seedling development was described; *gpt2* seedlings lacking *GPT2* expression exhibit a delayed establishment and greening of the cotyledons (Dyson *et al*, 2014).

To explore whether GPT2 also has a pivotal role in the sucrose-induced stimulation of cell proliferation, we subjected *gpt2-1* mutant seedlings to the experimental sucrose assay. *gpt2-1* seedlings were grown together with their corresponding wild-type seedlings on control medium for nine days, after which they were then transferred to control or sucrose-supplemented media for 24 h, after which leaf area, cell size and pavement cell number were determined. Generally, third leaves of *gpt2-1* seedlings were significantly smaller than wild-type leaves at 10 DAS ($P < 0.05$; Fig. 6A), due to a significant decrease in pavement cell number ($P < 0.05$; Fig. 6B). Sucrose significantly increased third leaf size with 20% in wild-type seedlings, also due to a significantly increased total pavement cell number ($P < 0.05$). Remarkably, *gpt2-1* seedlings showed a completely insensitive cell proliferation response to the transfer to sucrose ($P = 0.81$; Fig. 6B), and no change of third leaf size (Fig. 6A). Cell sizes remained unchanged between both wild-type and *gpt2-1* leaves, independent of the transfer to sucrose-containing or control medium ($P = 0.44$; Fig. 6C).

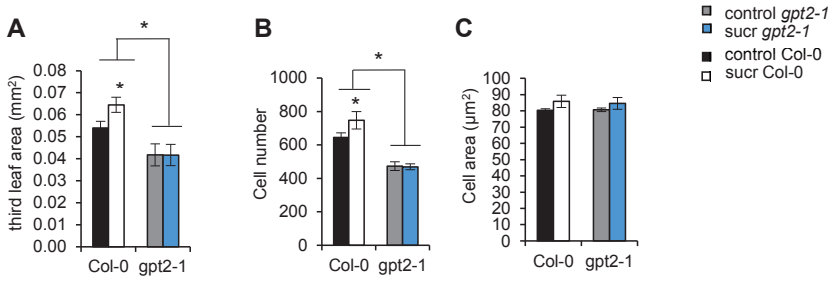

Figure 6. *gpt2* mutant seedlings show an insensitive cell proliferation response to the transfer to sucrose. *gpt2-1* mutant seedlings were grown together with their corresponding wild-type on medium without sucrose for nine days and, subsequently, transferred to medium supplemented with 15 mM sucrose (sucr) or without sucrose (control). A-C, At 10 DAS, 24 hours after transfer, the third leaf area (A), pavement cell number (B) and cell area (C) were determined and compared. Values are the means of three biological repeats with their SE. Leaf area was measured for 9 to 40 leaves in each repeat. Cellular data are from three to fourteen leaves in each repeat. *, adjusted $P < 0.05$ for log-transformed values in (A), mixed models (see Supplemental Methods).

In conclusion, GPT2 has an essential role in the short-term stimulation of cell proliferation by sucrose. Seedlings without functional GPT2 have less cells and completely abolish the expected cellular response to sucrose leading to growth promotion.

GPT2 is Required for the Sucrose-Mediated Repression of Plastome Transcription

In the absence of GPT2, no stimulation of cell proliferation by sucrose could be observed. Subsequently, to investigate whether *GPT2* expression is also required for the downstream sucrose-induced transcriptional responses, a comparative analysis was done between our transcriptomics dataset and the published micro-array dataset of Dyson *et al* (2015). In latter study, transcriptome analysis was performed on mature leaves of the *gpt2.2* mutant and Ws-4 wild-type (Dyson *et al*, 2015). The dataset of Dyson *et al* (2015) was first filtered, using the same criteria as the transcriptome analysis described here (i.e. $Log_2FC > 0.58$ and $P < 0.05$). Consequently, comparison between the differentially expressed genes in the *gpt2.2* mutant with the 66 sucrose-repressed genes 24 h after transfer to sucrose, revealed a significant overlap of 20 genes (P = 8.37E-8, Chi-square test; Supplemental Fig. S6A). Remarkably, 19 of the 20 overlapping genes were chloroplast-encoded transcripts, representing a mixture of genes coding for different photosynthesis-related proteins, and only one mitochondria-encoded transcript, *rpl16* (Supplemental Fig. S6B). Furthermore, 18 of the 20 transcripts demonstrated an opposite effect in gene expression, namely, up-regulated in the *gpt2.2* mutant compared with the wild-type, and down-regulated upon transfer to sucrose. The two transcripts that did not show this opposite effect were the mitochondria-encoded transcript, *rpl16,* and the chloroplast-encoded transcript, *rps14*.

Consequently, this significant overlap between sucrose-repressed and *gpt2.2* up-regulated chloroplast-encoded transcripts, as well as the insensitivity of the *gpt2-1* mutant to stimulate cell proliferation upon transfer to sucrose, prompted us to test the expression of several sucrose-responsive genes with qRT-PCR in micro-dissected third leaves of wild-type and *gpt2-1* mutant seedlings, 24 h after transfer to control or sucrose-supplemented medium. The expression levels of ten chloroplast-encoded genes (*psbA*, *petD*, *psaA*, *psaB*, *ycf3*, *ndhI*, *atpH, rps18*, *rbcL* and *rpoC2*) were determined and compared between sucrose-transferred and control leaves in the wild-type and the *gpt2-1* mutant separately (Supplemental Fig. S6C). These ten chloroplast-encoded genes were selected based on the above-described transcriptome analysis and were also used for the confirmation of the sucrose-responsive repression (Supplemental Fig. S3). Notwithstanding that no significant

differences for each gene could be detected ($P > 0.05$), a clear contrasting trend in relative expression levels was observed between wild-type and *gpt2-1* seedlings. All chloroplast transcripts were expressed at lower levels upon transfer to sucrose in wild-type leaves, whereas 8 of 10 of these transcripts were up-regulated in *gpt2-1* mutant leaves (Supplemental Fig. S6C).

Taken together, these results demonstrate that chloroplast DNA transcription is affected in the *gpt2* mutant, but that this transcriptional response is opposite to wild-type plants transferred to sucrose, suggesting a central role for GPT2 in mediating the sucrose-induced repression of chloroplast DNA transcription.

DISCUSSION

The aim of this study was to identify the underlying cellular and transcriptional mechanisms of sugars in regulating early leaf growth in Arabidopsis. For this, we developed an experimental setup in which the sugar status was changed during the proliferating phase of the third leaf, which normally depends on other photosynthetically active leaves for carbon and energy supply. This is in contrast with the two first leaves which probably mainly depend on carbon provided by the cotyledons for their growth. We showed that sucrose had a pronounced effect on the leaf pavement cell proliferation phase. This observation is in agreement with previously described roles of sucrose in cell cycle regulation. In higher plants, the cell cycle is controlled by cyclin-dependent kinases (CDKs) and their interacting cyclins (CYCs), which in turn respond to developmental and environmental signals (Inzé & De Veylder, 2006; Komaki & Sugimoto, 2012). A link between sucrose and the cell cycle was first demonstrated through the use of sucrose to synchronize Arabidopsis cell suspension cultures (Goetz & Roitsch, 1999). Sucrose-starvation induces a reversible arrest in the G1 or G0 phase of the cell cycle and after re-supplementing sucrose to the growth medium, the cell cultures are synchronized. Sucrose mainly regulates the expression of D-type CYCs involved in the G1-to-S phase progression (Riou-Khamlichi *et al*, 2000). However, none of these major cell cycle regulators were differentially expressed in our transcriptome analysis, 3 or 24 h after transfer of

seedlings to sucrose. Hence, these findings suggest that sucrose has no impact on the transcription of the cell cycle machinery during leaf growth stimulation. Nonetheless, at 13 DAS, we clearly observed a difference in cell proliferation between control and sucrose-transferred plants through staining of the pCYCB1;1::CYCB1;1-D-box:GUS reporter line. Sucrose-treated leaves demonstrated a cell cycle arrest front closer to the tip, which suggest an increase in cell proliferation. This effect on cell proliferation could result from (post-)translational regulation. Sucrose starvation-induced translational control of cell division and cell growth has already been described in Arabidopsis cell cultures (Nicolaï *et al*, 2006; Rahmani *et al*, 2009). Several transcripts involved in protein synthesis, cell cycle and growth were less abundant in polysomal RNA compared with their total RNA and, thus, translationally repressed by sucrose starvation.

It is well known that sugars trigger conserved signalling systems regulating plant growth and development (Lastdrager *et al*, 2014; Smeekens *et al*, 2010). One of these major regulators is the conserved Sucrose Non-Fermenting1 (SNF1)-related Protein Kinases 1 (SnRK1s) in plants, SNF1 in yeast and AMP-activated kinase (AMPK) in animals, which are heterotrimeric serine/threonine kinases consisting of a catalytic α-subunit and two regulatory β- and γ-subunits (Ghillebert *et al*, 2011; Hardie *et al*, 2012; Polge & Thomas, 2007). These proteins act as metabolic sensors activated when environmental stress conditions deplete carbon and energy supply (Baena-González *et al*, 2007; Baena-González & Sheen, 2008). Interestingly, PV42a (AT1G15330), one of the cystathionine-β-synthase domain-containing proteins that belong to the γ-type subunits of SnRK1, was significantly repressed 3 h after transfer of seedlings to sucrose. Trehalose-6-phosphate (T6P) and glucose-6-phosphate (G6P) are known to inhibit SnRK1 activity (Nunes *et al*, 2013; Paul *et al*, 2008; Toroser *et al*, 2000; Zhang *et al*, 2009) and are tightly correlated with the cellular sucrose levels (Lunn *et al*, 2006). T6P is synthesized from G6P and UDP-glucose by trehalose-6-phosphate synthase 1 (TPS1) (Gómez *et al*, 2010), demonstrating a close link between G6P and T6P levels. G6P allosterically activates sucrose phosphate synthase (Huber & Huber, 1996), whereas inorganic phosphate (Pi) inhibits this enzyme, by which sucrose synthesis is buffered upon increased sucrose levels. T6P has been described to act as a sucrose signal, via the inhibition of SnRK1, in the regulation of many different aspects of plant development, such as

growth, flowering and senescence (reviewed by Lunn *et al*, 2014). Arabidopsis plants lacking TPS1 are embryo-lethal, due to an embryonic developmental arrest between the transition from cell proliferation to cell expansion (Eastmond et al, 2002). Plants overexpressing TPS1, and, thus, with a higher T6P content and lower G6P levels, have small dark green leaves, whereas plants overexpressing trehalose-phosphate hydrolase result in large pale green leaves due to low T6P and high G6P levels (Schluepmann *et al*, 2003). These phenotypes are in line with our results in which higher sucrose and, thus, G6P/T6P levels affect chloroplast differentiation.

The effect of sugars during early leaf growth has not yet been investigated and understanding the molecular processes that regulate growth requires the micro-dissection of these growing tissues. The use of whole young seedlings for transcriptome experiments mainly reveals differential gene expression in expanding tissues (Skirycz *et al*, 2010). To elucidate the sucrose-regulated transcriptional responses during growth of a young leaf, we developed a setup integrating plant developmental timing. Surprisingly, only 19 and 69 genes were differentially expressed in the developing leaf 3 and 24 h, respectively, after transfer of the seedlings to sucrose-containing medium, whereas previously published transcriptomics datasets generally resulted in approximately hundreds of sugar-induced, differentially expressed transcripts (Gonzali *et al*, 2006; Müller *et al*, 2007; Osuna *et al*, 2007; Price *et al*, 2004; Usadel *et al*, 2008). This difference in the abundance of the differentially expressed genes might be explained by the differences in the harvested samples (micro-dissected proliferating leaves performed here compared with whole seedlings or mature plants in other studies) as well as differences in sugar concentrations. Nevertheless, similar sugar-responsive genes were found in our transcriptional analysis of micro-dissected third leaves. A significant repression of specific plastid-encoded genes was already reported in response to sugar treatment of whole seedlings grown in liquid cultures or whole rosettes (Price *et al* 2004; Osuna *et al* 2007; Gonzali *et al* 2006). However, we found that the complete plastome transcriptome was significantly repressed without a change in plastome copy number per cell. At 3 h after exposure to sucrose, most of these chloroplast-encoded transcripts were already down-regulated, albeit not significantly (FDR > 0.05). These findings clearly demonstrate an effect of sucrose on plastome expression. Almost all proteins that are present in the chloroplasts are encoded by the nuclear genome. These proteins assembly in large complexes, such

as the photosynthetic systems PSI and PSII, around core protein components encoded by the plastid (Jarvis & Lopez-Juez, 2013). Hence, repressing chloroplast transcription can disturb the establishment of these important complexes, and, consequently might impair further chloroplast differentiation. Indeed, transfer of seedlings to sucrose-supplemented medium resulted in significant differences in chloroplast morphology. Generally, the chloroplasts were smaller and showed less differentiated thylakoid membranes and starch granules compared with control leaves. Besides that, chloroplasts were significantly larger in the tip compared with the base of control leaves. It has been shown that before cells start to expand at the leaf tip, transcripts involved in photosynthesis and retrograde signaling are up-regulated, which suggests a profound role of chloroplast differentiation in controlling the onset of cell expansion (Andriankaja *et al*, 2012). Moreover, leaves treated with NF, a chemical inhibitor of chloroplast differentiation, show a delay in the onset of cell expansion (Andriankaja *et al*, 2012). In concert, we found that leaves transferred to medium without sucrose, but with NF show a similar increase in total pavement cell number as seedlings transferred to media with sucrose. This phenotype was even more enhanced when both sucrose and NF were present, suggesting that both molecules would act additively to stimulate cell proliferation. NF directly acts on the chloroplasts itself, blocking chloroplast differentiation at an early stage, whereas transfer to sucrose affects plastome expression, which probably leads to less differentiated chloroplasts. By this, less retrograde signals are sent to stimulate the onset of cell expansion. Leaves of seedlings transferred to sucrose contained less chloroplasts in transverse sections compared with control leaves, showing that sucrose treatment not only results in smaller chloroplasts with reduced differentiation, but also negatively affects chloroplast division.

Besides the effects of sucrose on chloroplast-encoded transcripts and chloroplast morphology, sucrose induced the expression of *GPT2*. GPT2, together with its homolog GPT1, acts as a plastid phosphate antiporter involved in the transport of G6Ps between the cytosol and plastids in exchange for Pi (Knappe *et al*, 2003). Mutants lacking *GPT1* have been described to be embryo lethal (Andriotis *et al*, 2010), whereas a disruption of *GPT2* does not result in obvious growth defects in final growth stages (Niewiadomski *et al*, 2005). However, recently, several studies

identified GPT2 as an important regulator in seedling development and during acclimation to high light (Athanasiou *et al*, 2010; Dyson *et al*, 2014). In addition, micro-array analysis revealed higher transcript levels for photosynthesis-related and chloroplast-encoded genes in *gpt2* mutants (Dyson *et al*, 2015). A significant overlap showing opposite gene expression profiles was found between this micro-array analysis and the sucrose-repressed chloroplast-encoded transcripts 24 h after transfer and almost all selected chloroplast-encoded genes were not repressed upon transfer to sucrose in the *gpt2* mutant background. Furthermore, no increase in pavement cell number by sucrose could be observed in *gpt2* mutant seedlings. These observations further indicate the involvement of GPT2 in the sucrose-induced promotion of cell proliferation as well as in mediating the sucrose-induced repression of the chloroplast-encoded transcripts. Sucrose could directly induce the transcription of *GPT2* by which possibly more G6P, which is correlated with cellular sucrose levels (Lunn *et al*, 2006), is imported in the plastid. Alternatively, *GPT2* expression could also be regulated by glucose, which is cleaved from sucrose and which is further converted in G6P. Metabolic regulation of *GPT2* transcription has been reported in different studies. Microarray analysis of the *pho3* mutant, impaired in the *SUC2* gene encoding a sucrose transporter for phloem loading of sucrose, revealed a remarkable up regulation of both *GPT1* and *GPT2* expression, but the causative metabolic signal was not investigated in detail (Lloyd & Zakhleniuk, 2004). Another study analysing *GPT2* expression in dark-grown Arabidopsis seedlings treated for 6 h with a broad range of different sugar concentrations (0–200 mM), showed that mainly sucrose induces *GPT2* expression, whereas glucose treatment only affects its transcription moderately (Gonzali *et al*, 2006). Our results also show that low concentrations of glucose do not stimulate leaf growth, probably because glucose is not transported through the phloem (Liu *et al*, 2012). Whether intracellular conversion of sucrose to G6P in the sink tissue is needed to regulate *GPT2* transcription, remains elusive. Further experiments using a different kind of experimental setup in which sugars can be directly supplied to the sink tissue, for example using plant cell cultures, would be interesting to identify the causative metabolic signal.

Taken together, GPT2 imports G6P in the chloroplast, which signals the sucrose status of the cytosol to the chloroplasts to adjust their development and, thus, to regulate photosynthesis according to the carbon demand of the growing leaf. We

hypothesize that during early leaf development, exogenously applied sucrose or sucrose produced by source leaves, delays chloroplast differentiation in sink leaves, by which cell expansion is postponed and cell proliferation stimulated (Fig. 7). Conversely, when sucrose levels are limiting, a faster transition from sucrose-requiring sink tissue to sucrose-producing photosynthetically active source tissue will ensure sufficient energy supply and proper plant development. In the sink cells, sucrose will be cleaved to fructose and glucose, which will result in higher levels of cytosolic G6P, which can then be transported into the chloroplasts by the sucrose-induced plastid transporter GPT2. Furthermore, higher sucrose levels result in repression of chloroplast transcription, leading to a stop in chloroplast differentiation, which might be due to higher levels of G6P inside the chloroplast stroma. Consequently, less retrograde signals because of less differentiated chloroplasts could delay the transition to cell expansion, making it possible to use sugars for further stimulation of cell proliferation until the leaf becomes too large and needs autonomous sugar production to sustain growth.

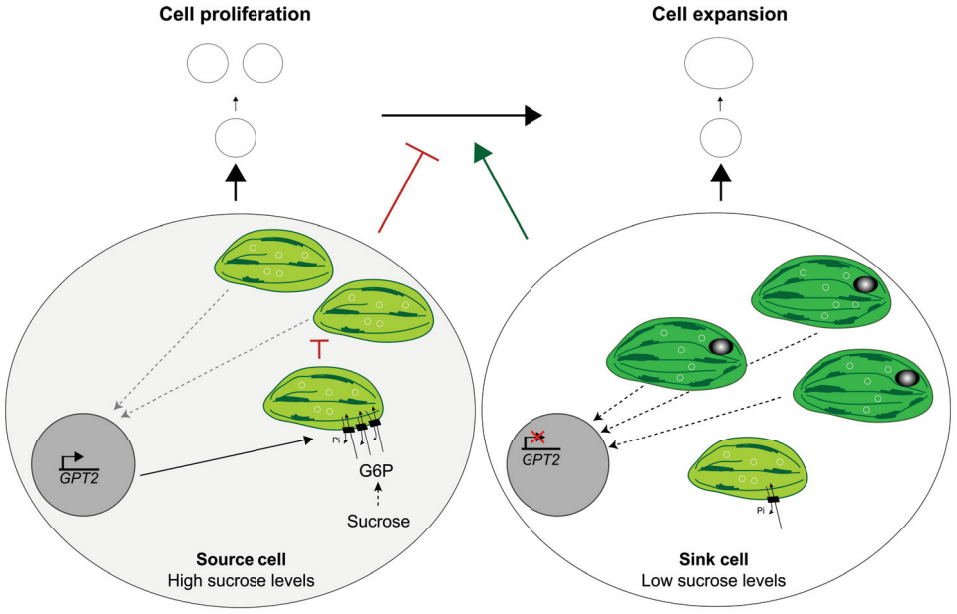

Figure 7 . Model of the central role of chloroplasts in the sucrose-induced stimulation of cell proliferation. Low sucrose levels in sink cells (right) trigger a rapid formation of photosynthetically active chloroplasts. Consequently, these chloroplasts sent retrograde signals to the nucleus to start the transition to cell expansion. However, higher sucrose levels in source cells (left) result in higher levels of glucose-6-phosphates (G6Ps) that are transported into the chloroplasts by the sucrose-induced plastid transporter GPT2 (black square). In addition, high sucrose levels repress chloroplast transcription (plastome represented by white circles), causing the chloroplasts to stop differentiating and dividing. Consequently, less retrograde signals are sent to the nucleus, which postpones the onset of the transition to cell expansion and stimulates cell proliferation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All experiments were performed on *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0). Seedlings were grown *in vitro* on half-strenght MS medium (Murashige & Skoog, 1962) without sucrose for 9 days (9 DAS) under a 16-h day (50 µmol m^{-2} s^{-1}) and 8-h night regime, unless specified differently. Plates were overlaid with nylon mesh of 20-µm pore size. At 9 DAS, seedlings were transferred to plates containing control medium without sucrose or medium supplemented with different concentrations of sucrose and glucose (6 mM, 15 mM and 30 mM). In the norflurazon (NF) experiments, seedlings were transferred at 9 DAS to half-strenght MS medium with or without 15 mM sucrose supplemented with 5 µM NF. Homozygous seeds of the *gpt2-1* mutant, a T-DNA insertion GABI-kat line in the Col-0 background (GK-454H06-018837), were a kind gift of Dr. Giles Johnson (University of Manchester; Dyson *et al*, 2014).

Growth Analysis

For the leaf area analysis, leaves were cleared in 100% ethanol, mounted in lactic acid on microscope slides, and photographed. Leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov//ij/).

Abaxial epidermal cells of the leaves were drawn with a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective. Drawings were scanned and analyzed using automated image analysis algorithms (Andriankaja *et al*, 2012). Subsequently, drawings were used to measure average pavement cell area, from which the total pavement cell number was calculated. The stomatal index was defined as the percentage of stomata compared with all cells.

GUS Staining and Analysis

Seedlings of two biological repeats were harvested at 13 DAS, four days after transfer to control or 15 mM sucrose-containing medium, incubated in heptane for 10 min and subsequently left to dry for 5 min. Then, they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50mM NaCl buffer (pH 7.0), 2mM K3[Fe(CN)6], and

4mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C overnight. Seedlings were cleared in 100% ethanol and then kept in 90% lactic acid. The third leaf was micro-dissected, mounted on slides and photographed under a light microscope. Leaf length and GUS staining was measured with the ImageJ software (http://rsb.info.nih.gov/ij/). The position of the cell cycle arrest front along the length of each leaf was calculated based on the color intensities between stained (proliferation zone) and non-stained (expanding zone) regions according to the method described by (Vercruyssen *et al*, 2014). The average cell cycle arrest front was determined by taking the average gray-scale intensities of the expansion zones of the control leaves.

RNA Extraction and expression analysis by qRT-PCR

Seedlings were harvested in liquid nitrogen, put in RNAlater ice solution and incubated at -20°C for at least one week, after which the third leaf was micro-dissected under a binocular light microscope. Leaves were frozen in liquid nitrogen and RNA was extracted using Trizol (Invitrogen) and the RNeasy Plant Mini Kit (Qiagen). DNase treatment was done on columns with RNase-free DNase I (Promega). The iScript cDNA synthesis kit (Bio-Rad) was used to prepare cDNA from 200 ng RNA and qRT-PCR was done on the LightCycler 480 with SYBR Green I Master (Roche) according to the manufacturer's instructions. Normalization was done against the average of three housekeeping genes AT1G13320, AT2G32170, AT2G28390. Primer sequences are listed in Supplemental Table S2.

RNA-Sequencing Analysis

Library preparation was done using the TruSeq RNA Sample Preparation Kit v2 (Illumina). Briefly, polyA-containing mRNA molecules were reverse transcribed, double-stranded cDNA was generated and adapters were ligated. After quality control using 2100 Bioanalyzer (Agilent), clusters were generated through amplification using the TruSeq PE Cluster Kit v3-cBot-HS kit (Illumina), followed by sequencing on an Illumina HiSeq2000 with the TruSeq SBS Kit v3-HS (Illumina). Sequencing was performed in Paired-End mode with a read length of 100 nt. The quality of the raw data was verified with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, version 0.9.1). Next,

quality filtering was performed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/, version 0.0.13): reads where globally filtered, in which for at least 75% of the reads, the quality exceeded Q20 and 3' trimming was performed to remove bases with a quality below Q10, ensuring a remaining minimum length of 90 nt. Re-pairing was performed using a custom Perl script. Reads were subsequently mapped to the Arabidopsis reference genome (TAIR10) using GSNAP (Wu et al, 2010, version 2012-07-20), allowing maximum five mismatches. These steps were performed through Galaxy (Goecks *et al*, 2010). The concordantly paired reads that uniquely mapped to the genome were used for quantification on the gene level with HTSeq-count from the HTSeq.py python package (Anders *et al*, 2015). The analysis was performed with the R software package edgeR ((Robinson *et al*, 2010), R core team (2014), R version 3.1.2). TMM normalization (Robinson & Oshlack, 2010) was applied using the calcNormFactors function. Differentially expressed genes were analyzed with the exact binomial test. False discovery rate adjustments of the *P*-values were done with the method described by Benjamini and Hochberg (1995).

Next-generation sequence data from this article were deposited in ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4262.

Gene Set Enrichment Analysis

Gene set enrichment analysis was performed with the R package Piano (Väremo *et al*, 2013) based on *P*-values and $\log_2$ fold changes. Three methods were compared: Fisher's combined probability test, Stouffer's method and the Tail strength method. Permutation-based null distributions were calculated by permuting the genes 1,000 times. *P*-values were adjusted with the FDR method (Benjamini & Hochberg, 1995).

Transmission Electron Microscopy

Leaves were immersed in a fixative solution of 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M Na-cacodylate buffer, placed in a vacuum oven for 30 min and then left rotating for 3 h at room temperature. This solution was later replaced with fresh fixative and samples were left rotating overnight at 4°C. After washing, samples were post-fixed in 1% OsO4 with $K_3Fe(CN)_6$ in 0.1 M Na-cacodylate buffer, pH 7.2.

Samples were dehydrated through a graded ethanol serie, including a bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in Spurr's resin. In order to have a larger overview of the phenotype, semi-thin sections were first cut at 0.5 µm and stained with toluidine blue. Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by post-staining with uranyl acetate and lead citrate in a Leica EM AC20 and collected on Formvar-coated copper slot grids. Two leaves of control and three leaves of sucrose-treated seedlings were viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan), operating at 80 kV, using Image Plate Technology from Ditabis (Pforzheim, Germany). For each leaf, 17 to 68 mesophyll cells of the leaf tip and 21 to 87 mesophyll cells of the base of the leaf, representing, respectively, expanding and proliferating cells, were analyzed.

SUPPLEMENTAL DATA

The following supplemental materials are available at the end of this chapter.

Supplemental Figure S1. Glucose treatment did not result in an increase in final leaf size increase.

Supplemental Figure S2. Relative leaf growth rate.

Supplemental Figure S3. Repression of chloroplast transcripts by sucrose.

Supplemental Figure S4. Plastome copy numbers per cell of the third leaf upon sucrose transfer.

Supplemental Figure S5. Increased starch accumulation in control leaves.

Supplemental Figure S6. Transcriptional responses in *gpt2* mutant.

Supplemental Table S1. Differentially expressed genes 3 h and 24 h after transfer to sucrose.

Supplemental Table S2. qRT-PCR primer sequences of selected chloroplast-encoded transcripts.

Supplemental Methods. Plastome copy number determination and Statistical analysis of growth experiments and chloroplast measurements

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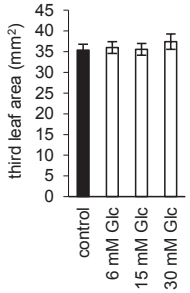
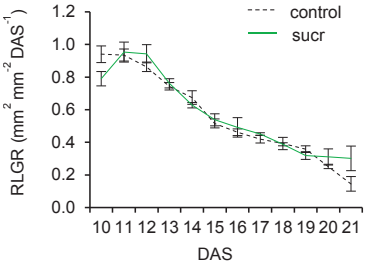
SUPPLEMENTAL DATA

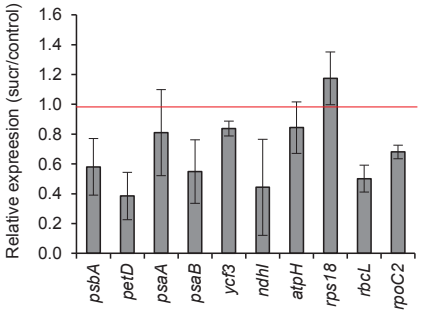
Supplemental Figure S1. Glucose treatment did not result in an increase in final leaf size. Plants were grown on MS media supplemented with four different glucose (Glc) concentrations (0 mM, 6 mM, 15 mM, 30 mM). At 21 DAS, the third leaf area was measured and compared between concentrations. Values are the means of three biological repeats with their SE. Leaf area was measured for 10 to 30 leaves in each repeat.



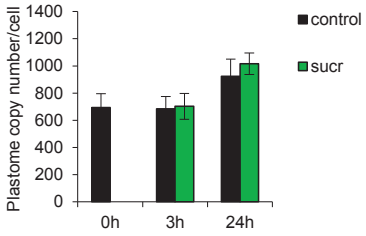
Supplemental Figure S2. Relative leaf growth rate. Wild-type plants were subjected to the experimental sucrose setup and harvested daily after transfer to sucrose (sucr) and control medium. Relative leaf growth rate (RLGR) is expressed as the increase in leaf area (mm^2) relative to the initial leaf area per unit of time (day). Values are the means of three biological repeats with their SE. Leaf area was measured for 5 to 20 leaves in each repeat.



Supplemental Figure S3. Repression of chloroplast-encoded transcripts by sucrose. Relative expression of several chloroplast-encoded genes in leaves 24 h after transfer to sucrose compared with control medium. Values are the means of the ratios (sucrose/control) of three biological repeats with their SE.



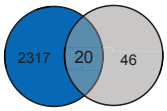
Supplemental Figure S4. Plastome copy numbers per cell of the third leaf upon sucrose transfer. Plastome copy numbers determined by qRT-PCR in the third leaf of seedlings before transfer (0 h), three hours (3 h) and, 24 hours (24 h) after transfer to control or sucrose-supplemented medium. Values are the means of three biological repeats with their SE.



Supplemental Figure S5. Increased starch accumulation in control leaves. Wild-type seedlings were transferred at 9 DAS to medium with or without sucrose (sucr) and, after 3 days (at 12 DAS), the third leaf was stained with Lugol's solution to visualize starch.

Supplemental Figure S6. Transcriptional responses in the *gpt2* mutant. A, Overlap between *gpt2*-induced and sucrose-responsive genes. Values indicated in the Venn diagram represent the number of genes differentially expressed in *gpt2.2* mutant compared to wild type Ws-4 from the dataset of Dyson et al., 2015 (FC > 1.5 and P-value < 0,05) and the 66 repressed genes 24 hours after transfer to sucrose. B, List of the 20 overlapping genes with corresponding fold changes (FC) and P-values. C, Relative expression of sucrose-responsive chloroplast transcripts in the third leaf of seedlings 24 hours after transfer to sucrose compared with control leaves, both in wild-type (black) and *gpt2* (blue) seedlings. Values are the means of the ratios (sucrose/control) of five to six biological repeats with their SE.

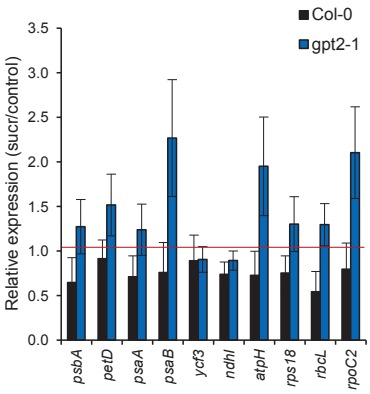
A



B

		gpt2.2		sucr 24h	
Gene ID	Name	Log_2FC	*P*-value	Log_2FC	*P*-value
ATCG00150	***atpI***	1.02	0.00	-1.78	0.00
ATCG00170	***rpoC2***	1.48	0.00	-1.87	0.00
ATCG01110	***ndhH***	0.73	0.00	-2.08	0.00
ATCG00380	***rps4***	0.67	0.00	-2.19	0.00
ATCG00500	***accD***	0.74	0.01	-2.32	0.00
ATCG00420	***ndhJ***	1.08	0.00	-2.46	0.00
ATCG00330	***rps14***	-1.01	0.02	-2.48	0.00
ATCG00680	***psbB***	1.44	0.00	-2.52	0.00
ATMG00080	***rpl16***	-0.80	0.01	-2.53	0.00
ATCG00270	***psbD***	1.92	0.00	-2.54	0.00
ATCG00160	***rps2***	1.18	0.00	-2.55	0.00
ATCG00140	***atpH***	0.75	0.15	-2.68	0.00
ATCG00350	***psaA***	3.13	0.00	-2.93	0.00
ATCG00490	***rbcL***	1.00	0.12	-2.99	0.00
ATCG01100	***ndhA***	0.78	0.08	-3.02	0.00
ATCG00520	***ycf4***	2.42	0.00	-3.07	0.00
ATCG00280	***psbC***	0.61	0.12	-3.12	0.00
ATCG00730	***petD***	3.19	0.00	-3.27	0.00
ATCG00020	***psbA***	2.76	0.00	-3.37	0.00
ATCG00360	***ycf3***	1.32	0.00	-3.38	0.00

C



Supplemental Table S1. Differentially expressed genes 3 h and 24 h after transfer to sucrose.

3 h				24 h			
GENE_ID	GENE NAME	LOGFC	FDR	GENE_ID	GENE NAME	LOGFC	FDR
AT1G61800.1	GPT2	2.04	0.05	AT2G33790.1	AGP30	-7.04	0.00
AT5G58390.1		1.42	0.02	AT3G22235.1		-6.41	0.00
AT3G49110.1		0.90	0.02	AT4G30170.1		-6.09	0.02
AT3G15630.1		-0.64	0.00	AT2G41800.1		-6.00	0.03
AT1G28330.5	DRM1	-0.64	0.00	AT1G66280.1	BGLU22	-4.95	0.00
AT1G04280.1		-0.65	0.00	AT1G66270.1	BGLU21	-4.38	0.00
AT1G68440.1		-0.70	0.00	ATMG01360.1	COX1	-3.66	0.00
AT1G80920.1		-0.71	0.00	ATCG01090.1	ndhI	-3.57	0.04
AT3G48360.1	BT2	-0.78	0.00	ATCG00360.1	ycf3	-3.38	0.01
AT2G05380.1		-0.78	0.00	ATCG00020.1	psbA	-3.37	0.00
AT5G56550.1		-0.80	0.03	ATCG00730.1	petD	-3.27	0.00
AT3G15450.1		-0.87	0.00	ATCG00340.1	psaB	-3.14	0.00
AT2G33830.2	DRM2	-1.69	0.00	AT5G26260.1		-3.12	0.00
AT2G39400.1		-1.80	0.02	ATCG00280.1	psbC	-3.12	0.00
AT2G32150.1		-2.16	0.00	AT2G07698.1		-3.10	0.03
AT2G05540.1		-2.25	0.02	AT1G54000.1	GLL22	-3.09	0.00
AT4G35770.1	SEN1	-2.34	0.01	ATCG00520.1	ycf4	-3.07	0.00
AT1G15330.1	PV42a	-2.73	0.00	ATCG00720.1	petB	-3.03	0.00
AT3G47340.1	DIN6	-3.49	0.01	ATCG01100.1	ndhA	-3.02	0.01
				ATCG00490.1	rbcL	-2.99	0.00
				ATCG00350.1	psaA	-2.93	0.00
				ATCG00540.1	petA	-2.89	0.00
				ATMG00090.1		-2.79	0.00
				ATCG00650.1	rps18	-2.76	0.03
				ATCG01040.1	ycf5	-2.76	0.05
				ATCG00140.1	atpH	-2.68	0.04
				ATMG00640.1		-2.67	0.02
				ATMG00160.1	COX2	-2.65	0.02
				AT4G35770.1	SEN1	-2.60	0.00
				ATCG00040.1	matK	-2.55	0.00
				ATCG00160.1	rps2	-2.55	0.03
				ATCG00270.1	psbD	-2.54	0.00
				ATMG00080.1	RPL16	-2.53	0.01
				ATCG00680.1	psbB	-2.52	0.00
				ATCG00330.1	rps14	-2.48	0.01
				ATCG00420.1	ndhJ	-2.46	0.02
				ATMG00060.1	NAD5	-2.35	0.05
				AT3G28550.1		-2.32	0.04
				ATCG00500.1	accD	-2.32	0.05
				ATCG01050.1	ndhD	-2.26	0.02
				AT2G01008.1		-2.24	0.01
				AT3G41762.1		-2.21	0.01
				ATCG00380.1	rps4	-2.19	0.04
				ATCG00120.1	atpA	-2.13	0.00
				ATCG01110.1	ndhH	-2.08	0.05
				AT1G49200.1		-1.91	0.03
				ATCG00170.1	rpoc2	-1.87	0.02
				ATCG01130.1	ycf1.2	-1.81	0.01
				ATCG00150.1	atpI	-1.78	0.05
				AT3G47340.1	DIN6	-1.70	0.00
				AT2G33830.2	DRM2	-1.53	0.00
				AT2G05540.1		-1.51	0.00
				AT1G73830.1	BEE3	-1.50	0.02
				AT1G10070.1	BCAT-2	-1.42	0.03
				AT3G01500.2	CA1	-0.98	0.00

AT5G23010.1	MAM1	-0.83	0.04
AT5G14740.1	CA2	-0.81	0.00
AT4G26260.2	MIOX4	-0.81	0.00
AT3G15770.1		-0.80	0.03
AT5G27350.1	SFP1	-0.78	0.02
AT5G03350.1		-0.77	0.00
AT3G48360.1	BT2	-0.73	0.00
AT3G15630.1		-0.72	0.00
AT2G19800.1	MIOX2	-0.59	0.00
AT1G23390.1		-0.59	0.00
AT2G34490.1	CYP710A2	0.62	0.04
AT5G06400.1		0.72	0.03
AT1G43800.1	FTM1	0.87	0.02

Supplemental Table S2. qRT-PCR primer sequences of selected chloroplast-encoded transcripts

Gene ID	Name	Forward primer	Reverse primer
ATCG01090	***ndhI***	ACCCTACGAGCTGCAAGGTA	CCAATCAACAACAGGCAAAT
ATCG00020	***psbA***	TATCGCATTCATTGCTGCTC	CATAAGGACCGCCGTTGTAT
ATCG00730	***petD***	TGGTACCATTGCCTGTAACG	CCGCTGGTACTGAAACCATT
ATCG00340	***psaB***	TCTTGGCCCGGTGAATATAG	CCCCGAATAGTCCTGACAAA
ATCG00490	***rbcL***	CAAAGGACGATGCTACCACA	CAGGGCTTTGAACCCAAATA
ATCG00350	***psaA***	GGGCGGTGAGTTAGTAGCAG	TCACAAGGGAAACGAAAACC
ATCG00360	***ycf3***	AAGGAAATTATGCGGAAGCA	TGTGGTAAAAAGGGGTTTCG
ATCG00650	***rps18***	CAAGCGATCTTTTCGTAGGC	TCGACTCACTTCTTTCAAATTGTT
ATCG00140	***atpH***	ATCCACTGGTTTCTGCTGCT	TGCTACAACCAGGCCATAAA
ATCG00170	***rpoC2***	AAAGCAATTTACGCGAAGGA	ACCGAAATCCCTCGGATAGT
ATCG00920	***rrn16S***	GCGTCTGTAGGTGGCTT	GCCGTTGGTGTTCTTTCC

Supplemental Methods. Plastome copy number determination

Total cellular DNA was extracted from micro-dissected third leaves using CTAB-protocol (Doyle and Doyle, 1997). DNA samples were diluted to 0.4 ng and chloroplast DNA copy number was calculated as described in Zoschke et al. (2007). Differences in nuclear DNA content were measured by CyFlow flow cytometer with the FloMax Software (Partec). Relative DNA content was analyzed by qRT-PCR for *ndhI, psbA, rbcL, rpoC2* and *rrn16S* using the primer sequences listed in Supplemental Table S2. Data was normalized to the nucleur 18S rRNA gene (AT2G01010) using following primer pair 5'-AAACGGCTACCACATCCAAG-3' and 5'-ACTCGAAAGAGCCCGGTATT-3'.

Supplemental Methods. Statistical analysis of growth experiments and chloroplast measurements

All analyses were performed with SAS (Version 9.4 of the SAS System for windows 7 64bit. Copyright © 2002-2012 SAS Institute Inc. Cary, NC, USA (www.sas.com).

Growth experiments

All growth experiments involved one, two or three factors and consisted of three independent biological repeats. For the representation of the ratios, measurements of the sucrose-treated leaves were compared to the measurements of the control leaves of the same repeat. Averages were then taken over the three independent repeats and represented in the graphs with their standard error.

When needed, raw measurements were log-transformed to stabilize the variance prior to statistical analysis; this is specified in the figure legends. For all growth experiments, a linear mixed model was fitted to the variable of

interest with all main factors and their interaction, in case of two factors, as fixed effects using the mixed procedure. The biological repeat term was included in each model as a random factor to take into account the correlation between observations done at the same time. In the presence of a significant F-test (for the main effect in case of one factor, for the interaction term in the case of two factors), appropriate post-hoc tests were performed. When the interest was in comparison with a control, multiple testing correction was done according to Dunnett. When the interest was in all-pairwise comparisons, a Tukey adjustment was performed. For the time course experiment, simple tests of effects were performed at each day separately with the plm procedure.

Leaf series experiment

The area measurements of the leaf series experiment was analyzed with a linear mixed model taking into account correlations between measurements done on leaves originating from the same plant. Leaf data was available for both conditions up and till leaf 8. Model building started with a saturated mean model containing the main effects of the sucrose concentrations (6 mM, 15 mM, and 30 mM) and leaf, and, the interaction term and a random effect for the biological repeat. Several structures were tested for the variance-covariance matrix: unstructured, (heterogeneous) compound symmetry, (heterogeneous) autoregressive, and (heterogeneous) banded toeplitz. Based on the AIC values, an autoregressive structure was assumed. The main biological interest was in the effect of the transfer of seedlings to the sucrose concentrations on the sizes of the different leaves. Type III tests of fixed effects were calculated to verify that there was a significant interaction term at the 0.05 significance level. Simple F-tests of effect for the sucrose concentrations were carried out at each leaf separately. For the leaves that showed a significant F-test ($P < 0.05$), pairwise comparisons were estimated between each concentration of sucrose and the control level. At each leaf, correction for multiple testing was done, applying the Dunnett method.

Chloroplast number and area measurements

A generalized linear mixed-effect model was fitted to the number of chloroplasts with the glimmix procedure of SAS assuming a Poisson distribution and a log-link function. The fixed effects were the treatment and leaf part and their interaction effect . A random effect was included in the model to take into account the correlations between observations originating from the same leaf. Significance of the random effect was assessed with the covtest statement and left out of the model whenever $P < 0.05$. Significance of the interaction effect was tested with a type 3 Wald test. Significance of the interaction effect was tested with a type 3 wald test at the 5% significance level. In the absence of a significant interaction term, the significance of the main effects was subsequently tested. Differences in Least-Square means were calculated between groups of interest.

A random intercept model was fitted to the mesophyll area data using the mixed procedure with the same fixed and random effects as for the chloroplast number.

For the log-transformed chloroplast area data, a random intercept model was fitted using the mixed procedure. Two random effects were included in the model to take into account the correlations between observations originating from the same leaf, and originating from the same mesophyll cell within the leaf. The model with only a random intercept for mesophyll cell was preferred over a model with two random intercepts based on AIC values. All-pairwise comparisons between the factor level combinations of the factors, treatment and leaf part, were estimated. P-values were corrected for multiple testing with the Tukey adjustment method.

In all analyses the Kenward-Roger method was used for computing the denominator degrees of freedom for the tests of fixed effects. For each analysis, residual diagnostics were carefully examined.

Supplemental Literature Cited.

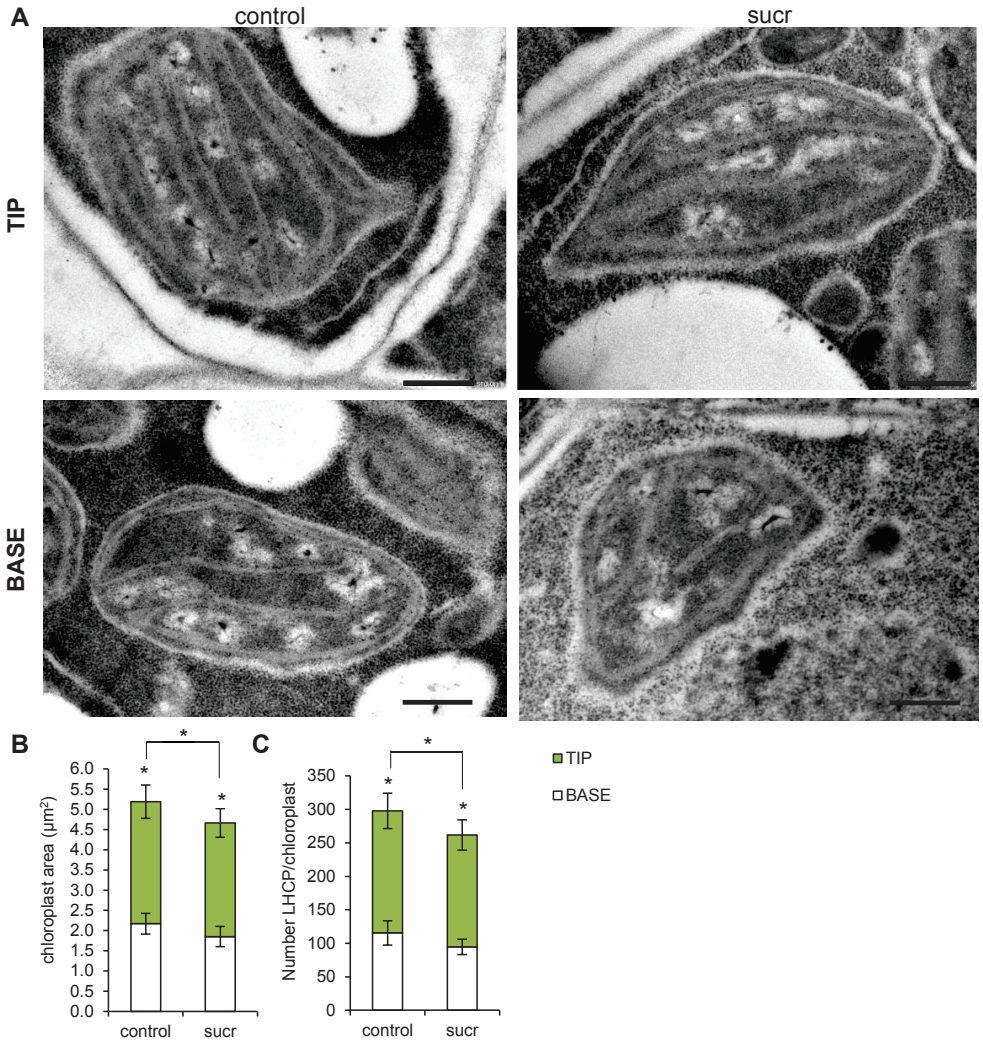
Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin.* **19**:11-15.

SUPPLEMENTAL INFORMATION

An additional experiment was performed in the period of submission of the paper. Because the manuscript was accepted as it is, I decided to not include the figure in the main chapter but will briefly discuss it here.

RESULT

The transfer of seedlings to sucrose-containing medium resulted in the formation of mesophyll cells with fewer, smaller and less differentiated chloroplasts with limited formation of thylakoid membranes and less starch granules. To further quantify the differences in chloroplast differentiation, the number of light harvesting chlorophyll proteins (LHCPs) per chloroplast was examined by transmission electron microscopy. LHCP are encoded by the nucleus and their synthesis was found to coincide with the progression of photosynthetic chloroplast development (Dekeyser *et al*, 1990; Jansson 1994). LHCPs are located at the thylakoid membranes and are necessary for the light-dependent reactions of photosynthesis. Transverse sections of leaves, 24 hours after transfer to control or sucrose-containing medium, were cross-reacted with antibodies against pea LHCP and subsequently treated with gold particles for visualization (Fig. SI1A; Dekeyser *et al*, 1990). The tip and the base of two leaves per condition were examined and chloroplast size and number of LHCPs per chloroplast were analyzed (Fig. SI1B and C). Similar results as described above (Fig. 5) were found, i.e. chloroplasts were smaller in sucrose-transferred leaves compared with control leaves independently of the tip or base ($P < 0.05$; Fig. SI1B). In general, chloroplasts were larger at the tip compared with the base of the leaves in both control and sucrose-transferred leaves ($P < 0.05$; Fig. SI1B). Furthermore, chloroplasts of sucrose-transferred leaves contained on average less LHCPs compared with control leaves ($P < 0.05$; Fig. SI1C) and chloroplasts in the tip of the leaf contained more LHCPs compared to the base, independently of the condition ($P < 0.05$; Fig. SI1B). These findings further demonstrate that chloroplasts of sucrose-transferred leaves are less differentiated than control leaves. Sucrose-treated leaves have smaller chloroplasts with less LHCPs.



Supplemental Information Figure SI1. Differences in chloroplast differentiation in the tip and the base of sucrose-transferred and control leaves. A, Transmission electron micrographs of tip and base of the 10-d-old third leaf, 24 hours after transfer to control or 15 mM sucrose (sucr) supplemented medium. Transverse sections were cross-reacted with antibodies against pea LHCP and treated with protein A-gold. The bar represents 500 nm. B, Average chloroplast size in the tip and base of the third leaf of control and sucrose-treated seedlings. C, Average number of LHCPs per chloroplast counted in approximately 15 chloroplasts of two leaves of control and sucrose-transferred seedlings. Values are means ± SE from 15 chloroplasts of the tip and the base of two independent leaves. *, $P < 0.05$, mixed models.

METHOD

Leaves were cut into small pieces and immersed in a fixative solution of 2,5 % paraformaldehyde and 0,3% glutaraldehyde in 0.1 M NaCacodylate buffer, pH 7.2. Samples were dehydrated through a graded ethanol series and infiltrated stepwise over 3 d at 4°C in LR-White, hard grade (London Resin), followed by embedding in capsules. Polymerization was done by UV illumination for 24 h at 0°C followed gradually by 24 h at 20°C ending at 37 °C for 72 h. Ultrathin sections of gold interference colour were cut with an ultramicrotome (Leica EM UC6) and collected on formvar-coated copper slot grids. All steps of immunolabeling were performed in a humid chamber at room temperature. Grids were floated upside down on 25 µl of blocking solution 5% (w/v) bovine serum albumin (BSA), for 30 min followed by washing five times for 5 min each times with 1% BSA in PBS. Incubation in a 1:50 dilution (1% BSA in PBS) of primary antibodies anti-Cab (rabbit, 1:20000) for 60 min was followed by washing five times for 5 min each time with 0.1% BSA in PBS. The grids were incubated with PAG10 nm 1:50 dilution (1% BSA in PBS) (Cell Biology, Utrecht University, The Netherlands) and washed twice for 5 min each time with 0.1% BSA in PBS, PBS, and double-distilled water. Sections were post-stained in a Leica EM AC20 for 40 min in uranyl acetate at 20°C and for 10 min in lead citrate at 20°C. Grids were viewed with a JEM 1400plus transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV.

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CHAPTER 5

Characterization of Putative Sugar-dependent Leaf Growth Regulators

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ABSTRACT

Leaf growth is a tightly controlled and complex process that strongly depends on the environmental conditions. In our previous study presented in Chapter 4, we studied young proliferating leaves from Col-0 after transfer to sucrose, at phenotypic and molecular level. Upon transfer to sucrose, final size of the third leaf was increased and we demonstrated an essential function of the *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER*2 (*GPT2*) in this response to sucrose. Here, we selected nine sucrose-responsive genes from the transcriptomic datasets to further characterize their function in leaf growth regulation. Knock-out and knock-down mutants of these nine genes as well as constitutive overexpression lines of several of the candidate genes were evaluated for leaf size phenotype *in vitro* and *in soil*. Preliminary data show that mutants of *DRM2* and *AT5G26260* have increased and reduced leaf sizes, respectively, suggesting that these genes might act as a negative and positive regulator of leaf growth. In conclusion, this study highlights that still additional genes can be found to expand the leaf growth regulatory network.

INTRODUCTION

Global demand and prices for food and feed are rising dramatically and more extreme climate conditions are likely to aggravate the situation. To be able to cope with this, crop yield and productivity will have to increase (Edgerton, 2009). Therefore it is important to unravel the underlying regulatory mechanisms controlling plant growth. Plant growth is a highly complex process regulated at the cellular, organ and whole organism level integrating the inherent genetic signaling pathways with environmental signals (Gonzalez *et al*, 2010; Gonzalez & Inze, 2015; Skirycz *et al*, 2010).

Leaves are important plant organs since they are the primary source of photosynthesis providing the energy for nearly all living organisms. Currently the question on how leaves control their size is far from resolved. Leaf growth is determined by two spatial and temporal overlapping processes, cell proliferation and cell expansion (Andriankaja *et al*, 2012; Beemster *et al*, 2014; Donnelly *et al*, 1999). In Arabidopsis, leaf primordium originates from the peripheral zone of the shoot apical meristem as a group of undifferentiated cells rapidly dividing. In the proliferating phase, young leaves develop by cell growth and division. Next, during the secondary morphogenesis phase, leaf growth is mainly sustained by cell

expansion. The transition between both phases is tightly coordinated and during this period, a cell cycle arrest front progresses in a basipetal (from the tip to the base) direction (Kazama *et al*, 2010). This transition to cell expansion was shown to proceed in a non-gradual manner since the cell cycle arrest front abruptly disappears (Andriankaja *et al*, 2012). Besides these two main processes, also division of meristemoids, stomatal precursor cells in the epidermis, results, after several round of asymmetric divisions, in the production of additional pavement cells contributing to final leaf size (Peterson *et al*, 2010).

The characterization of mutants and transgenic lines exhibiting increased or decreased leaf sizes helped with the identification of different genetic factors involved in the regulation of leaf growth and acting on one or several of the cellular processes during leaf development (Gonzalez *et al*, 2012; Hepworth & Lenhard, 2014). However, leaf growth is not solely regulated at the genetic level; environmental cues have to be integrated in the developmental program to control growth. By using forward genetic screens important environmental triggered pathways controlling plant growth might be missed because mostly optimal growth conditions are used. Photosynthesis in the leaf is one of the processes that strongly depends on environmental growth conditions and which has a major impact on how plants grow if altered (Schurr *et al*, 2006). Indeed, changes in the photosynthetic capacity of the leaf alters the endogenous sugar status of the plant which affects different aspects of growth and development. Sugars not only act as major structural components of the cell but they can also elicit sugar signalling pathways which are strongly interconnected with plant growth regulatory mechanisms (Lastdrager *et al*, 2014; Sheen, 2014).

In this study, we selected nine sucrose-responsive genes from the transcriptome analyses described in Chapter 4 (Van Dingenen *et al*, 2016). Transcriptional responses were determined in young growing leaves, 3 hours and 24 hours, after transfer of seedlings to sucrose-supplemented media. Eight of the nine selected genes were repressed upon transfer to sucrose and one gene was upregulated by sucrose, i.e. the *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER2* (*GPT2*). As wild type plants transferred to sucrose showed an increased final rosette and leaf size, the sucrose-repressed genes were good candidates to screen for their

potential involvement in leaf growth linking sugar signals with the leaf growth machinery.

RESULTS

Selection of Sucrose-responsive Genes

Candidate genes were selected from the RNA-sequencing datasets described in Chapter 4 (Van Dingenen *et al*, 2016). Briefly, transcriptome profiling was done on RNA extracted from proliferating third leaves 3 and 24 hours after transfer of nine-day-old Col-0 seedlings to sucrose-supplemented or control (without sucrose) media. Nineteen genes and 69 genes were found to be differentially expressed 3 and 24 hours after transfer to sucrose, respectively, from which the majority was repressed (Supplemental Table 1, Chapter 4). To find novel genes involved in leaf growth regulation, we chose several genes based on the following criteria: (1) significant repression by sucrose both at 3 and 24 hours or strong down- or up-regulation independent of the time point, (2) genes involved in different metabolic processes (3) genes with a yet unreported or detailed plant growth phenotype based on literature search and (4) availability of transfer DNA (T-DNA) insertion mutants. This resulted in the selection of nine sucrose-responsive genes for which potential involvement in leaf growth regulation was then determined (Table 1).

Table 1. Selected sucrose-responsive genes in young growing leaves for functional characterization. FC = fold change, FDR = false discovery rate

	Gene ID	Name	Description	3h		24h	
				Log_2FC	FDR	Log_2FC	FDR
1	AT3G47340	**DIN6/ASN1**	Dark-inducible gene/glutamine-dependent asparagine synthetase	-3.490	0.006	-1.702	0.003
2	AT4G35770	**DIN1/SEN1**	Dark-inducible gene/Senescence-associated gene	-2.342	0.011	-2.599	0.000
3	AT2G33830	**DRM2**	Dormancy/auxin associated family protein	-1.690	0.000	-1.525	0.000
4	AT3G48360	**BT2**	BTB and TAZ domain protein 2, essential component of the TAC1-mediated telomerase activation	-0.778	0.001	-0.730	0.000
5	AT2G05540	**GRP3**	Glycine-rich protein family	-2.158	0.000	-1.514	0.000
6	AT1G61800	**GPT2**	Glucose-6-phosphate/Phosphate Transporter 2	2.037	0.049	0.494	0.839
7	AT3G15630	/	unknown protein	-0.643	0.000	-0.722	0.000
8	AT5G26260	/	TRAF-like family protein	1.241	0.894	-3.125	0.002
9	AT3G22235	/	unknown protein	-0.334	1.000	-6.413	0.003

Six of these nine genes (*AT3G47340*, *AT4G35770*, *AT2G33830*, *AT3G48360*, *AT1G61800, and AT3G15630*) were previously described as sugar-responsive genes (Fujiki *et al*, 2000; Gonzali *et al*, 2006). Five of these six genes were repressed upon transfer to sucrose in our datasets. *AT3G47340* encodes a glutamine-dependent asparagine synthase and its expression is controlled by sugars and nitrogen levels (DIN6/ASN1; Lam *et al*, 1994). Initially, *DIN6/ASN1* was identified as a *DARK-INDUCIBLE* gene, induced by sugar-starvation (Fujiki *et al*, 2000). *AT4G35770* or *DIN1/SEN1* has also been identified as a *DARK-INDUCIBLE* gene (Shimada *et al*, 1998) and is regulated by different nutrient stress conditions and senescence, hence its second name *SENESCENCE-ASSOCIATED GENE*, *SE*N1 (Chung *et al*, 1997; Oh *et al*, 1996; Yu *et al*, 2005). The third selected gene encodes a dormancy-associated/auxin-repressed protein DRM2 (AT2G33830), which was characterized, together with its homologue DRM1, to be involved in bud dormancy (Stafstrom *et al*, 1998). *DRM* expression is rapidly repressed in pea axillary buds upon decapitation (Stafstrom *et al,* 1998). The expression of the fourth sucrose-repressed gene, *BT2* (*AT3G48360*), is regulated by a variety of hormones, nutrients, stresses and the circadian clock with a peak in the dark (Mandadi *et al*, 2009). The last sucrose-repressed transcript is *AT3G15630,* encoding an unknown protein and found to be rapidly repressed after sucrose treatment in complete Arabidopsis seedlings (Osuna et al, 2007) and under zinc-deficiency conditions (van de Mortel *et al*, 2006). *GPT2* (*AT1G61800*) was the only gene selected that was significantly up-regulated upon transfer to sucrose. *GPT2* expression is commonly found to be induced with increasing sugar levels (Gonzali *et al*, 2006) as well as in starch biosynthesis mutants (Kunz et al, 2010). Finally, the last three genes *AT2G05540*, *AT5G26260* and *AT3G22235*, encode proteins of unknown function and are assigned to a glycine-rich family protein GRP3 (Mangeon *et al*, 2010), a TRAF (tumor necrosis factor receptor-associated factor)-like family protein and a hypothetically protein.

These nine genes were selected to be screened for their potential role in leaf growth regulation since no leaf growth phenotype was previously reported.

Selection of Loss-of-Function mutants and Screen for Sucrose-responsiveness

To investigate the involvement of the nine selected genes in the regulation of growth, T-DNA insertion mutants were ordered from the SALK (Alonso *et al*, 2003) or GABI-Kat collection (Kleinboelting *et al*, 2012). T-DNA insertions in the exons of *DIN6* (GABI_829B05), *BT2* (SALK_002306) and *GPT2* (*gpt2*, GABI_454H06) as well as in the first intron of *SEN1* (SALK_020571) and in the 5' untranslated region (UTR) or promoter of *DRM2* (SALK_098437) completely abolished the expression of the targeted gene (Supplemental Fig.S1). Also the expression of *AT5G26260* was strongly but not completely reduced in the SALK_107244 mutant line having an insertion in the second exon. Transcript levels of *GRP3* (GABI_075G07, insertion in 5'UTR) and *AT3G22235* (GABI_893A09, insertion in 3'UTR) were only mildly knocked-down by T-DNA insertion since residual expression of 66% and 44% of the targeted gene was found, respectively. No line with reduced *AT3G15630* expression was found (SALK_139803), although, harboring an insertion in the first exon, so this line was excluded from the analysis.

The eight remaining mutants were grown *in vitro* using the experimental setup described in Chapter 4 (Van Dingenen *et al*, 2016), to study their potential importance during the sucrose-induced regulation of early leaf growth (Van Dingenen *et al*, 2016). Seedlings were first grown on meshes covering MS medium without carbon source under low light conditions (50 $\mu mol/m^{-2}s^{-1}$) for 9 days. At 9 DAS, seedlings were transferred to control medium without sucrose or to 15 mM sucrose-supplemented medium. At 21 DAS, final rosette areas were calculated from the individual leaf areas obtained by making leaf series (Fig. 1). In almost all lines, transfer to sucrose resulted in a similar significant increase in rosette area compared to wild type plants (20 ± 10%; $P < 0.05$; Fig. 1A). In *bt2* mutant plants, transfer to sucrose resulted in a significant larger increase in final rosette area of on average 34% ($P < 0.05$) compared to 19% for WT (SALK) ($P < 0.05$). Furthermore, rosette size of the *gpt2* mutant was not significantly increased (12%, $P = 0,06$), in line what has been shown before (Chapter 4). Interestingly, comparison of the rosette sizes of plants only transferred to control media showed that the *drm2* and *at5g26260* mutants were significantly larger (25%, $P < 0.05$) and smaller (17%, $P < 0.05$), respectively, than their corresponding wild type plants, whereas the other mutants were indistinguishable from wild type ($P > 0.05$; Fig. 1B).

As *GPT2* expression was found to be essential for the positive effect of sucrose on leaf growth (Van Dingenen *et al*, 2016), the growth phenotype of *gpt2* plants was investigated in further details. The measurements of the individual leaf areas at 21 DAS showed that all leaves of wild type and *gpt2* seedlings transferred to control medium were equal in size (Fig. 1C). Transfer to sucrose resulted in an increase in size for all leaves (with the exception of the cotyledons and the two first leaves) of wild type plants, whereas *gpt2* leaves were not or less affected (Fig. 1C). Detailed measurements of the third leaf area showed a significant increase of 36% ($P < 0.05$) by sucrose for wild type plants, and no significant increase for the *gpt2* mutant (14 %, $P = 0.41$; Fig. 1C right).

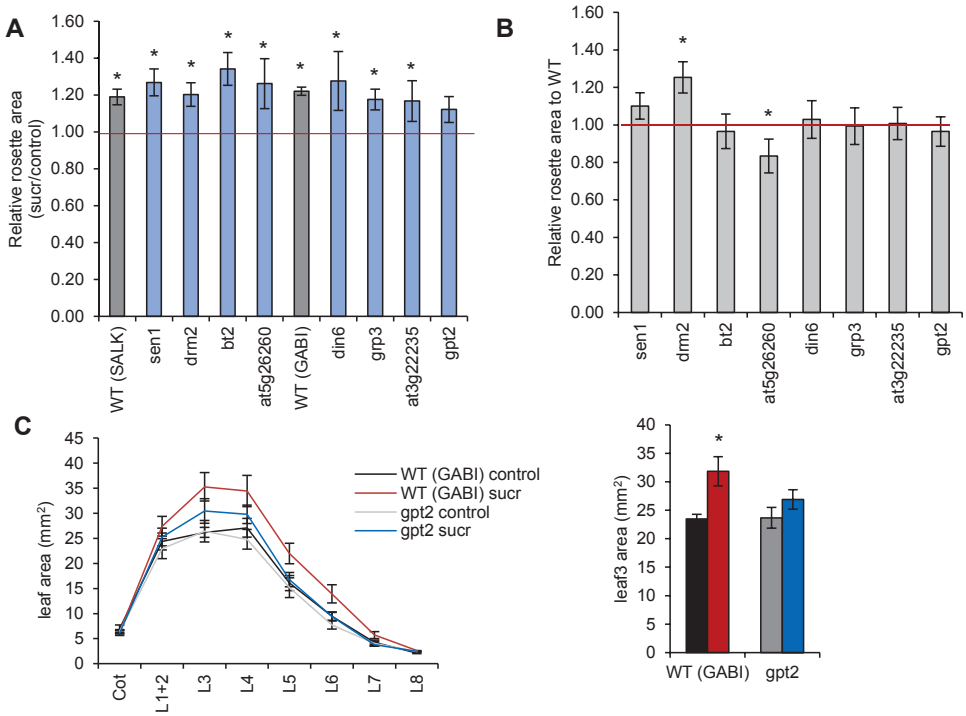

Figure 1. Relative rosette and leaf sizes of loss-of-mutants grown *in vitro*. Seedlings were first grown on medium without sucrose (control) and, at 9 DAS, transferred to medium supplemented with or without 15 mM sucrose (sucr). A, Relative rosette area increase upon transfer to sucrose was compared to control plants at 21 DAS. B, Control average rosette sizes were measured and normalized to the corresponding wild type, WT (SALK) for *sen1*, *drm2*, *bt2* and *at5g26260* or WT (GABI) for *din6*, *grp3*, *at3g22235* and *gpt2* lines, at 21 DAS. C, Average individual leaf area (left) and third leaf area (right) of control and sucrose-transferred wild type and *gpt2* plants measured at 21 DAS. Cot = cotyledons; Lx = leaf position x in the order of appearance on the rosette. Values are means of three to four repeats with their SE. Rosette and leaf area was measured for 5 to 7 plants in each repeat. *, $P < 0.05$, mixed models.

In conclusion, the *bt2* mutant was found to be hypersensitive to sucrose and only one of the eight mutants, *gpt2*, was less sensitive to transfer to sucrose resulting in no significant increase in final rosette area at 21 DAS. These measurements further confirmed the essential role of *GPT2* in sucrose-induced leaf growth as demonstrated in Chapter 4. Furthermore, two mutants, *drm2* and *at5g26260*, produce larger and smaller rosettes, respectively, than wild type plants when grown on control medium, suggesting a role in leaf growth regulation.

An in soil Phenotypic Screen further validates *DRM2* and *AT5G26260* as potential Leaf Growth Regulators

In order to further investigate the involvement of *SEN1*, *DRM2*, *BT2*, *AT5G26260*, *DIN6*, *GRP3*, *AT3G22235* and *GPT2* in leaf growth regulation, independently from sugar addition, rosette size of the loss-of-function mutants was characterized in soil. Plants were grown in soil on the phenotyping platform 'Multi Camera in vivo Rosette Growth Imaging System' (MIRGIS, see Material and Methods), which allows daily measurements of the projected rosette area from 8 until 21 DAS. At 22 DAS, average rosette area, based on the sum of the individual leaf sizes, was calculated (Fig. 2A). At this time point, all mutants were indistinguishable from their corresponding wild type ($P > 0.05$; Fig.2A), with the exception of *drm2* and *at5g26260.* Rosettes of *drm2* and *at5g26260* mutants were 24% larger ($P < 0.05$) and 25% smaller ($P < 0.05$), respectively, with a consistent effect on all leaves (Fig. 2B). Projected rosette area measurement over time showed that *at5g26260* mutant plants tend to grow slower compared to wild type as they are similar in size at early time points, i.e. 8-10 DAS, but have reduced rosette growth compared to wild type at later time points (Fig. 2C). Contrastingly, *drm2* mutants already have larger rosettes at 8 DAS and remain larger until 22 DAS (Fig. 2C). In addition, we quantified *drm2* seed weight and found a significant increase in average weight of 200 seeds (Fig. 2D). To further identify whether the positive growth phenotype was specific for DRM2, an insertion mutant with T-DNA inserted in the last exon of its homologues gene *DRM1* (GABI_085G06) was ordered. Reduction in *DRM1* expression was verified with qRT-PCR analysis (Supplemental Fig. S2A). Plants were grown in soil for 22 days as described above

and at this time point *drm1* rosettes were not different from wild type plants (Supplemental Fig. S2B).

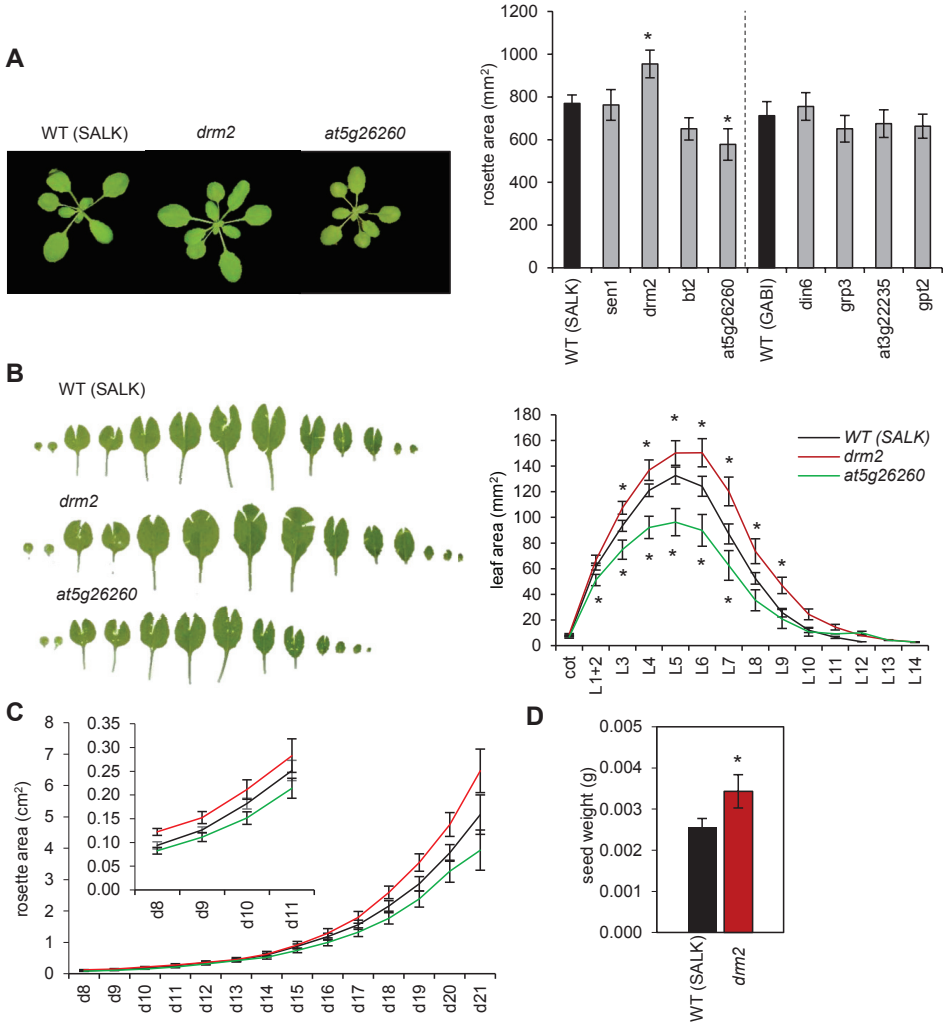

Figure 2. Rosette and individual leaf area of the loss-of-function mutants grown in soil. Plants were grown in soil under long-day (16-h day/8-h night) conditions for 22 days. At 22 DAS, average rosette and individual leaf areas were measured. A, Average rosette area of the eight selected mutants compared to their corresponding wild type, WT (SALK) for SALK and WT (GABI) for GABI-Kat T-DNA insertion mutants. At the left, pictures of the rosettes of WT (SALK), *drm2* (SALK_098437) and *at5g26260* (SALK_107244) at 22 DAS. B, Average individual leaf area of WT (SALK), *drm2* and *at5g26260* with representative pictures of leaf series. C, Rosette area over time of WT (SALK), *drm2* and *at5g26260* plants. Inset is a close-up of early time points 8-11 DAS. D, Average seed weight measured by weighting 200 seeds of three independent seed stocks. Cot = cotyledons; Lx = leaf position x in the order of appearance on the rosette. Values are means from three independent repeats with their SE. Rosette and leaf area was measured for 8 to 12 plants in each repeat. *, $P < 0.05$, mixed models for (A) to (C) and Student's t-test for (D).

Taken together, both *in vitro* and *in soil* experiments demonstrate that a mutation in *DRM2* positively affects growth, whereas an impaired *AT5G26260* expression results in smaller leaves. In addition, preliminary experiments suggest that this growth advantage is specific for a mutation in *DRM2,* and not in the *DRM1* homolog.

To uncover the underlying cellular mechanisms of the larger and smaller leaves of *drm2* and *at5g26260* mutants, pavement cell number and cell size of the third leaf of soil-grown plants were measured (Fig. 3). Third leaf area of *drm2* plants was significantly increased with 15% ($P < 0.05$) and for *at5g26260* leaves, decreased with 18% ($P < 0.05$) compared with wild type plants (Fig. 3). The enlarged leaf size of the *drm2* mutant was mainly due to more pavement cells with an average increase of 11% ($P = 0.06$), whereas average cell size remained unchanged. Leaves of *at5g26260* mutants were smaller due to smaller and less pavement cells presenting a reduction of 9% ($P = 0.05$) and 12% ($P = 0.13$), respectively (Fig. 3).

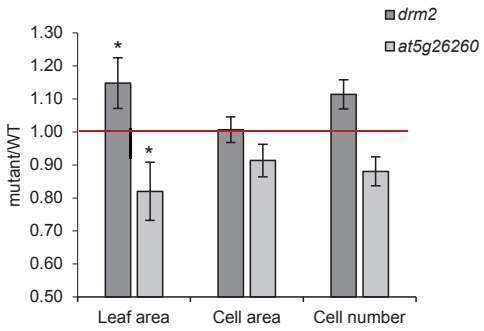

Figure 3. Cellular measurements of *drm2* and *at5g26260* T-DNA insertion lines. Average third leaf area and total pavement cell number and size of *drm2* and *at5g26260* mutants compared with wild type plants grown in soil for 22 DAS. Values are means from three independent repeats with their SE. Leaf area was measured for 5 to 7 plants in each repeat. Cellular data are from three leaves in each repeat. *, $P < 0.05$, mixed models.

Preliminary Screen for Rosette Growth phenotype of Overexpression lines grown in soil

Gain-of-function lines were generated by overexpressing the gene of interest under the control of the 35S promoter of the cauliflower mosaic virus and were additionally N- or C-terminally fused to GFP. Homozygous lines for a single-locus insertion of the transgene were selected and overexpression of the gene was verified in 10-day-old seedlings with qRT-PCR analysis (Supplemental Fig. S2). We were able to generate

overexpression lines for four candidate genes: *35S::GFP-DRM2*, *35S::SEN1-GFP*, *35S::GRP3-GFP*, *35S::GPT2-GFP* and two independent lines of N-terminal GFP fused *GPT2* overexpression line, *35S::GFP-GPT2 A* and *B*. Segregation analysis was based on the Fluorescence-Accumulating-Seed Technology (FAST) by which homozygous transformed seeds can be selected based on fluorescence (Shimada *et al*, 2010). Interestingly, homozygous *35S::DRM2-GFP* transgenic lines were not able to germinate and viable *35S::GFP-DRM2* transgenic lines did not demonstrate *DRM2* overexpression (Supplemental Fig. S3), suggesting that ectopic expression of *DRM2* might be embryo lethal. *SEN1* and *GRP3* expression was more than 10-fold increased in the *35S::SEN1-GFP* and *35S::GRP3-GFP* transgenic seedlings, respectively, whereas *GPT2* was very strongly overexpressed with 500 to 2000 times higher transcript levels compared with wild type seedlings (Supplemental Fig. S3).

To investigate the effect of overexpression of the genes of interest on rosette growth, all lines were grown simultaneously with their appropriate wild type plants, WT (OE) for *35S::SEN1-GFP* and *35S::GRP3-GFP* and WT (GPT2) for *35S::GPT2-GFP*, *35S::GFP-GPT2 A* and *B,* in soil for 22 days using the MIRGIS platform. Similarly as for the mutant plants, rosette sizes were measured by making leaf series at 22 DAS. Rosettes of *35S::SEN1-GFP* and *35S::GRP3-GFP* were significantly larger than wild type with an average increase of 19% and 27% ($P < 0.05$), respectively (Fig. 4A). Contrastingly, *35S::GPT2-GFP* and only one of the *35S::GFP-GPT2* lines (*35S::GPT2-GFP B*) were significantly smaller compared with their corresponding wild types with an average reduction of 9% and 29% ($P < 0.05$), respectively (Fig. 4A). Projected rosette area over time from 8 until 22 DAS showed that *35S::SEN1-GFP* and *35S::GRP3-GFP* plants are already larger at early time points, i.e. 8 to 11 DAS and remained larger compared to wild type (Fig. 4B and inset).

Taken together these data show that overexpression of *SEN1* and *GRP3* stimulated growth, whereas *GTP2* overexpression results in growth repression. Interestingly, we were not able to generate transgenic lines with high *DRM2* transcript levels.

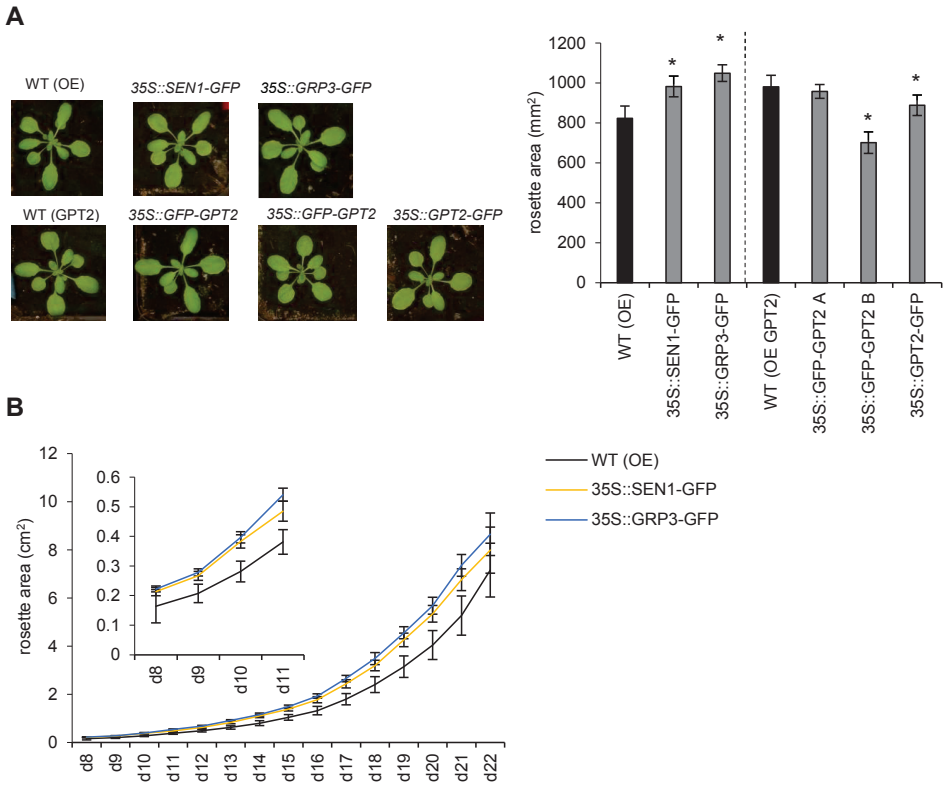

Figure 4. Rosette growth of overexpression lines of *SEN1*, *GRP3* and *GPT2*. Plants were grown in soil under long long-day (16-h day/8-h night) conditions for 22 days. A, Average rosette size measured by making leaf series at 22 DAS of *35S::SEN1-GFP* and *35S::GRP3-GFP* plant with their appropriate wild type, WT (OE), and *35S::GPT2-GFP*, *35S::GFP-GPT2 A* and *B* with their appropriate wild type, WT (OE GPT2). At the right, representative pictures of plants in graph. B, Rosette area over time of *35S::SEN1-GFP* and *35S::GRP3-GFP* plant with their appropriate wild type, WT (OE). Inset is close-up of early time points 8-11 DAS. Values are means from three independent repeats with their SE. Rosette area was measured for 8 to 12 plants in each repeat. *, $P < 0.05$, mixed models.

DISCUSSION

This study presents a preliminary screen aiming at identifying novel genes involved in the regulation of leaf growth. Candidate genes were selected from the differentially expressed genes in young proliferating leaves upon transfer of seedlings to sucrose-supplemented media as described in Chapter 4. All selected genes, with the exception of *GPT2*, were repressed shortly after transfer to sucrose (Table 1). We screened mutant and overexpression lines of eight candidate genes *in vitro* as well as *in soil*. A role for these genes in sucrose-induced early leaf growth might be

suspected if the loss-of-function mutants would demonstrate a sucrose-induced hypersensitive response on leaf size, which was the case for *bt2* plants. Furthermore, we identified *DRM2* and *AT5G26260* as negative and positive leaf growth regulator, respectively. These findings are solely based on the phenotype of one mutant line and should be verified with additional knock-out/transgenic lines to exclude effects due to the site of the T-DNA insertion in the genome.

This said, we were not able to obtain transgenic lines strongly overexpressing *DRM2* C-terminally tagged to GFP, further indicating that *DRM2* indeed plays a role as negative growth regulator. Moreover, homozygous lines of *DRM2* under the control of the 35S promoter and N-terminally fused to GFP were even not able to germinate. DRM2 occurs in two splice isoforms in Arabidopsis with the presence or absence of an N-terminal Molecular Recognition Feature (MoRF) probably involved in protein-protein interaction (Vacic *et al*, 2007). The phenotypes of the N-terminally GFP tagged DRM2 transgenic lines might thus suggest a yet unknown function of DRM2 in embryo development and/or germination but, as the use of the 35S promoter might result in undesired pleiotropic effects as discussed in Vanhaeren *et al*, (2016), this phenotype should be first investigated with more care in future experiments. For example, the use of the endogenous *DRM2* promoter to drive *DRM2* expression could shed a more realistic image of the role of DRM2 during plant growth.

In Arabidopsis, two homologues *DRM* genes share 60% of identity in amino acid sequence: DRM1 (AT1G28330) and DRM2 (Tatematsu *et al*, 2005). These homologues proteins are routinely used as dormancy release factors and are highly conserved between plant species (Rae *et al*, 2013). Expression of both DRMs has been previously reported to be induced by different abiotic stresses, such as cold, salt, drought and light (Lee *et al*, 2013; Park & Han, 2003; Rae *et al*, 2014) and repressed after sucrose treatment (Gonzali *et al*, 2006; Rae *et al*, 2013) in various plant species. Also in our transcriptome datasets described in Chapter 4, transcription of both *DRM1* and *2* was repressed 3 hours after transfer to sucrose, while only *DRM2* remained strongly repressed after 24 hours (Van Dingenen *et al*, 2016). Arabidopsis DRMs were not previously reported to be linked with plant growth, whereas overexpression of the *Brassica rapa* orthologue of DRM1 in *Arabidopsis* resulted in reduced vegetative growth and lower seed yield, suggesting a role of the

DRMs in growth arrest (Lee *et al*, 2013). This is consistent with our results wherein we have shown that a mutation in the 3'UTR of *DRM2* increases leaf growth and constitutive overexpression of *DRM2* negatively affects plant growth. The enlarged leaf size of the *drm2* mutant was due to more cells, suggesting a role for DRM2 in the inhibition of cell proliferation. In addition, rosettes of *drm2* seedlings were already larger compared to wild type at early developmental time points and preliminary measurements of *drm2* seed weight showed an increased average seed weight. These observations suggest that the increased final leaf size of the *drm2* mutant might also be due to initial larger seeds. However, additional experiments are needed, such as quantifications of total seed yield, seed size and number. Contrastingly, insertion in the last exon of the *DRM1* did not affected leaf growth. Alternative splicing of *DRM1* has been shown to result in multiple splice variants with diverse C-terminal protein sequences which have different functions (Rae *et al*, 2014). The T-DNA insertion mutant we used, was predicted to have an insert in the last intron/exon boundary, which might affect the formation of all splice variants, but this has to be further examined with splice variant specific primers. In addition, the multiple splice variants demonstrated different responsiveness to various treatments compared to *DRM2* as well as different tissue-specific protein expression patterns in Arabidopsis seedlings. These findings further suggest that DRM1 and DRM2 have distinct roles during leaf growth.

Besides DRM2, also a mutation in *AT5G26260* was found to affect growth. *AT5G26260* leaves were smaller because of less and smaller cells suggesting a role of this gene in cell proliferation and cell expansion regulation during leaf development. *AT5G26260* is predicted to encode a protein with a domain homologue to the TRAF (tumor necrosis factor receptor-associated factor) domain in animals. TRAFs play a role in a wide variety of processes, such as cell survival, proliferation, differentiation and in numerous stress responses (Chung *et al*, 2002). Also here, we demonstrated that mutation in *AT5G26260* influences different cellular processes, positively affecting cell proliferation and expansion.

Finally, we further validated the essential role of the *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER2* (*GPT2*) in the regulation of leaf growth by sucrose. Also at the final time point 21 DAS, both rosette size and third leaf area were not significantly increased for the *gpt2* mutant transferred to sucrose-

containing medium, further confirming the critical role of GPT2 during sucrose-induced early leaf growth. Loss-of-function mutants of *GPT2* were previously described to grow as wild types without obvious effects on plant growth and development (Niewiadomski *et al*, 2005). This is consistent with our results since rosettes of *gpt2* mutants were unaltered compared to wild type plants both when grown *in vitro* and in soil. However, constitutive overexpression of *GPT2* resulted in smaller plants. Transgenic potato plants overexpressing the *PsGPT* gene of pea in tubers was previously reported not to result in differences in tuber starch content and tuber yield (Zhang *et al*, 2008). Our findings suggest that *GPT2* overexpression negatively affects growth and to further unravel its function it would be interesting to determine the underlying cellular mechanisms linking *GPT2* with cell proliferation or expansion.

In conclusion, leaf growth is a strictly regulated and complex process and, although multiple important players have been already characterized, a lot of additional regulators are still missing. Leaf growth is controlled by different molecular networks integrating developmental and environmental cues, such as sugars status. In this study, we started from sucrose-responsive genes and we were able to find additional molecular players with a potential role in cell proliferation and/or cell expansion during leaf development.

MATERIALS AND METHODS

Transgenic Lines and Mutants

All experiments were performed on *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0). Mutants for *din6* (GABI_829B05), *grp3* (GABI_075G07), *at3g22235* (GABI_893A09) were obtained from the GABI-Kat collection (Kleinboelting *et al*, 2012). *gpt2* mutant line was a kind gift of Dr. Giles Johnson (University of Manchester). *bt2* (SALK_002306), *sen1* (SALK_020571), *drm2* (SALK_098437) and at5g26260 (SALK_107244) mutants were ordered from the SALK collection (Alonso *et al*, 2003). Through Gateway cloning *35S::GFP-DRM2*, *35S::SEN1-GFP*, *35S::GRP3-GFP*, *35S::GPT2-GFP* and *35S::GFP-GPT2* constructs were made using the pFAST-R06 and pFAST-R05 destination vectors (Karimi *et al*,

2007a; Karimi *et al*, 2007b) and transformed *in Arabidopsis thaliana* Col-0 by floral dip using the *Agrobacterium tumefasciens* strain C58C1 (pMP90). *35S::GFP-DRM2*, *35S::SEN1-GFP*, *35S::GRP3-GFP* and *35S::GPT2-GFP* and *35S::GFP-GPT2* constructs, were independently transformed and for each transformation pipeline a different segregating wild type was used for performing growth experiments, WT (OE) for the first three lines and WT (GPT2) for the GPT2 overexpression lines.

Growth Conditions *in vitro*

For the *in vitro* experiments, seedlings were grown on half-strenght MS medium (Murashige & Skoog, 1962) under a 16-h day (50 µmol m^{-2} s^{-1}) and 8-h night regime. Seedlings were grown for 9 days (9 DAS) on nylon mesh of 20-µm pore size overlaying growth medium without sucrose. At 9 DAS, seedlings were transferred to plates containing control medium without sucrose or medium supplemented with 15 mM sucrose.

Growth Conditions in soil

For the in soil experiments, plants were grown for 22 days at 21℃ under a 16-h day (80-100 µmol m^{-2} s^{-1}) and 8-h night regime. For the in-vivo growth analysis, four plants per pots were sown on soil. After one week, the seedling with a projected rosette area closest to median area of that genotype was selected per pot. While the growth of these seedlings was followed during further development, the other seedlings were removed.

Image acquisition, image processing and data analysis

For the in-vivo growth analysis, image acquisition was performed using Canon EOS 550D slr cameras equipped with a Canon EF 35mm f/2 objective. Pictures were automatically captured on a daily basis by a Perl script (www.perl.org) using the gPhoto2 library (www.gphoto.org). Image preprocessing and segmentation for the seedling selection and growth analysis was performed with C++ scripts using the OpenCV image analysis library (www.opencv.org). Parsing of quantitative

measurements and further data analysis was performed with Perl scripts (www.perl.org). Graphs of the calculated data were automatically plotted making use of the graphing utility gnuplot (www.gnuplot.info). More details on the image and data analysis procedures can be found elsewhere (Dhondt *et al*, 2014).

Growth Measurements

Leaf series were made by cutting each individual leaf of the rosette and ranking them from old to young on a square agar plate. Plates were photographed and pictures were subsequently analysed using ImageJ software (http://rsb.info.nih.gov/ij/) to measure the size of each individual leaf.

For the leaf area analysis, leaves were cleared in 100% ethanol, mounted in lactic acid on microscope slides, and photographed. Leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov//ij/). Abaxial epidermal cells of leaves were drawn with a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective. Drawings were scanned and analyzed using automated image analysis algorithms (Andriankaja *et al*, 2012). Subsequently, drawings were used to measure average cell area, from which the total pavement cell number was calculated. The stomatal index was defined as the percentage of stomata compared with all cells.

SUPPLEMENTAL DATA

Supplemental figures are available at the end of this chapter.

Supplemental Figure S1. T-DNA insertion mutants and expression of the nine selected candidate genes.

Supplemental Figure S2. Phenotyping *drm1* mutant plants.

Supplemental Figure S3. Expression levels of *DRM2*, *SEN1*, *GRP3* and *GPT2* in overexpression lines.

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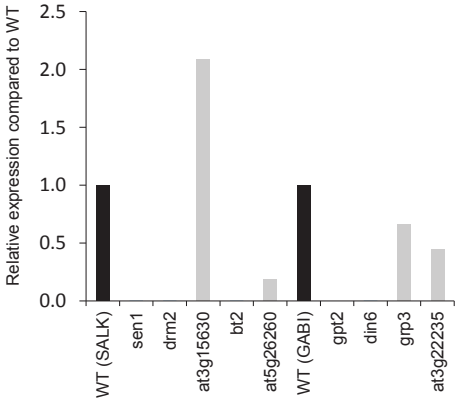
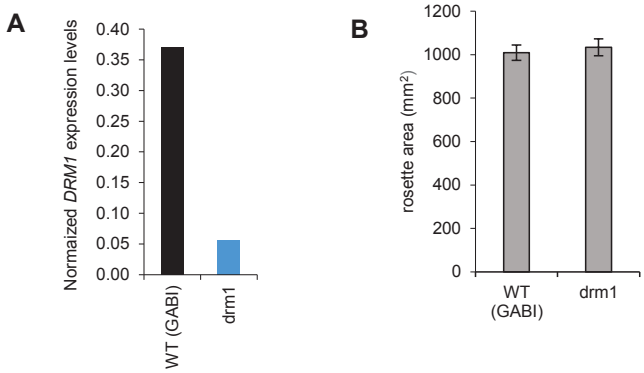
SUPPLEMENTAL DATA

Supplemental Figure S1. T-DNA insertion mutants and expression of the nine selected candidate genes. Loss-of-function mutants were ordered from the SALK or GABI-Kat collection. Table at the left represents the name of the gene, the insertion mutant and the region of the transfer DNA insertion. Homozygous plants were selected and the expression level of the specific genes was tested with qRT-PCR analysis in 10-day-old seedlings (right). Expression was normalized against the average of three housekeeping genes *AT1G13320, AT2G32170, AT2G28390*.

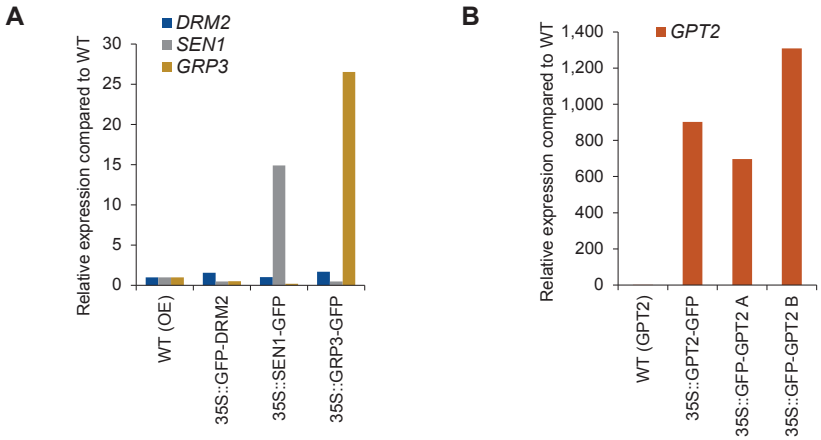
Name	T-DNA mutant	Insertion
din6	GABI_829B05	exon
grp3	GABI_075G07	5' UTR
sen1	SALK_020571	intron
drm2	SALK_098437	5'UTR
bt2	SALK_002306	exon
at3g15630	SALK_139803	exon
at5g26260	SALK_107244	exon
at3g22235	GABI_893A09	3'UTR
gpt2	GABI_454H06	exon



Supplemental Figure S2. Phenotyping *drm1* mutant plants. A, *DRM1* expression was tested with qRT-PCR analysis in 10-day-old wild type WT (GABI) and *drm1* T-DNA insertion mutant (GABI_085G06) seedlings grown in vitro. Expression was normalized against the average of three housekeeping genes *AT1G13320, AT2G32170, AT2G28390*. B, Plants were grown in soil under long-day (16-h day/8-h night) conditions for 22 days. At 22 DAS, average rosette areas were measured for *drm1* and appropriate wild type plants. Values in (A) are means of three biological repeats with their SE.



Supplemental Figure S3. Expression levels of *DRM2*, *SEN1*, *GRP3* and *GPT2* in overexpression lines. A, Expression of *SEN1* and *GRP2* was checked by qRT-PCR in 10-day old seedlings from homozygous transgenic lines expressing *35S::GFP-DRM2, 35S::SEN1-GFP* or *35S::GRP3-GFP*. B. Expression of *GPT2* in *35S::GPT2-GFP* and two independent *35S::GFP-GPT2* (A and B) lines. Expression was normalized against the average of three housekeeping genes *AT1G13320, AT2G32170, AT2G28390*.



CHAPTER 6

The Role of HEXOKINASE1 during Arabidopsis leaf development

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AUTHOR CONTRIBUTIONS: J.V.D. performed experiments, analyzed data and is the main author of the chapter; J.V.L, N.D.W. and G.D.J. performed the TAP experiments; M.V., L.D.M. and N.D.W. assisted in experiments; W.D. performed confocal imaging; F.R., N.G., S.D. and J.V.L. were involved in discussions throughout the project; N.G. and S.D. contributed to the writing of the chapter. G.D.J. and D.I. supervised the project.

ABSTRACT

In the last decade, extensive efforts have been made to unravel the underlying regulatory mechanisms of the role of the conserved glucose sensor HEXOKINASE1 (HXK1) in the control of plant growth. HXK1-signaling has been shown to exert both growth-promoting and growth-inhibitory effects depending on the environmental conditions as well as plant species. In a previous study we developed an experimental *in vitro* assay in which the sucrose availability can be altered during early Arabidopsis (*Arabidopsis thaliana*) leaf development we found that sucrose induced early leaf growth by stimulating cell proliferation. Here, we used a *hxk1* mutant in the Col-0 accession to investigate the role of HXK1 in early leaf growth in more detail. *hxk1* knock-out mutants show similar growth defects and glucose-insensitive responses as previously described for *glucose insensitive2* (*gin2*) mutant deficient in HXK1 in the Ler accession. We found that *hxk1* mutants are affected in both cell proliferation and cell expansion early during leaf development. Furthermore, *hxk1* mutants were less sensitive to sucrose-induced cell proliferation with no significant increase in final leaf growth after treatment. Upon transfer to sucrose, *GPT2* expression was still induced but chloroplast differentiation was not repressed by sucrose in the *hxk1* mutant suggesting a GPT2-independent regulation of early leaf growth. Finally, we used tandem affinity purification of HXK1 protein complexes from cell cultures in the presence or absence of externally applied sucrose to identify novel HXK1-interacting proteins. We identified several putative HXK1 protein partners for which a role in growth-regulation can be postulated.

INTRODUCTION

Leaf growth is a strictly controlled and complex process that strongly depends on changing environmental conditions that can alter light, energy and nutrient availability. Leaves are the major energy factories of the plant using light during the photosynthetic process to produce the essential components necessary to sustain plant growth. Interestingly, different studies have illustrated a link between photosynthesis, and thus sugar production, and the cellular processes during early leaf growth (Andriankaja *et al*, 2012; Lastdrager *et al*, 2014; Van Dingenen *et al*, 2016).

Leaves first form at the shoot apical meristem as primordia and subsequently grow by cell proliferation and cell expansion (Donnelly *et al*, 1999). These two cellular processes occur simultaneously during the so-called transition phase in leaf development when cell expansion starts at the tip of the leaf and a cell division arrest

front moves in a tip-to-base direction (Andriankaja *et al*, 2012). Establishment of the photosynthetic machinery and chloroplast differentiation is coupled with the onset of cell expansion during early leaf growth (Andriankaja *et al*, 2012). In accordance, when sink leaves receive enough sugars, chloroplast differentiation is postponed and cell proliferation is stimulated (Chapter 4, Van Dingenen *et al,* 2006). *GLUCOSE-6-PHOSPHATE TRANSPORTER2* (*GPT2*) expression was found to play a central role in mediating this sucrose-responsive promotion of cell proliferation as well as the repression of plastome expression, probably resulting in the less differentiated chloroplasts when seedlings are transferred to sucrose-containing medium (Van Dingenen *et al*, 2016). Sugars are used as substrate in respiration and important biosynthesis pathways, such as the oxidative pentose phosphate pathway, cell wall biosynthesis and starch biosynthesis, but they can also act as signaling molecules to trigger major regulatory mechanisms controlling plant growth (Lastdrager *et al*, 2014; Rolland *et al*, 2006; Sheen, 2014). These sugar-mediated regulatory networks have to be integrated with the growth molecular networks to monitor and adjust plant development depending on the environmental conditions.

Several proteins respond to changes in the cellular sugar status to monitor growth and development. One of these central regulators is the protein kinase SnRK1/SNF1/AMPK which is activated when carbon and energy sources are scarce. Its activation promotes energy-producing catabolic reactions and represses energy-consuming biosynthetic processes through transcriptional and post-transcriptional regulation (Baena-Gonzalez, 2010; Baena-Gonzalez *et al*, 2007). SnRK1 has a conserved heterotrimeric structure comprising one catalytic α-subunit and two regulatory β- and ɣ-subunits (Ghillebert *et al*, 2011). Three subgroups of ɣ-like proteins have been described in plants based on their structure: KINβɣ-, KINɣ- and PV42-type proteins, and two classes of β-subunits have been reported (Polge & Thomas, 2007; Emmanuelle *et al*, 2016). However, only KINβɣ was found to take part in the canonical heterotrimeric SnRK1 complex and KINɣ could not interact with any other subunit (Ramon *et al,* 2013; Emannuelle *et al,* 2015). The plant catalytic α-subunit, KIN10, plays a pivotal role in plant growth and development. Over-expression of KIN10 confers enhanced tolerance to energy deprivation, delays senescence and flowering and promotes plant organ growth, whereas the *kin10kin11* double mutant exhibits severe growth inhibition (Baena-Gonzalez et al, 2007).

Another important sugar sensing protein is HEXOKINASE1 (HXK1), a glycolytic enzyme involved in glucose metabolism in yeast, animals and plants, converting glucose to glucose-6-phosphate (Moore *et al*, 2003). The *glucose insensitive2* (*gin2*) mutant is impaired in the HEXOKINASE1 (HXK1) protein and can survive on high concentrations of glucose, whereas wild-type plants exhibit developmental arrest, such as repression of cotyledon expansion and chlorophyll accumulation (Zhou *et al*, 1998). HXK1-signaling mediates glucose-induced repression of photosynthesis-related genes, such as *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) and the small subunit of *RUBISCO (RBCS*) (Moore *et al* 2013). The *gin2* mutant generally exhibits severe growth defects, such as small dark green leaves and a reduced root system when grown under high light conditions (Moore *et al* 2003). Based on the distance between neighboring trichomes it was proposed that the small *gin2* leaves result from reduced cell expansion. Complementation of the *gin2* mutant with catalytically inactive *HXK1* alleles resulted in recovering of the susceptibility to high glucose concentrations as well as enlarged leaves when grown under high light. These findings further support a central role of HXK1 in regulating sugar signaling during plant growth (Moore *et al*, 2003). Remarkably, over-expressing HXK1 in Arabidopsis and its orthologues in tomato and rice plants also results in growth defects, with reduced chlorophyll accumulation and photosynthesis as well as early onset of senescence (Dai *et al*, 1999; Kelly *et al*, 2012; Kim *et al*, 2013). Furthermore, silencing of *NtHXK1* in tobacco also leads to growth-inhibitory effects and pale bleached leaves due to degraded chloroplasts in the source leaves and starch accumulation in the sink leaves (Kim *et al*, 2013). Taken together, these observed growth defects of both the *hxk1* mutants and HXK1-overexpression lines suggest that, depending on the environmental conditions and developmental stages, HXK1 can exhibit growth-promoting as well as growth-inhibiting effects. Obviously, a better understanding of how HXK1 monitors growth as well as the underlying cellular mechanisms of HXK1-mediated signaling is necessary.

Due to the presence of a N-terminal hydrophobic membrane anchor domain, the predominant localization of HXK1 is on the outer membrane of mitochondria where it exerts its metabolic function during glycolysis (Claeyssen & Rivoal, 2007; Granot, 2008). Several GFP-fusion studies in Arabidopsis, tomato, spinach and tobacco revealed HXK1-association with the mitochondria and showed that a deletion of the

N-terminal domain resulted in localization in the cytosol (Balasubramanian et al, 2007; Damari-Weissler et al, 2007; Heazlewood et al, 2004). Furthermore, to be able to regulate transcription, it has been suggested that a small portion of the HXK1 proteins is translocated to the nucleus where it interacts with the vacuolar H(+)-ATPase B1 (VHA-B1) and the 19S regulatory particle of the proteasome subunit (RPT5B) (Cho *et al*, 2006). However, how this nuclear HXK1 complex is exactly established and whether additional HXK1-interacting protein partners are involved in the HXK1-mediated regulation of transcription is still not well understood.

In this study, we characterized a *hxk1* mutant in the Col-0 background and studied the role of HXK1 in sucrose-mediated leaf growth. Furthermore, tandem affinity purification experiments allowed for the identification of several novel HXK1 interacting proteins.

RESULTS

Characterization of a Sugar-Insensitive *hxk1* mutant in the Col-0 genetic background

To date, most of the studies on HXK1 in Arabidopsis used the *gin2* mutant identified from a genetic screen of glucose insensitive mutants in the Landsberg *erecta* (L*er*) background (Rolland *et al*, 2002; Zhou *et al*, 1998). To study the role of HXK1 during early leaf development, we selected a T-DNA mutant in the Col-0 background from the SALK collection (Alonso *et al*, 2003). This insertion mutant harbors a T-DNA at the end of the first intron of the *HXK1* gene (SALK_018086; Supplemental Fig. S1A). Homozygous plants were selected and the *HXK1* expression level was checked with qRT-PCR analysis which demonstrated a complete abolishment of *HXK1* expression (Supplemental Fig. S1B).

The shoot growth of the *hxk1* mutant was studied by growing plants on soil exposed to high light intensity (≥100 µmol/$m^{-2}s^{-1}$) for 22 days after stratification (DAS). At this time point, *hxk1* had a significant decrease of 77% in rosette area compared with Col-0 plants (P < 0.0001; Fig. 1A). Therefore, in general, the *hxk1* mutant demonstrated a rosette growth defect as previously described for the *gin2* mutant (Moore *et al*, 2003). All individual rosette leaves were significantly smaller and the

third leaf area of *hxk1* was only 25% of the area of Col-0 plants ($P < 0.0001$; Fig. 1B). This decrease in leaf size was due to both a significant reduction of 61% in the number of pavement cells and significant smaller pavement cells being on average 54% of the size of Col-0 cells ($P < 0.0001$; Fig. 1C).

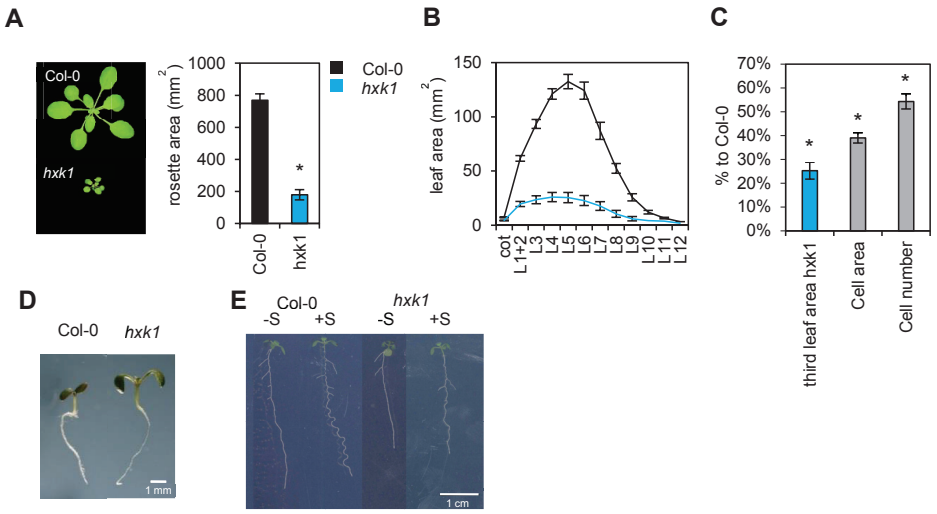

Figure 1. *hxk1* plants exhibit growth defects and are insensitive to sugars. *hxk1* mutant and Col-0 wild type plants were grown in soil for 22 days after stratification (DAS) under a high light intensity of approximately 100 µmol/m^{-2}s^{-1}. A and B, Rosette area (A) and individual leaf area (B) were measured at 22 DAS. Cot = cotyledons; Lx = leaf position x in the order of appearance on the rosette. C, Ratio of the third leaf size, pavement cell area and total cell number of *hxk1* compared with Col-0. D, 8-day-old seedlings grown on MS medium supplemented with 6% glucose. E, 10-day-old seedlings grown on vertical plates without sucrose (-S) or 1% sucrose (+S). Values are means of three biological repeats with their SE. Rosette and leaf area was measured for 10 to 12 plants in each repeat. Cellular data are from three leaves in each repeat. *, $P < 0.05$ compared to Col-0, mixed models.

The sugar-insensitivity of the *hxk1* mutant was verified at shoot and root level. First, *hxk1* seedlings did not show a developmental arrest on Murashige and Skoog (MS) medium supplemented with 6% glucose, whereas Col-0 wild type seedlings exhibited reduced cotyledon expansion and root growth, and anthocyanin production under this growth condition (Fig. 1D; Moore *et al*, 2003). Secondly, sugar responsiveness of the primary root was checked based on sucrose-induced waving when plants are grown on vertical plates with MS media supplemented with 1% sucrose (+S; Fig. 1E; Oliva and Dunham 2007). The primary root of 10-day-old wild type seedlings showed a typical waving pattern which is not present when seedlings were grown without sucrose (-S; Fig. 1E). Sucrose addition did not result in waving of the primary root of

hxk1 seedlings (Fig. 1E). Besides this absence of waving, the *hxk1* mutant seedlings generally had a reduced root system.

Taken together, we show that the identified *hxk1* mutant in the Col-0 background exhibits a decrease in shoot and root growth and insensitivity to sugars similar to the previously described *gin2* mutant in the L*er* accession. Furthermore, the decreased leaf size of *hxk1* plants grown in soil is due to less and smaller pavement cells.

hxk1 leaves have More Pavement Cells in the Young Leaves but undergo Faster Onset of Cell Differentiation compared with Wild type plants

To investigate further the underlying cellular mechanisms responsible for the reduced leaf size of *hxk1* plants and to study the potential role of HXK1 during sucrose-induced early leaf growth stimulation, the *in vitro* experimental setup described in Chapter 4 (Van Dingenen *et al,* 2006) was used in all following experiments. In this setup, sugar status can be altered during the cell proliferation phase of the third leaf which allows for studying the function of HXK1 in a developmental context. Seedlings were first grown on meshes covering MS medium without carbon source under low light conditions (50 µmol/$m^{-2}s^{-1}$) for 9 days. At 9 DAS, seedlings were transferred to control medium without sucrose or to 15 mM sucrose-supplemented medium after which rosette size, leaf area, pavement cell number and cell area of the third leaf were measured from 9 to 16 DAS and at the final time point 21 DAS. In the following paragraph, we describe the phenotypes of the seedlings transferred to control medium without sucrose to determine the cellular differences between *hxk1* mutant and Col-0 plants.

Using above *in vitro* growth conditions, the average final rosette size and the third leaf size at 21 DAS were similar between the *hxk1* mutant and wild type plants (Fig. 2A and B). In accordance, no difference was observed in pavement cell area and cell number between *hxk1* and wild type leaves grown on control medium (Supplemental Fig. S2). However, early during development, from 9 until 14 DAS, the third leaf area of *hxk1* seedlings was significantly larger than Col-0 leaves ($P < 0.05$; Fig. 2C). At 10 DAS, *hxk1* leaf was 43% larger due to a significant increased number of pavement cells that were slightly smaller (69%, $P < 0.05$ and -13%, $P = 0.21$ respectively; Fig. 2D).

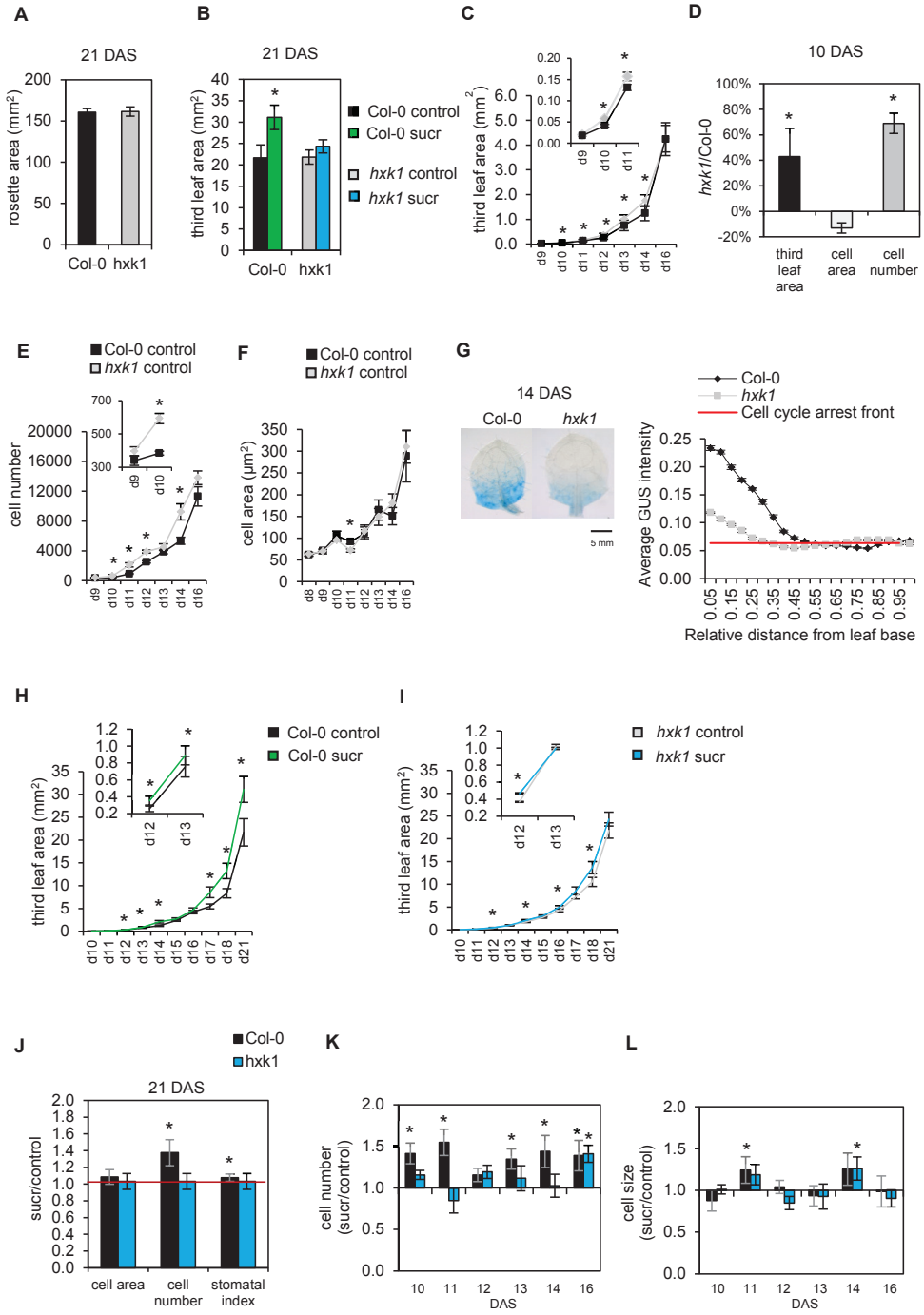
A
21 DAS
rosette area (mm²)
Col-0 hxk1
B
21 DAS
third leaf area (mm²)
Col-0 hxk1
Col-0 control
Col-0 sucr
hxk1 control
hxk1 sucr
C
third leaf area (mm²)
d9 d10 d11 d12 d13 d14 d16
D
10 DAS
hxk1/Col-0
third leaf area
cell area
cell number
E
Col-0 control
hxk1 control
cell number
F
Col-0 control
hxk1 control
cell area (µm²)
G
14 DAS
Col-0
hxk1
5 mm
Col-0
hxk1
Cell cycle arrest front
Average GUS intensity
Relative distance from leaf base
H
Col-0 control
Col-0 sucr
third leaf area (mm²)
I
hxk1 control
hxk1 sucr
third leaf area (mm²)
J
Col-0
hxk1
21 DAS
sucr/control
cell area
cell number
stomatal index
K
cell number (sucr/control)
DAS
L
cell size (sucr/control)
DAS

Figure 2. *hxk1* leaves have more cells and undergo faster differentiation but are insensitive to sucrose early during leaf development. Col-0 and hxk1 mutant seedlings were grown on medium without sucrose and, at 9 DAS, transferred to medium without (control) or supplemented with 15 mM sucrose (sucr). A, Rosette area at 21 DAS of Col-0 and *hxk1* control plants. B, Third leaf area at 21 DAS of Col-0 and *hxk1* plants transferred to control and sucrose. C, Third leaf area from 9 to 16 DAS of Col-0 and *hxk1* control seedlings. D, Ratio of leaf area, pavement cell area and total cell number of the third leaf of *hxk1* control seedlings compared with Col-0 seedlings 10 DAS. E, Pavement cell number and cell area (F) from 9 to 16 DAS of Col-0 and *hxk1* leaves from control seedlings. G, GUS-stained third leaves at 14 DAS of *pCYCB1;1::CYCB1;1-D-box:GUS* in Col-0 and *hxk1* background seedlings transferred to control medium and GUS intensity quantification along the leaf. GUS staining was quantified in a defined region from the base to the tip of each leaf. The red line indicate the cell cycle arrest front. H and I, Leaf area from 10 DAS until 21 DAS of Col-0 plants (H) transferred to control or sucrose-supplemented medium (from Chapter 4, Van Dingenen *et al.*,2016) and *hxk1* plants (I). The insets are close-ups of 12 and 13 DAS. J, Ratio of pavement cell area, cell number and stomatal index of the third leaf of Col-0 (black) and *hxk1* (blue) plants transferred to sucrose relative to the control, at 21 DAS. K and L, Ratio of epidermal cell number (K) and cell area (L) from 10 until 16 DAS of the third leaf of Col-0 and *hxk1* plants transferred to sucrose relative to the control. Values from (A) to (D) and (H) to (J) are the means of three biological repeats with their SE. Rosette and leaf area was measured for on average 12 leaves in each repeat. Cellular parameters were measured for four to five leaves in each repeat. Values in (E) and (F) are the means of four to eight leaves with their SE. Values in (G) are the means of two repeats with their SE. GUS intensity was measured for 8 to 10 leaves in each repeat. Values in (K) and (L) are the means from four to nine leaves. *, $P < 0.05$ for log-transformed values in (B) to (D) for *hxk1* compared to Col-0 and (H) to (L) for sucrose compared to control, mixed models and *, $P < 0.05$

To further examine the underlying cellular effect, pavement cell number and size were calculated daily from 9 until 16 DAS (Fig. 2E and F). At 9 DAS, *hxk1* leaf primordia already contained more cells than Col-0 (17%, $P = 0.21$; Fig. 2E inset). This increase in cell number was significantly different from wild type plants from 10 DAS to 14 DAS (Fig. 2E). No difference in the average cell size was observed between Col-0 and *hxk1.*

The positive effect on total pavement cell number was further investigated with the *pCYCB1;1::CYCB1;1-D-box:GUS* reporter that allows for the visualization of mitotic active cells, in Col-0 and *hxk1* mutant backgrounds. At 9 DAS, seedlings were transferred to control medium and harvested for GUS staining at 14 DAS, five days after transfer. Almost no GUS staining could be detected for the *pCYCB1;1::CYCB1;1-D-box:GUS/hxk1* mutant line, whereas *pCYCB1;1::CYCB1;1-D-box:GUS* wild type seedlings demonstrated a strong staining at the base of the third leaf (Fig. 2G). The GUS intensity was quantified in a defined region from the base to the tip of the leaf as described in Chapter 4 and in Vercruyssen *et al* (2014). *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* leaves showed weaker GUS activity as well as a cell cycle arrest front closer to the leaf base compared with *pCYCB1;1::CYCB1;1-D-box:GUS*/Col-0 control plants (Fig. 2G). These observations suggest that, at this time point, almost all cells of *hxk1* have reduced cell divisions and/or stopped dividing, whereas Col-0 cells are still actively dividing at the base of the leaf. Similar GUS staining profiles were observed at earlier time point 12 DAS

corresponding to three days after transfer (Supplemental Fig. S3). To further examine cellular differentiation in *hxk1* mutant leaves, ploidy levels were measured by flow cytometry in the third leaves of *hxk1* and Col-0 seedlings harvested daily from 11 to 17 DAS. *hxk1* leaves had an increased endoreduplication index, i.e. the average number of endocycles a cell undergoes, between 11 and 13 DAS, which suggests that *hxk1* cells have higher ploidy levels compared to wild type (Supplemental Fig. S4). In accordance, at the same time points decreased 2 C levels and increased 4 C levels were observed (Supplemental Fig. S4).

In conclusion, early during development, leaves of *hxk1* seedlings grown *in vitro* without sucrose are larger due to more pavement cells and *hxk1* leaf cells stop dividing and start differentiating earlier than in wild type plants.

hxk1 seedlings are Insensitive to the Sucrose-Induced Cell Proliferation during Early Leaf Development

In Chapter 4 (Van Dingenen *et al*, 2016), we showed that transfer of young seedlings to sucrose-containing media stimulates cell proliferation and increases final leaf size in wild type plants. To investigate whether HXK1 plays a role in this process, *hxk1* and Col-0 seedlings were transferred at 9 DAS to 15 mM sucrose-supplemented medium or control medium and area, pavement cell number and cell size of the third leaf were measured.

At 21 DAS, the third leaf area of Col-0 plants transferred to sucrose was increased by 43% ($P < 0.05$) compared to control plants grown without sucrose, whereas leaf area of the *hxk1* plants was not significantly increased by sucrose (11%, $P = 0.57$; Fig. 2B). To further examine the partial sucrose insensitivity of *hxk1* leaves, leaf sizes were measured at three consecutive time points, i.e. 20, 21 and 22 DAS. At these time points, addition of sucrose (at 9 DAS) resulted in a significant increase in the third leaf size of 30%, 45% and 39% ($P < 0.05$), respectively, for Col-0, whereas the leaf area of *hxk1* mutant plants remained unaffected by sucrose ($P > 0.05$; Supplemental Fig. S5).

Subsequently, a time-course experiment was performed by harvesting the third leaf daily after transfer, from 10 DAS until 21 DAS, and measuring its area. In Col-0 seedlings, the third leaf of sucrose-transferred plants was significantly larger than that

of control plants starting from 12 DAS, corresponding to 3 days after transfer (37%, $P < 0.05$), and remained larger until 21 DAS (Fig. 2H). Also in the *hxk1* mutant, leaf size was significantly increased by sucrose at 12 DAS (27%, $P < 0.05$) but this effect was less pronounced than in Col-0 plants and remained less pronounced until 21 DAS (Fig. 2I). The increased leaf area of Col-0 plants at 21 DAS was mainly due to a significant higher number of pavement cells (37%, $P < 0.05$; Fig. 2H and Supplemental Fig. S6). In the *hxk1* mutant, cell number was not significantly increased with 11% ($P = 0.70$; Fig. 2J and Supplemental Fig. S6). Average cell size of Col-0 and *hxk1* leaves remained unchanged by sucrose ($P > 0.05$). Stomatal index was slightly increased by sucrose in Col-0 leaves (8%, $P < 0.05$) but did not change upon transfer to sucrose in the *hxk1* mutant ($P > 0.05$). The positive effect of sucrose on cell proliferation was already found to be significant at 10 DAS, corresponding to 24 hours after transfer, in Col-0 seedlings (41%; $P < 0.05$; Fig. 2K). In *hxk1* mutant leaves, transfer to sucrose increased pavement cell number to a much lesser extend (16%; $P = 0.13$). At later time points no positive effect on cell number, such as was observed in wild type leaves, was found in *hxk1* mutant leaves, except at 16 DAS (41%, $P < 0.05$; Fig. 2K). In addition, similar to Col-0 seedlings, no consistent effect in the average cell size was found between control and sucrose-transferred *hxk1* seedlings (10-16 DAS; Fig. 2L).

As described previously, expression of *GPT2* is necessary for the sucrose-induced stimulation of cell proliferation at 10 DAS (Chapter 4; Van Dingenen *et al* 2016). To examine whether *GPT2* expression was compromised in *hxk1* seedlings, transcript levels were determined with qRT-PCR analysis in shoots of sucrose-transferred and control seedlings 24 hours after transfer (Supplemental Fig. S7). Interestingly, shoots of Col-0 and *hxk1* control seedlings had equal amount of *GPT2* transcript levels and also upon transfer to sucrose, *GPT2* expression was equally up-regulated in both lines.

In conclusion, *hxk1* seedlings are less sensitive to the sucrose-induced stimulation of cell proliferation at early time points. This effect was not due to a change in the sucrose-induced *GPT2* expression.

hxk1 have Larger Chloroplasts compared with Wild Type Upon Transfer to Sucrose

As described previously (see Chapter 4, Van Dingenen *et al*, 2016), transfer of wild type seedlings to sucrose-supplemented medium increases cell number and cells have fewer, smaller and less differentiated chloroplasts at 10 DAS. To examine the effect of sucrose on *hxk1* seedlings, chloroplast number and size were determined by transmission electron microscopy. Transverse cross-sections of Col-0 and *hxk1* leaves of seedlings transferred to control or sucrose-containing media were made two days after transfer (11 DAS). Mesophyll cell area, chloroplast number and chloroplast size were measured taking into account the differences between the tip and the base of the leaf.

The average mesophyll cell size did not differ between Col-0 and *hxk1* control leaves and in both lines cells were larger at the tip compared to the base of the leaf ($P < 0.05$; Fig. 3A). Sucrose treatment led to a significant decreased mesophyll cell area of 26% ($P < 0.05$) in Col-0 leaves at the tip and to no change in *hxk1* leaves (Fig. 3A). No clear difference in chloroplast ultrastructure and shape could be observed between Col-0 and *hxk1* seedlings and, sucrose and control conditions (Fig. 3B). However, chloroplasts at the tip of sucrose-transferred *hxk1* leaves have slightly increased grana stacking compared to all other conditions (Fig. 3B). For both lines and conditions, chloroplasts were larger at the tip compared with the base of the leaves ($P < 0.05$; Fig. 3C). Furthermore, in *hxk1* leaves, transfer to sucrose resulted in the formation of approximately 50% larger chloroplasts, although not significantly. Finally, chloroplast number per cell did not differ between Col-0 and *hxk1* control leaves and transfer to sucrose resulted in significant less chloroplasts in both lines (Fig. 3D).

In summary, transfer of seedlings to sucrose resulted in smaller mesophyll cells with less and smaller chloroplasts in Col-0 leaves, whereas no difference in mesophyll cell size but larger chloroplasts were found in *hxk1* leaves after transfer to sucrose.

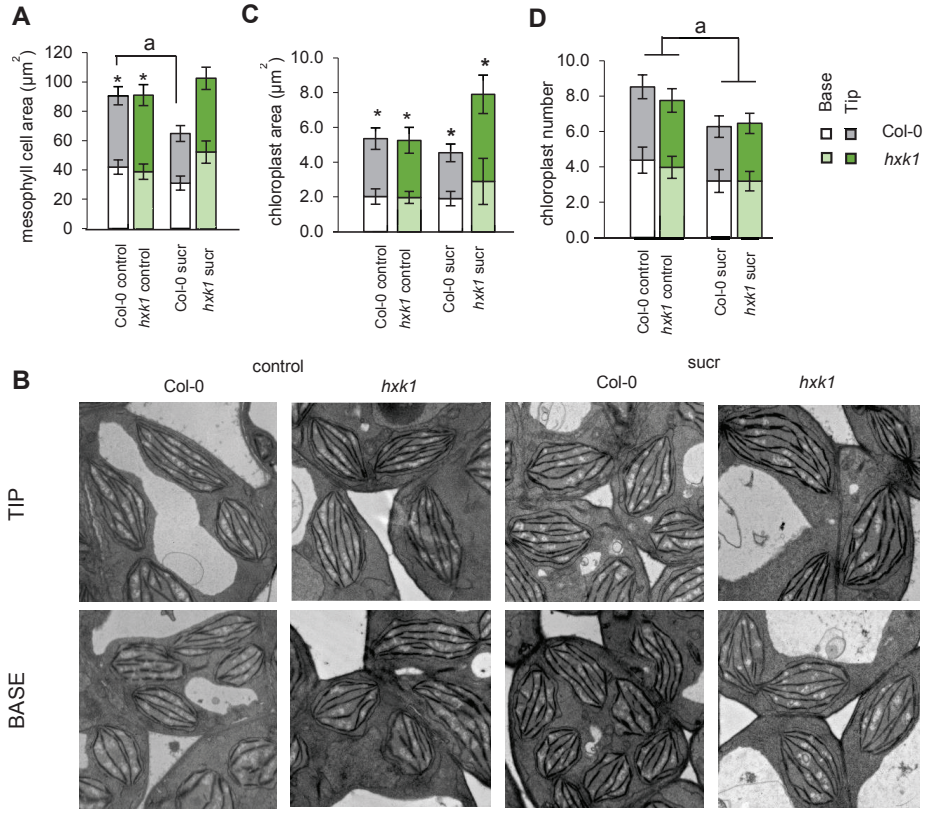

Figure 3. Mesophyll cell area, chloroplast size and chloroplast number in Col-0 and *hxk1* leaves upon transfer to sucrose. Col-0 and hxk1 leaves were grown on control medium without sucrose for 9 days, and subsequently transferred to control or 15 mM sucrose (sucr)-supplemented media for two additional days. Average mesophyll cell area (A), average chloroplast size (C) and chloroplast number (D) in the tip and base of the third leaf of control and sucrose-treated Col-0 (whit=base and grey=tip) and hxk1 (light green=base and dark green=tip) seedlings at 11 DAS. B, Transmission electron micrographs of tip and base of the 11-d-old third leaves of Col-0 and hxk1 seedlings after transfer to control or 15 mM sucrose (sucr) supplemented medium. The bar represents 1 µm. Values are means ± SE from 27-87 mesophyll cells of the tip and the base of two independent leaves. *, $P < 0.05$ for tip compared to base and a, $P < 0.05$ for sucrose compared to

Expression of 35S::HXK1-GS^{green} vector in *hxk1* mutant plants restores growth and sugar-responsiveness

To identify novel interaction partners of HXK1, tandem affinity purification (TAP) experiments were performed with the HXK1 GS^{rhino}- or GS^{green} tagged fusion proteins. These tags are derivatives of the TAP tag GS (Van Leene *et al*, 2008). GS^{rhino} consists of the protein-G (G) tag and the streptavidin-binding peptide (S) separated by rhinovirus 3C protease cleavage sites (Van Leene *et al*, 2015). In the GS^{green} TAP-tag, the protein-G tag in GS^{rhino} has been replaced by GFP, allowing also

in vivo visualization of the bait protein. Both tags were C-terminally fused to HXK1 because the N-terminal domain contains a mitochondrion-anchor domain (Balasubramanian *et al*, 2007). Both constructs were expressed under the control of the 35S promoter. Prior to the TAP experiments, the functionality of the construct was tested by transforming *hxk1* mutant plants with the 35S::HXK1-GS^{green} vector. The abundance of the GS^{green}-tagged HXK1 protein was tested in 9-day-old seedlings by western blot and showed to be high in two independent T2 transgenic lines (Fig. 4A). One of the lines was selected for further upscaling and in the next generation (T3), the overexpression of *HXK1* was confirmed in 10-day-old seedlings with qRT-PCR analysis (Fig. 4B). Next, the effect of the gain-of-function of HXK1 in the *hxk1* background was verified (Fig. 4C and D).

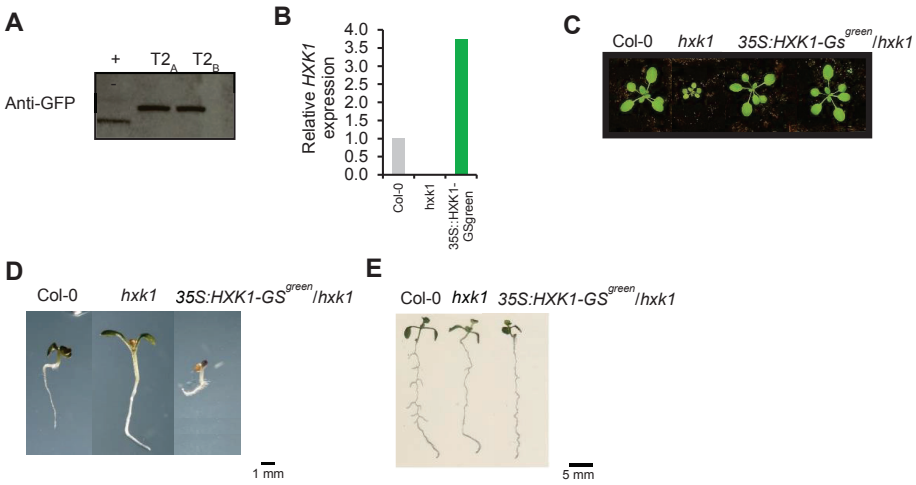

Figure 4. Complementation of *hxk1* mutation with 35S::HXK1-GS^{green} fusion protein. A, Western blot of 9-day-old T2 hxk1 seedlings expressing the 35S::HXK1-GSgreen fusion protein using primary antibody against GFP. +, positive control and -, negative control Col-0 seedlings. B, Relative HXK1 expression in 10-day-old *35S::HXK1-GS^{green}/hxk1* T3 seedlings. C, 22-day old Col-0, *hxk1* and *35S::HXK1-GS^{green}/hxk1* plants grown in soil under high light intensity of approximately 100 µmol/m^{-2} s^{-1}. D, 8-day-old Col-0, *hxk1* and *35S::HXK1-GS^{green}/hxk1* seedlings grown on vertical plates supplemented with 6% glucose. E, 10-day-old Col-0, *hxk1* and *35S::HXK1-GS^{green}/hxk1* seedlings grown on vertical plates with 1% sucrose.

As described above, the average rosette size of the *hxk1* mutant was reduced when grown under high light intensity. Interestingly, mutant plants expressing the 35S::HXK1-GS^{green} vector demonstrated a normal rosette growth which was similar to the rosette of Col-0 wild type plants (Fig. 4C). To investigate the sugar

responsiveness of the 35S::HXK1-GS^{green}/*hxk1* line, the seedlings were grown on 6% glucose. The transgenic line showed a hypersensitive response with almost complete inhibition of cotyledon expansion and chlorophyll accumulation, with high anthocyanin production and reduced root growth compared to *hxk1* mutant seedlings which were insensitive to the high glucose concentrations (Fig. 4D). Finally, the root waving pattern of 10-day old seedlings grown on vertical plates with 1 % sucrose was checked. The typical waving pattern which was absent in the *hxk1* mutants was partially rescued in the *hxk1* mutant expressing the 35::HXK1-GS^{green} vector (Fig. 4D).

Next, the localization of the HXK1-GS^{green} fusion protein was verified in roots and cotyledons of 5-day old 35S::HXK1-GS^{green}/*hxk1* transgenic seedlings. Fluorescence was detected in both roots and cotyledons, with an apparent localization in mitochondria (Fig. 5A). Mitochondrial localization was confirmed by co-localization with the mitochondrial specific dye Mito tracker red (Fig. 5B, C). Interestingly, in the cotyledons the two signals of the HXK1-GS^{green} fusion protein and the Mito tracker red, mainly merged in larger foci which were also described before in Balasubramanian *et al*. (2007), and probably represent aggregated mitochondria.

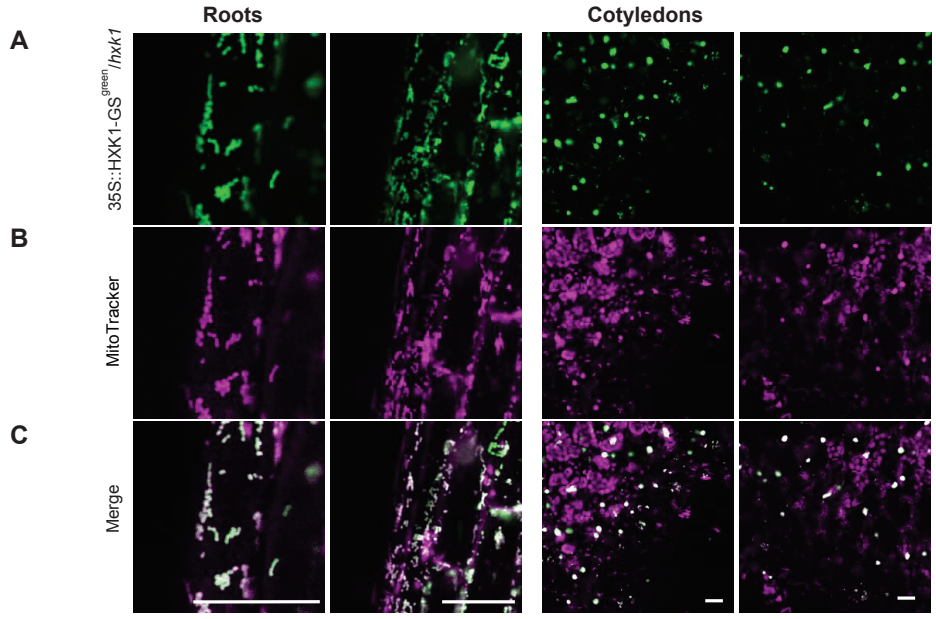

Figure 5. Localization of the 35S::HXK1-GS^{green} fusion protein in Arabidopsis seedlings. Confocal images of 5-day-old 35S::HXK1-GS^{green}/*hxk1* Arabidopsis seedlings grown on vertical plates on MS medium. A, HXK1-GS^{green} fluorescence in roots and cotyledons. B, Mitochondrial staining with Mito tracker red. C, Merged images of A and B. Scale bar is 20 µm.

In conclusion, expressing of the GS^{green}-tagged HXK1 under the control of the 35S promoter in the *hxk1* mutant background rescued the growth inhibition and the sugar insensitivity of the *hxk1* mutant line and demonstrated normal HXK1 localization to the mitochondria.

Finding Sucrose-responsive Interaction Partners of HXK1

To identify novel and sugar-dependent HXK1 interactors, HXK1 was used as bait for tandem affinity purification (TAP) experiments and consecutive mass spectrometry analysis. As described above, HXK1 was C-terminally fused to the GS^{rhino} TAP tag for expression in cell cultures and to the GS^{green} TAP tag for expression in seedlings (Van Leene *et al*, 2008).

TAP experiments were performed in two biological repeats from Arabidopsis cell suspension cultures grown in three different growth conditions. For the first condition, Arabidopsis suspension cells were continuously grown in the presence of 3% sucrose, for the second condition cell cultures were sucrose-starved for 24 hours and for the third condition the sucrose-starved cell cultures were resupplied with 3% sucrose for 15 minutes. From the three different growth conditions, a total of 23 co-purified proteins could be retained after subtraction of aspecific background proteins based on the procedure described in Material and Methods (Table 1). Five proteins were pulled-down with HXK1 from cells continuously grown in the presence of sucrose but only in one experiment (Table 1). TAP performed on sucrose-starved cell cultures resulted in the purification of eleven other proteins, from which five were also found after re-supplementation of sucrose. Two of these five overlapping proteins are mitochondrial inner membrane proteins, the alternative oxidase 1A (AOX1A; AT3G22370) and a leucine zipper-EF-hand-containing transmembrane protein (LETM1; AT3G59820; Zhang et al 2012). A similar purification pattern was found for two plasma membrane proteins, a non-race-specific disease resistance (NDR)-like protein/tobacco hairpin-induced gene (HIN)-like protein (AT5G06320) and a senescence/dehydration-associated protein EARLY-RESPONSIVE TO DEHYDRATION7 (ERD7; AT2G17840). The fifth protein found to interact with HXK1 in both conditions was a histone H2A protein, HTA6 (AT5G59870). Five of the six other proteins exclusively purified after sucrose starvation were only found in one

experiment (Table 1). KINγ1 (AT3G48530) was the only protein isolated twice after sucrose starvation. Re-supplementation of sucrose for 15 minutes resulted in the identification of seven additional proteins, besides the five isolated in both conditions (Table 1). Six of these seven proteins were only purified once and were assigned to distinctive protein families (Table 1). However, one protein, a quinone reductase family protein (AT4G36750), was exclusively isolated after sucrose addition and found in both independent repeats. Finally, one TAP experiment was performed *in planta* on *35S::HXK1-GS^{green}/hxk1* transgenic seedlings subjected to the experimental *in vitro* sucrose assay (Chapter 4, Van Dingenen *et al*, 2016). Unfortunately, interactors identified with TAPs from seedlings transferred for 1 hour to control or sucrose-supplemented media did not reveal any overlap with the proteins co-purified with HXK1 from cells. Nevertheless, several other interesting interaction partners were found which could be of interest for future research (Supplemental Table S1).

Taken together, by using different sugar treatments on cell cultures, 23 different proteins could be purified together with HXK1. However, only two of these 23 proteins were isolated twice independently in specific growth conditions, a quinone reductase family protein which was only isolated after re-addition of sucrose and KINγ1 which co-purified with HXK1 from sucrose-starved cell cultures.

Role of the Putative HXK1 Interaction Protein KINγ1 in Sucrose-Induced Responses

From the TAP experiment, we found that KINγ1 is a possible interesting HXK1 protein partner putatively involved in sugar responses (Table 1). KINγ1 was, based on its structure, suggested to belong to the regulatory subunits of the heterotrimeric SnRK1 complex (Gissot et al, 2006), although no direct involvement in SnRK1 sugar-mediated signaling was demonstrated (Ramon *et al*, 2013). We found that KINγ1 copurifies with HXK1 when cell cultures are sucrose-starved and it was not isolated when cell cultures were treated with sucrose (Table 1).

Table 1. Proteins identified in the purification experiments with HXK1 in cell cultures grown in three different growth conditions.

			sucr starvation	3% sucr	cell cultures
Gene ID	**Name**	**Description**			
AT3G48530	**KINγ1**	SNF1-related protein kinase regulatory subunit gamma 1	**2**		
AT1G76850	**SEC5A**	exocyst complex component sec5	**1**		
AT2G33040	**ATP3**	gamma subunit of Mt ATP synthase	**1**		
AT2G42790	**CSY3**	citrate synthase 3	**1**		
AT2G44530		Phosphoribosyltransferase family protein	**1**		
AT5G49830	**EXO84B**	exocyst complex component 84B	**1**		
AT5G59870	**HTA6**	histone H2A 6	**2**	**1**	
AT4G36750		Quinone reductase family protein		**2**	
AT2G17840	**ERD7**	Senescence/dehydration-associated protein-related	**1**	**2**	
AT3G22370	**AOX1A**	alternative oxidase 1A	**1**	**2**	
AT3G59820	**LETM1**	LETM1-like protein	**1**	**2**	
AT5G06320	**NHL3**	NDR1/HIN1-like 3	**1**	**2**	
AT1G07180	**NDI1**	alternative NAD(P)H dehydrogenase 1		**1**	
AT1G48900		Signal recognition particle		**1**	
AT2G26990	**CSN2**	Proteasome family protein/COP signalosome subunit		**1**	
AT4G20830		FAD-binding Berberine family protein		**1**	
AT4G23630	**BTI1**	VIRB2-interacting protein 1		**1**	
AT5G43900	**MYA2**	myosin 2		**1**	
AT3G08947		ARM repeat superfamily protein			**1**
AT4G26110	**NAP1;1**	Nucleosome assembly protein1;1			**1**
AT2G19480	**NAP1;2**	Nucleosome assembly protein 1;2			**1**
AT5G58410		HEAT/U-box domain-containing protein			**1**
AT1G68680		unknown protein			**1**

* TAPs were performed on *Arabidopsis* cell cultures continuously grown in the presence of sucrose (cell cultures), on cell cultures that were sucrose-starved for 24 hours (sucr starvation) and on sucrose-starved cells resupplied with 3% sucrose for 15 minutes (3% sucr). The numbers indicate the number of experiments in which the protein was identified. TAPs were performed in two independent repeats.

If HXK1 and KINγ1 are part of the same complex, they should have similar cell localization. According to the webtool, SUBA3 (http://suba3.plantenergy.uwa.edu.au/), localization of KINγ1 is predicted to be in the cytosol. By transient expression of C-terminal and N-terminal RFP translation fusion proteins in tobacco leaves, we could confirm the cytosolic localization of KINγ1 (Fig. 6A). Transient co-expression of the HXK1-GS^{green} fusion protein with either 35S::KINγ1-RFP or 35S::RFP-KINγ1 did not reveal clear overlap, although, a possible co-localization at the outside of mitochondria might be possible (Fig. 6A).

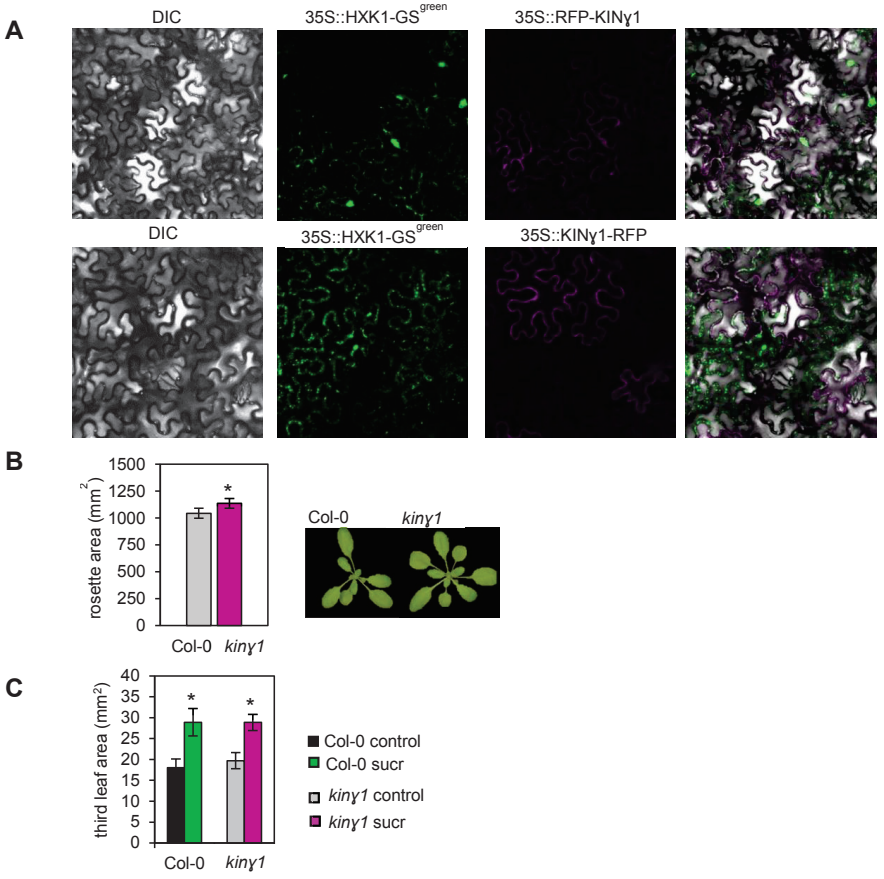

Figure 6. Localization of KINγ1 fusion protein and growth phenotypes of *kinγ1* mutant. A, Transient co-expression of KINγ1-RFP or RFP-KINγ1 with HXK1-GSgreen fusion proteins in leaves of *Nicotiana benthamiana*. Infiltrated leaves were imaged three days after Agrobacterium-mediated transformation in lower epidermal cells. B, Rosette size and images of Col-0 and *kinγ1* plants grown in soil for 22 days under a high light intensity of approximately 100 µmol/m-2s-1. C, Col-0 and *kinγ1* mutant seedlings were grown in vitro for 9 days without sucrose and transferred at 9 DAS to medium without (control) or with 15 mM sucrose (sucr). Leaf area was measured at 21 DAS. Values are means of three biological repeats with their SE. Leaf area was measured for 5 to 10 leaves in each repeat. *, P < 0.05 in (A) compared to Col-0 and in (B) for sucrose compared to control; mixed models.

To verify the direct interaction of HXK1 and KINγ1, a Bimolecular Fluorescence Complementation (BiFC) experiment was performed in tobacco leaves, but failed to produce fluorescent signal (data not shown). Additionally, a Yeast-Two-Hybrid (Y2H) assay was done using yeast growth media supplemented with glucose or with galactose/raffinose to rule out a possible inhibitory effect of glucose on the protein interaction. However, transformation of yeast with KINγ1 expressed in the pDEST32 destination vector resulted in strong auto-activation and the HXK1-KINγ1 interaction could not be confirmed (Supplemental Fig. S8). Localization and interaction was also checked in 8-days-old Arabidopsis cell cultures which were sucrose starved for 24 hours. Similarly as shown before in tobacco leaves, GFP fluorescence of the HXK1-GS^{green} fusion protein was detected in small and large foci possibly representing mitochondria, whereas C- and N-terminal KINγ1 fusion proteins were localized to the cytosol (Supplemental Fig. S9). No co-localization could be detected for HXK1-GS^{green} and KINγ1-RFP fusion proteins, whereas RFP-KINγ1 fusion protein co-localized with HXK1-GS^{green} in some cells.

Finally, a BiFC experiment was performed in sucrose-starved cell cultures expressing HXK1 C-terminally fused to head GFP (hGFP) and KINγ1 N-terminally fused to tail GFP (tGFP). Weak GFP fluorescence signal was detected, which was stronger than the background fluorescence signal of untransformed wild type PSB-L cell cultures (Fig. 7). Transformation with tGFP-KINγ1 fusion alone or the BiFC combination before sucrose starvation did not result in a detectable fluorescence signal (data not shown).

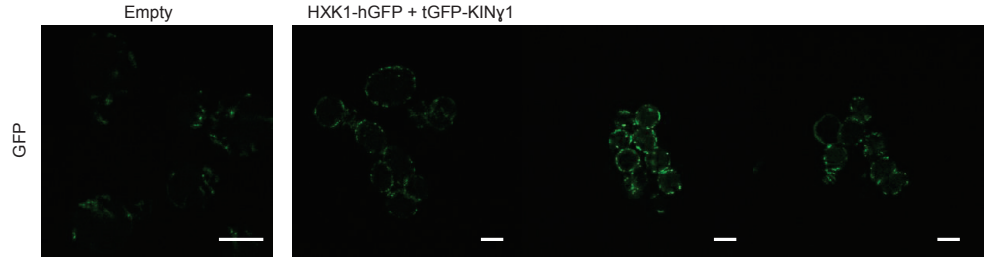

Figure 7. Bimolecular Fluorescence Complementation in sucrose-starved cell cultures demonstrates weak HXK1-KINγ1 interaction. Arabidopsis cell cultures were stable co-transformed with HXK1-head GFP (hGFP) and KINγ1-tail GFP (tGFP) fusions. Empty represents autofluorescence in untransformed wild type PSB-L cells. Scale bar represents 20 µm.

To study whether KINγ1 is involved in the regulation of growth, rosette size of homozygous *kinγ1* knock-out mutant plants grown in soil under high light intensity of approximately 100 µmol/$m^{-2}s^{-1}$ was measured. At 22 DAS, rosettes of *kinγ1* knock-out plants were significantly larger than wild type plants indicating a role of KINγ1 in growth regulation under these environmental conditions (9%, $P < 0.05$; Fig. 6B). To explore whether KINγ1 is involved in the sucrose-induced leaf growth stimulation, *kinγ1* knock-out mutant seedlings were subjected to the experimental sucrose assay developed previously (Chapter 4, Van Dingenen *et al*, 2016). At 21 DAS, the average third leaf area was significantly increased upon transfer to sucrose by 60% and 46% in Col-0 plants and *kinγ1* mutant plants, respectively ($P < 0.05$). However, no difference in the effect on final third leaf size could be observed between wild type Col-0 and *kinγ1* mutant plants ($P = 0.74$; Fig. 6C).

DISCUSSION

The *gin2* mutant in the Landsberg *erecta* (L*er*) genetic background was selected from a large ethyl methanesulfonate (EMS)-mutagenized seed collection as a mutant that can still grow on 6-7% glucose (Zhou et al, 1998). We found a similar glucose insensitive response for the T-DNA insertion mutant *hxk1* in the Col-0 background. Furthermore, down-regulation of *HXK1* expression results in inhibition of growth under high light intensities both in the *Ler* (Moore *et al*, 2003) and Col-0 background. Here we showed that the reduced leaf size of these soil-grown *hxk1* plants was due to less and smaller cells. In order to explore whether HXK1 is important for the increase in leaf growth upon transfer of seedlings to sucrose, the previously described experimental setup was used (Chapter 4, Van Dingenen *et al*, 2016). Under the control growth conditions, i.e. a low light intensity of approximately 50 µmol/$m^{-2}s^{-1}$ and no exogenously supplied carbon source, both final rosette size and third leaf area of the *hxk1* mutant were not affected compared with wild type plants. Also other sugar metabolism related mutants exhibit these light-dependent phenotypes. For example, double mutants of the *triose-phosphate/phosphate translocator* (*tpt*) with starch-deficient or starch metabolizing mutants, exhibit severe

growth retardation under high light intensities but are indistinguishable from wild type plants under low light conditions (Schmitz *et al*, 2014; Schmitz *et al*, 2012).

Since under the low light conditions without exogenously supplied sucrose, the *hxk1* mutant behaves as wild type in terms of growth, this creates an excellent condition to monitor the role of HXK1 during early leaf development without drastic metabolic disturbances. Differences in size between Col-0 and *hxk1* control leaves were found at early time points (9-14 DAS), whereas no difference could be detected at mature stage (21 DAS). At the early developmental stages, i.e. 9-10 DAS, leaves mainly grow by cell proliferation and act as sink tissues (Andriankaja *et al*, 2012). *hxk1* mutants contained more cells compared to wild type plants, suggesting that HXK1 might play a role in inhibiting cell proliferation in sink tissues to fine-tune growth. Increases in cell number are often accompanied by a delay in the transition to cell expansion (Gonzalez *et al*, 2012). However, *hxk1* leaves showed decreased intensity and a shorter region of *pCYCB1;1::CYCB1;1-D-box:GUS* marker gene expression at 12 and 14 DAS indicating a reduction in cell cycle activity. Furthermore, the increased endoreduplication index observed during the transition phase (12 -13 DAS) indicates that at least some cells of *hxk1* leaves stop dividing and are triggered to differentiate earlier than in wild type plants. We indeed observed a shorter GUS-stained length of the *pCYCB1;1::CYCB1;1-D-box:GUS* marker line in *hxk1* sink leaves, although no concomitant increase in cell size was seen. The onset of the transition to cell differentiation and expansion was found to be preceded by the establishment of the photosynthetic machinery (Andriankaja *et al*, 2012). It is well known that HXK1 represses nuclear-encoded photosynthesis-related gene expression inhibiting photosynthesis (Jang *et al*, 1997; Moore *et al*, 2003). Because sugars are unable to repress the transcription of photosynthesis-related genes in the *gin2* mutant (Moore *et al.*, 2003), it is possible that sink leaves undergo an accelerated transition to cell expansion. In addition, no difference in chloroplast shape, size or ultrastructure was observed between wild type and *hxk1* control leaves. These findings suggest that in sink cells, HXK1–mediated signaling does not affect the establishment of the photosynthetic machinery which, consequently, could affect the onset of cell expansion. Increased levels of sucrose, fructose and glucose are reported in rosette leaves of the *gin2* mutant (Heinrichs *et al*, 2012). One possibility to explain the increased cell number at early time points could be that

higher glucose levels in *hxk1* leaves stimulate cell cycle progression by inducing the expression of D-type cyclins as previously described (Riou-Khamlichi *et al*, 2000). Another possibility could be that the *hxk1* mutant already has more cells during leaf primordium formation. Recruitment of more cells to the shoot apical meristem (SAM) has been demonstrated to be one of the mechanisms that can contribute to increase leaf size (Eloy *et al*, 2012; Gonzalez *et al*, 2012).

At 9 DAS, when the third leaf was still fully proliferating, seedlings were transferred to sucrose-supplemented media. In wild type plants, these higher sucrose levels result in repression of transcription of the plastome, in a delay in chloroplast development and in stimulation of cell proliferation via the sucrose-induced expression of *GPT2* (Chapter 4, Van Dingenen *et al*, 2016). *GPT2* transcript levels were similar in Col-0 and *hxk1* control shoots and *GPT2* was equally induced by sucrose in both lines. Also in *Ler* background, sugar treatment did not result in a different response in *GPT2* expression between wild type and *gin2* plants (Heinrichs *et al*, 2012). These findings suggest that HXK1 does not play a role in the regulation of *GPT2* expression. Furthermore, sucrose was found to repress chloroplast differentiation in sink leaves since smaller chloroplasts with less differentiated thylakoid membranes and starch granules were observed (Chapter 4, Van Dingenen *et al*, 2016). Contrastingly, in *hxk1* leaves, transfer to sucrose resulted in larger chloroplasts, particularly in the tip of the leaf. Higher sucrose levels in the cell might result in more glucose and glucose-6-phosphate (G6P) levels in both wild type and *hxk1* leaves which can be imported in the chloroplasts via *GPT2* and which might result in a repression of plastome transcription. It has been described that the *gin2* mutant maintains half of the glucose phosphorylation capacity of wild type plants, but shows increased glucose-6-phosphate (G6P) levels (Moore *et al*, 2003). G6P can be generated by other HXKs, such as HXK2 and HXK3 (Karve *et al*, 2008), or can be formed from F6P by phosphoglucoisomerase (Fettke & Fernie, 2015). *hxk1* mutants are known to induce nuclear-encoded photosynthesis gene expression independent of the presence of sugars (Jang *et al*, 1997). In addition, transfer to sucrose did not stimulate cell proliferation in the *hxk1* mutant as much as in wild type which suggests that HXK-mediated inhibition of photosynthesis overrules the sucrose-induced repression of chloroplast differentiation through *GPT2*.

Interestingly, pavement cell number was found to be increased by sucrose at later time points in the *hxk1* mutant. At these time points, most cellular divisions result from the stomatal lineage (Gonzalez *et al*, 2012). So, this late increase in cell number might point to an induction of asymmetric meristemoid division by sucrose. Meristemoids are stomatal precursor cells that divide asymmetrically resulting in the formation of additional pavement cells during leaf development (Geisler *et al*, 2000). In wild type plants, sucrose addition increases the stomatal index in leaves (Chapter 4, Van Dingenen *et al,* 2016), which is not observed in the *hxk1* mutant. Higher sucrose levels might trigger HXK1-signaling to generate stomata from meristemoids. Impairment in HXK1-signaling might result in more asymmetric divisions forming additional pavement cells instead of stomata at later time points. A detailed analysis of meristemoid division is required to test above hypothesis.

Another goal of this study was to identify novel HXK1 protein interactors involved in sugar-mediated signaling to regulate growth. Recently, it was shown that glucose-bound and unbound HXK1 exhibit structural differences explaining the dual functions of HXK1 and, thus, different protein-protein interaction possibilities depending on the sugar availability (Feng *et al*, 2015). A total of 23 different proteins were found to co-purify with HXK1 in cell cultures. Because the proteins purified from cells continuously grown in the presence of sucrose were only found in one independent experiment and did not overlap with the sucrose-starved cell cultures and the cell cultures re-supplemented with sucrose, we only focus here on the proteins identified in the latter two conditions. Some of the putative protein interactors identified have a distinctive predictive localization (e.g. on plasma membrane, mitochondria or peroxisome) compared to the verified mitochondrial localization of HXK1 as determined in this study and the existence of a putative nuclear HXK1 complex (Cho *et al*, 2006). The different localizations suggest that some of these proteins might be false-positives possibly due to artificial interaction during the extraction procedure. However, two mitochondrial proteins, AOX1A and the LETM1-like protein, were found to interact with HXK1 when sucrose is supplemented to cell cultures. AOX1A is involved in the alternative respiration pathway to reduce ROS and ATP production (Millar *et al*, 2011). Different biotic and abiotic stresses are known to induce AOX1A expression and a role of AOX1A in mitochondrial retrograde signaling has been demonstrated (Van Aken *et al*, 2009). Recently, several studies have highlighted a

central role of AOX1A in optimizing photosynthesis (Gandin *et al*, 2012; Vishwakarma *et al*, 2014; Vishwakarma *et al*, 2015). LETM1 has been described to act redundantly with LETM2 in maintaining mitochondrial function (Zhang *et al*, 2012). Co-purification of these two mitochondrial proteins probably reflects the metabolic function of HXK1. Higher sucrose levels stimulate HXK1 to phosphorylate glucose and, subsequently, to generate pyruvate during glycolysis to fuel the mitochondrial respiration (Fernie *et al*, 2004). Another interesting protein which was purified twice together with HXK1, when sucrose was added, is ERD7. *ERD7* was identified, together with 16 other *ERD*s, as an early dehydration-responsive gene (Kiyosue *et al*, 1994) and its expression is also induced by high light, drought, cold and salt stress (Kimura *et al*, 2003). ERDs have been suggested to function in redistributing sugars to protect cells from biotic stresses (Kiyosue *et al*, 1998). Furthermore, *ERD7* expression was up-regulated in the *sweetie* mutant, a carbohydrate metabolism mutant which has elevated levels of trehalose, trehalose-6-phosphate and starch, and exhibits severe growth defects and early senescence (Veyres *et al*, 2008). *HXK1* over-expression and *gin2* mutant plants demonstrate an early onset and a delayed onset of senescence, respectively (Dai *et al*, 1999; Kelly *et al*, 2012). The interaction of HXK1 with ERD7 when sugar levels rise in the cell might suggests a role for ERD7 in HXK1-mediated senescence. Finally, only one protein was exclusively found after re-supplementation of sucrose, a quinone reductase family protein with oxidoreductase activity. Quinone reductase proteins are involved in the cyclic electron flow around photosystem I during the light reactions of photosynthesis (Joly *et al*, 2010). However, the identified quinone reductase protein was found to localize to the plasma membrane via GFP-fusion (Marmagne *et al*, 2004), suggesting a different biological function and indicating that the interaction with HXK1 might be artificial.

Two other interesting proteins were only found to interact with HXK1 when cells are sucrose-starved, HTA6 and KINγ1. The nucleus-localized HTA6 or H2A.W, one of the four histone 2A variants involved in compacting DNA in the chromatin (Kawashima *et al*, 2015). HTA6 is required for heterochromatin condensation, preventing accessibility of DNA and transcription (Yelagandula *et al*, 2014). Sucrose starvation induces the expression of photosynthesis genes via the HXK1-mediated signaling pathway and, in accordance, HXK1 mediates down-regulation of photosynthesis when sugar levels rise (Moore *et al*, 2003; Yu, 1999). Therefore,

HXK1 has been suggested to translocate to the nucleus to regulate transcription in a large nuclear complex, isolated from 18-days old soil-grown plants (Cho *et al*, 2006). Co-purification of HXK1 with HTA6 when sucrose levels are low, suggests that at least a fraction of the HXK1 proteins is localized in the nucleus to interact with HTA6, maybe resulting in suppression of heterochromatin formation at specific DNA regions, such as the ones harboring photosynthesis genes. When sucrose is added, the HTA6-HXK1 interaction was only found in one TAP experiment, suggesting a predominant localization of HXK1 to the mitochondria, which is in line with the co-purified mitochondrial proteins (Table 1). Thereby HTA6 is free to induce heterochromatin formation and inhibit transcription. Finally, the other sucrose-starvation specific interaction partner of HXK1 is KINγ1. This protein is described as a regulatory γ-subunit of the SnRK1 heterotrimeric complex based on its structure (Ghillebert *et al*, 2011). The SnRK1 complex is activated by sugar and nutrient depletion and upon different stresses stimulates cellular metabolism and represses general biosynthetic pathways to sustain growth (Baena-Gonzalez *et al*, 2007). The γ-subunits have been suggested to act as energy-sensing proteins, similarly as for the mammalian orthologue of SnRK1, AMPK, which binds adenosine nucleotides (Xiao *et al*, 2011). Transcripts of Arabidopsis KINγ1 were found to accumulate in the dark and KINγ1 was unable to complement the *snf4* mutant in yeast suggesting a different biological function (Bouly *et al*, 1999). Furthermore, KINγ1 cannot interact with β subunits to generate the functional SnRK1 heterotrimeric complex (Ramon *et al*, 2013). In addition, *kinγ1* knock-out mutants demonstrate starvation responses and do not affect SNRK1 target gene expression (Ramon *et al*, 2013). Also in this study, *kinγ1* seedlings were still sensitive to sucrose when grown *in vitro*. Transfer to sucrose resulted in a similar increase in final leaf size as in wild type plants. Cytosolic interaction between HXK1 and KINγ1 is possible because KINγ1 is located in the cytosol and HXK1 at the outside of mitochondria as shown in tobacco leaves as well as in cell cultures. However, both proteins only co-localized in some cells and the direct interaction could not be confirmed by BiFC in tobacco leaves or by Y2H assays. This absence could be due to the fact that both systems have intrinsic high sugar content (tobacco leaves that perform photosynthesis and general carbon metabolism in yeast cells), whereas the interaction was only identified when cells were sucrose starved. In this study, we were able to show, a weak HXK1-KINγ1

interaction by BiFC in sucrose starved cell cultures. KINγ1 might act as an additional nutrient sensor which is activated when soluble sugar levels are low or upon different stresses, such as described for the SnRK1 complex (Baena-Gonzalez *et al*, 2007). The *kinγ1* mutant was slightly but significantly larger than wild type plants under the same environmental conditions that lead to a growth reduction of the *hxk1* mutant. Hence, it is tempting to speculate that KINγ1 and HXK1 act antagonistically, as growth inhibitor and growth promoter, respectively, under these environmental conditions. Increased light intensity or sucrose addition could inactivate KINγ1 which results in release of the HXK1-KINγ1 interaction. Consequently, HXK1 is free to sense glucose and triggers HXK1-mediated repression of photosynthesis to maintain growth. Furthermore, inactivation of KINγ1 could result in further stimulation of biosynthetic processes, and, thus, also enhance growth. However, future experiments are needed to examine this putative HXK1/KINγ1-mediated growth-regulatory pathway in more detail. Because KINγ1 is activated upon different stresses, its role might be only apparent under specific environmental conditions. To identify whether KINγ1 and HXK1 could act together to regulate growth under suboptimal environmental conditions, double *hxk1 kinγ1* mutants as well as crosses between the 35S::HXK1^{GSgreen} overexpression line and the *kinγ1* mutant are currently generated. The growth of these lines will be measured under different environmental conditions, such as extreme high light intensities and high glucose concentrations, to further elucidate how these proteins are interconnected.

MATERIALS AND METHODS

Growth Conditions *in vitro* and soil

For the in soil experiments, plants were grown for 22 days at 21℃ under a 16-h day (100 µmol m-2 s-1) and 8-h night regime. For the *in vitro* experiments, seedlings were grown on half-strenght MS medium (Murashige & Skoog, 1962) under a 16-h day (50 µmol m^{-2} s^{-1}) and 8-h night regime. Seedlings were grown for 9 days (9 DAS) on nylon mesh of 20-µm pore size overlaying growth medium without sucrose. At 9 DAS, seedlings were transferred to plates containing control medium without sucrose or medium supplemented with 15 mM sucrose. In the high glucose experiments, seedlings were grown for 8 days on MS medium with 6% glucose. For the root waving experiments, seedlings were grown on vertical plates without sucrose or supplemented with 1% sucrose.

Transgenic Lines and Mutants

All experiments were performed on *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0). The *hxk1* mutant was obtained from the SALK collection (SALK_018086). The *kinγ1* mutant was described by Ramon *et al*. 2013 and was kindly provided by Prof. Fillip Rolland. The *pCYCB1;1::CYCB1;1-D-box:GUS* reporter line (Eloy *et al*, 2012b) was crossed with *hxk1* to obtain homozygous *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* lines. Through Multisite Gateway cloning the 35S::HXK1-GS^{green} and 35S::HXK1-GS^{rhino} construct were made (Karimi *et al*, 2007a; Karimi *et al*, 2007b). 35S::HXK1-GS^{green} was introduced into pH7m34GW-FAST vector (Shimada *et al*, 2010) and transformed *in Arabidopsis thaliana* Col-0 by floral dip using the *Agrobacterium tumefasciens* strain C58C1 (pMP90).

Growth Analysis

For the leaf area analysis, leaves were cleared in 100% ethanol, mounted in lactic acid on microscope slides, and photographed. Leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov//ij/). Abaxial epidermal cells of leaves were drawn with a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective. Drawings were scanned and analyzed using

automated image analysis algorithms (Andriankaja *et al*, 2012). Subsequently, drawings were used to measure average cell area, from which the total pavement cell number was calculated. The stomatal index was defined as the percentage of stomata compared with all cells.

GUS Staining and Analysis

Seedlings of two to three biological repeats were harvested at 12 and 14 DAS, incubated in heptane for 10 min and subsequently left to dry for 5 min. Then, they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50mM NaCl buffer (pH 7.0), 2mM K3[Fe(CN)6], and 4mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C overnight. Seedlings were cleared in 100% ethanol and then kept in 90% lactic acid. The third leaf was dissected, mounted on slides and photographed under a light microscope.

RNA Extraction and expression analysis by qRT-PCR

Seedlings or shoots were frozen in liquid nitrogen and RNA was extracted using Trizol (Invitrogen) and the RNeasy Plant Mini Kit (Qiagen). DNase treatment was done on columns with RNase-free DNase I (Promega). The iScript cDNA synthesis kit (Bio-Rad) was used to prepare cDNA from 500 ng µg RNA and qRT-PCR was done on the LightCycler 480 with SYBR Green I Master (Roche) according to the manufacturer's instructions. Normalization was done against the average of three housekeeping genes AT1G13320, AT2G32170, AT2G28390.

Western Blot

Protein extraction was done as described before (Van Leene *et al*, 2007). Western-blot was performed with primary rabbit anti-GFP antibodies (Santa Cruz; diluted 1:4000) for 1 hours and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibodies (1:10000) for 1 hours. Proteins were detected by chemiluminescence (NEN Life Science Products).

Yeast-two-hybrid assay

HXK1 and KINγ1 were N-terminal fused to the GAL4BD (DNA-binding domain, pDEST32) and to the GAL4AD (Activation domain, pDEST22) through Gateway cloning. GUS was fused to GAL4BD and GAL4AD as control. Fusion proteins were co-transformed in PJ69-4A competent yeast cells using poly-ethylene glycol lithium acetate-mediated transformation (Gietz & Schiestl, 2007). Individual co-transformed yeast colonies were selected through growth on solid Synthetic Defined (SD, Clontech) minimal media supplemented with an amino acid mix without leucine and tryptophan (DO supplement, Clontech). Four to five colonies were tested for interaction by growing them on SD selective medium (with glucose or with galactose/raffinose) without leucine, tryptophan and histidine and supplemented with 0 mM, 2.5 mM, 5 mM, 7.5 mM or 10 mM Amino-1,2,4-triazole (Sigma).

Flow Cytometry

Leaves were chopped with a razor blade in CyStain UV Precise P Nuclei extraction buffer (Partec) according to the manufacturer's instructions. Nuclei were analyzed with the CyFlow MB flow cytometer with FloMax software (Partec).

BiFC and transient expression in tobacco leaves/PSB-L cell cultures

35S::KINγ1-RFP or *35S::RFP-KINγ1* were made through Gateway cloning in K7RWG2 and K7WGR2, respectively. HXK1 was C-terminally fused to head GFP (hGFP) under control of the 35S promoter in pH7m34GW and KINγ1 C-terminally fused to tail GFP (tGFP) and the 35S promoter through Multisite Gateway Cloning in pK7m24GW2. Tobacco (*N. benthamiana*) plants were used for transient expression of constructs by *Agrobacterium tumefaciens*-mediated transient transformation of lower epidermal leaf cells and addition of a P19 expressing Agrobacterium strain to boost protein expression. Stable transformation in Arabidopsis cell suspension cultures (PSB-L) was performed by direct transformation mediated through co-cultivation as described before (Van Leene *et al*, 2007).

Confocal imaging

Imaging of seedlings, tobacco leaves and cell cultures was performed with an Olympus FV10 ASW confocal laser scanning microscope using a 20X lens (NA 0.75) or 60x water immersion lens (NA 1.2) with up to 3x digital zoom for some images.

Tandem Affinity Purification (TAP)

Cloning of transgenes encoding GS^{rhino} tag (Van Leene *et al*, 2015) fusions under control of the constitutive cauliflower mosaic virus 35S promoter and transformation of Arabidopsis cell suspension cultures (PSB-D) with direct selection in liquid medium were carried out as previously described (Van Leene *et al*, 2011). TAP experiments were performed with 100 mg of total protein extract from 3-day old cell cultures continuously grown in the presence of sucrose or from 8-day old sucrose starved cell cultures with or without supplementation of 3% sucrose and 30 mg of total protein extract (*in planta*) as input as described in Van Leene et al., 2015. Protein interactors were identified by mass spectrometry using an LTQ Orbitrap Velos mass spectrometer. Proteins with at least two matched high confident peptides were retained. Background proteins were filtered out based on frequency of occurrence of the co-purified proteins in a large dataset containing 543 TAP experiments using 115 different baits (Van Leene *et al,* 2015).

Transmission Electron Microscopy

Leaves were immersed in a fixative solution of 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M Na-cacodylate buffer, placed in a vacuum oven for 30 min and then left rotating for 3 h at room temperature. This solution was later replaced with fresh fixative and samples were left rotating overnight at 4°C. After washing, samples were post-fixed in 1% OsO4 with $K_3Fe(CN)_6$ in 0.1 M Na-cacodylate buffer, pH 7.2. Samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in Spurr's resin. In order to have a larger overview of the phenotype, semi-thin sections were first cut at 0.5 µm and stained with toluidine blue. Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by

post-staining with uranyl acetate and lead citrate in a Leica EM AC20 and collected on Formvar-coated copper slot grids. Two leaves of control and sucrose-treated Col-0 and *hxk1* seedlings were viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan), operating at 80 kV, using Image Plate Technology from Ditabis (Pforzheim, Germany). For each line (Col-0 and *hxk1*), two leaves were analyzed per condition and chloroplast size and number of 27 to 87 mesophyll cells of the tip and the base of the leaf were measured.

Statistical analysis

All analyses were performed with SAS (Version 9.4 of the SAS System for windows 7 64bit. Copyright © 2002-2012 SAS Institute Inc. Cary, NC, USA (www.sas.com).

All growth experiments involved one, two or three factors and consisted of three independent biological repeats. For the representation of the ratios, measurements of the sucrose-treated leaves were compared to the measurements of the control leaves of the same repeat. Averages were then taken over the three independent repeats and represented in the graphs with their standard error. When needed, raw measurements were log-transformed to stabilize the variance prior to statistical analysis; this is specified in the figure legends. For all growth experiments, a linear mixed model was fitted to the variable of interest with all main factors and their interaction, in case of two factors, as fixed effects using the mixed procedure. The biological repeat term was included in each model as a random factor to take into account the correlation between observations done at the same time. In the presence of a significant F-test (for the main effect in case of one factor, for the interaction term in the case of two factors), appropriate post-hoc tests were performed. Multiple testing correction was done according with Tukey adjustment. For the time course experiment, simple tests of effects were performed at each day separately with the plm procedure.

For chloroplast-related analysis, fixed effects were genotype, treatment, tip or base and all interaction effects. A random intercept model was fitted to the mesophyll area data and log transformed chloroplast data using the mixed procedure of SAS. The Kenward-Roger method was used for computing the denominator degrees of freedom for the tests of fixed effects. For mesophyll area, one random effect was

included in the model to take into account the correlations between observations originating from the same leaf. For chloroplast area, two random effects were included in the model to take into account the correlations between observations originating from the same leaf, and originating from the same mesophyll cell within leaf. The fixed part of the full model was reduced until all remaining factors were significant at the 0.05 significance level. For the mesophyll area data, the three-way interaction term was significant at the 0.05 significance level, and thus the model was not reduced. All-pairwise comparisons were calculated and p-values were adjusted using the Tukey adjustment method as implemented in SAS. For the chloroplast area, only TB had a significant effect. Least-square means estimates for both levels of TB were calculated as well as the difference between tip and base. A generalized linear mixed-effect model was fitted to the number of chloroplasts with the glimmix procedure of SAS assuming a Poisson distribution and a log link function. The Satterthwaite method was used for computing the denominator degrees of freedom for the tests of fixed effects. A random effect was included for leaf to take into account the correlations between observations originating from the same leaf. The fixed part of the full model was reduced until all remaining factors were significant at the 0.05 significance level. For the number of chloroplasts, only treatment had a significant effect. Least-square means estimates for both levels of treatment were calculated as well as the difference.

SUPPLEMENTAL DATA

All supplemental data is listed below and can be found at the end of this chapter.

Supplemental Figure S1. Characterization of *hxk1* mutant in Colombia-0 background.

Supplemental Figure S2. Cellular parameters of the third leaf of *hxk1* mutant compared with wild type.

Supplemental Figure S3. GUS staining of the third leaf of the *pCYCB1;1::CYCB1;1-D-box:GUS* reporter line in Col-0 and *hxk1* background.

Supplemental Figure S4. Ploidy analysis of *hxk1* and Col-0 leaves grown on control medium *in vitro*.

Supplemental Figure S5. Third leaf size change upon transfer to sucrose of Col-0 and *hxk1* plants.

Supplemental Figure S6. Raw data of the cellular measurements shown in Figure 2H.

Supplemental Figure S7. Expression of *GPT2* in Col-0 and hxk1 shoots.

Supplemental Figure S8. Y2H assay between HXK1 and KINY1 proteins.

Supplemental Figure S9. Localization of HXK1 and KINγ1 fusion proteins in Arabidopsis cell suspension cultures.

Supplemental Table S1. Tandem Affinity Purification from seedlings with *35::HXK1-GS^{green}* as bait.

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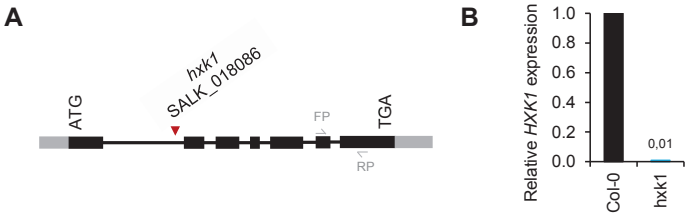
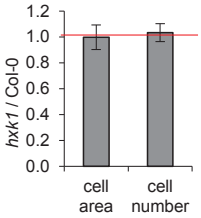
SUPPLEMENTAL DATA

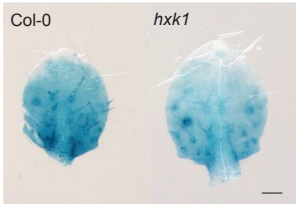
Supplemental Figure S1. Characterization of *hxk1* mutant in Columbia-0 background. A, Structure of *HXK1* gene with indication of T-DNA insertion by red triangle. Black rectangles represent exons, grey rectangles represent 5' and 3'UTR. Arrows represent forward primer (FP) and reverse primer (RP) used for qRT-PCR in (B). B, Relative HXK1 expression in 10-day old seedlings determined by qRT-PCR.



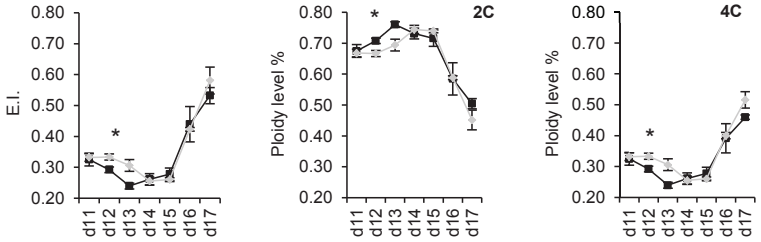
Supplemental Figure S2. Cellular parameters of the third leaf of *hxk1* mutant compared with wild type. Plants were grown *in vitro* without sucrose for 21 days. At 21 DAS pavement cell area and cell number was measured in third leaves of hxk1 and compared with Col-0.



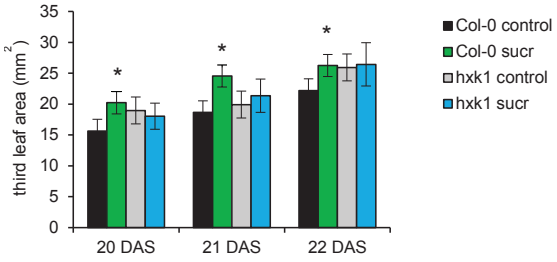
Supplemental Figure S3. GUS staining of the third leaf of the *pCYCB1;1::CYCB1;1-D-box:GUS* reporter line in Col-0 and *hxk1* background. Seedlings were grown *in vitro* on MS medium without sucrose for 12 days. The scale bar represents 1 mm.



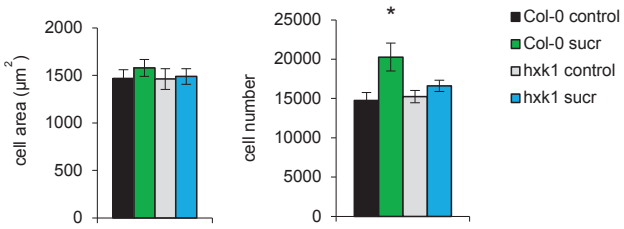
Supplemental Figure S4. Ploidy analysis of *hxk1* and Col-0 leaves grown on control medium *in vitro*. Ploidy levels were measured with flow cytometry. The endoreduplication index (E.I.) is calculated as % of 4C + 2 × % of 8C + 3 × % of 16C. Black line represents Col-0, grey line is *hxk1*. *, $P < 0.05$ compared to Col-0, Students t-test.



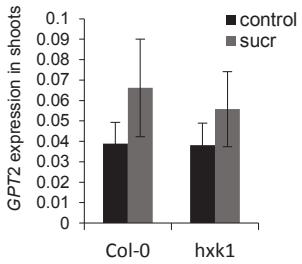
Supplemental Figure S5. Size of the third leaf upon transfer of Col-0 and *hxk1* seedlings to sucrose-containing media. Seedlings were grown *in vitro* on MS medium without sucrose for 9 days. At 9 DAS, seedlings were transferred to media without sucrose (control) or 15 mM sucrose (sucr) supplemented media. At 20, 21 and 22 DAS, third leaf size was measured. Values are averages of two to three independent repeats with their SE. *, $P < 0.05$ for sucrose compared to control, mixed models



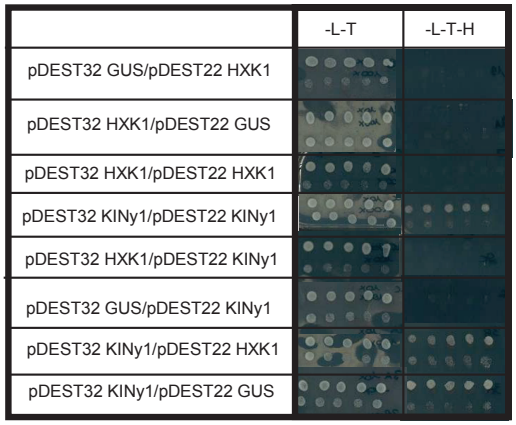
Supplemental Figure S6. Raw data of the cellular measurements shown in Figure 2H. Seedlings were grown *in vitro* on MS medium without sucrose for 9 days. At 9 DAS, seedlings were transferred to media without sucrose (control) or 15 mM sucrose (sucr) supplemented media. At 21 DAS, pavement cell area and total pavement cell number of third leaves of Col-0 and *hxk1* plants, transferred to control or sucrose-supplemented media, were measured. Values are averages of three independent repeats with their SE. *, $P < 0.05$ of sucrose compared to control, mixed models



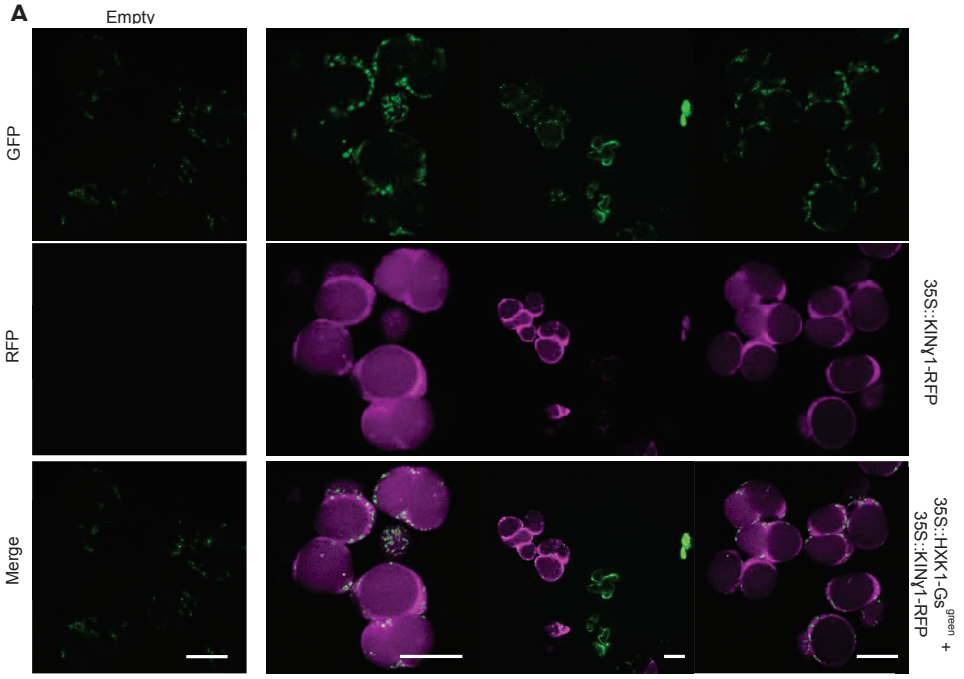
Supplemental Figure S7. Expression of *GPT2* in Col-0 and hxk1 shoots. Seedlings were grown *in vitro* on MS medium without sucrose for 9 days. At 9 DAS, seedlings were transferred to media without sucrose (control) or 15 mM sucrose (sucr) supplemented media. 24 hours after transfer, shoots were harvested for RNA extraction. *GPT2* expression was measured with qRT-PCR analysis and normalized against the average of three housekeeping genes *AT1G13320, AT2G32170, AT2G28390.*

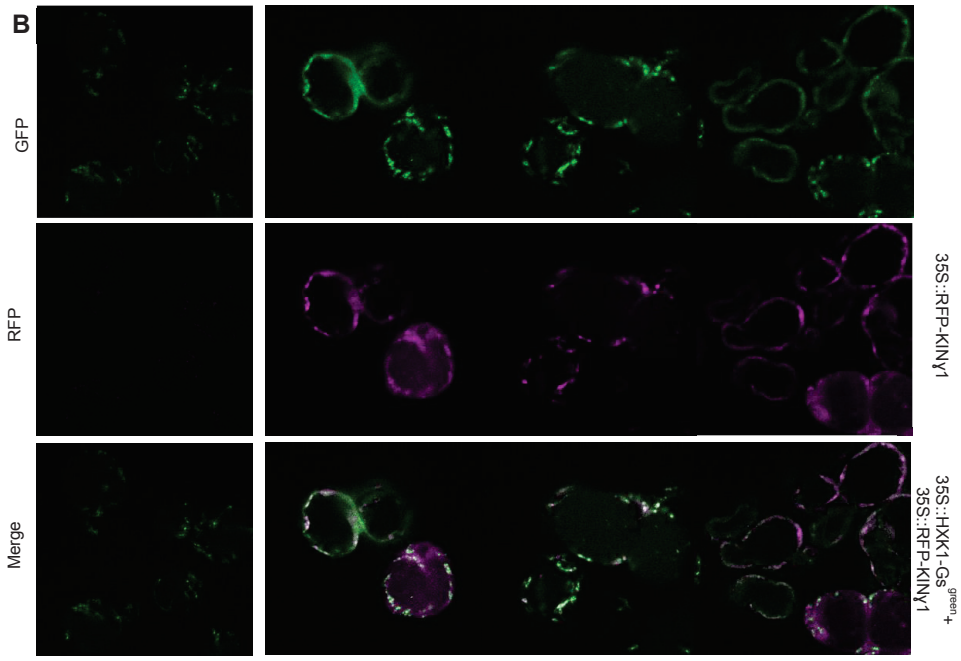


Supplemental Figure S8. Y2H assay between HXK1 and KINγ1 proteins. HXK1 and KINγ1 were fused to GAL4AD (Activation domain, pDEST22) or GAL4BD (DNA-binding domain, pDEST32) and co-transformed in yeast cells. GUS-AD and GUS-BD were used as control. Four to five independent colonies were grown for 2-4 days on non-selective medium (-Leucine –Tryptophan) or selective medium (-Leucine –Tryptophan – Histidine).



Supplemental Figure S9. Localization of HXK1-GS^{green} and KINγ1-RFP (A) or RFP-KINγ1 (B) fusion proteins in Arabidopsis cell suspension cultures. Cell cultures were stable co-transformed with 35S:: HXK1-GS^{green} and KINγ1-RFP or RFP-KINγ1. The scale bar represents 20 µm.




B
GFP
RFP
Merge
35S::RFP-KINγ1
35S::HXK1-Gs green +
35S::RFP-KINγ1

Supplemental Table S1. Tandem Affinity Purification from seedlings with *35::HXK1-GS^{green}* as bait. Seedlings were grown *in vitro* on MS medium without sucrose for 9 days. At 9 DAS, protein complexes were purified from seedlings transferred to media without sucrose (control) or 15 mM sucrose (sucr) supplemented media for 1 hour. The number indicates in which condition the protein is co-purified with HXK1, only one TAP experiment was performed in control and sucrose conditions. A list of non-specific background proteins was assembled by combining a previously compiled background list (Van Leene *et al*, 2010) with background proteins from control GS purifications on mock, GFP-GS, and GUS-GS cell culture extracts identified with LTQ Orbitrap Velos. To obtain the final list of interactors, these background proteins were subtracted from the list of identified proteins.

Gene ID	Description	control	sucr
AT4G29130	ATHXK1, GIN2, HXK1 | hexokinase 1	1	1
AT4G04640	ATPC1 | ATPase, F1 complex, gamma subunit protein	1	1
AT3G46780	PTAC16 | plastid transcriptionally active 16	1	1
AT1G61520	LHCA3 | photosystem I light harvesting complex gene 3		1
AT1G73110	P-loop containing nucleoside triphosphate hydrolases superfamily protein	1	1
AT1G09340	CRB, CSP41B, HIP1.3 | chloroplast RNA binding	1	1
ATCG00470	ATPE | ATP synthase epsilon chain		1
AT4G18480	CHLI1, CH42, CH-42, CHL11, CHLI-1 | P-loop containing nucleoside triphosphate hydrolases superfamily protein	1	1
AT1G32220	NAD(P)-binding Rossmann-fold superfamily protein	1	1
AT3G19390	Granulin repeat cysteine protease family protein		1
AT5G08650	Small GTP-binding protein	1	1
AT2G38040	CAC3 | acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit		1
AT2G20890	PSB29, THF1 | photosystem II reaction center PSB29 protein	1	1
AT1G56050	GTP-binding protein-related		1
AT4G35250	NAD(P)-binding Rossmann-fold superfamily protein	1	1
AT1G72150	PATL1 | PATELLIN 1	1	1
AT1G62750	ATSCO1, ATSCO1/CPEF-G, SCO1 | Translation elongation factor EFG/EF2 protein		1
AT1G67700	unknown protein		1
AT5G55220	trigger factor type chaperone family protein	1	1
AT3G10670	ATNAP7, NAP7 | non-intrinsic ABC protein 7		1
AT1G64190	6-phosphogluconate dehydrogenase family protein		1
AT1G16880	uridylyltransferase-related		1
AT2G22250	ATAAT, AAT, MEE17 | aspartate aminotransferase	1	
AT4G26300	emb1027 | Arginyl-tRNA synthetase, class Ic	1	
AT5G67030	ABA1, LOS6, NPQ2, ATABA1, ZEP, IBS3, ATZEP | zeaxanthin epoxidase (ZEP) (ABA1)	1	
AT3G01500	CA1, ATBCA1, SABP3, ATSABP3 | carbonic anhydrase 1	1	
AT2G45770	CPFTSY, FRD4 | signal recognition particle receptor protein, chloroplast (FTSY)	1	
AT1G17220	FUG1 | Translation initiation factor 2, small GTP-binding protein	1	
AT5G45930	CHLI2, CHL I2, CHLI-2 | magnesium chelatase i2	1	

CHAPTER 7

Strobilurins as yield-enhancing compounds:
How Stroby regulates Arabidopsis plant growth

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This chapter is a research article in preparation

AUTHOR CONTRIBUTIONS: J.V.D and C.A designed and performed experiments, analyzed data and are the main authors of the chapter. P.F., N.G. and S.D. were involved in discussions throughout the project. V.F. and D.I. supervised the project.

ABSTRACT

Strobilurins are an important class of agrochemical fungicides used throughout the world on a wide variety of crops to protect against a wide variety of pathogens. Their increasing popularity comes from the fact that, besides their protective role against pathogens, these compounds are reported to also positively influence plant physiology. In this study, we studied the effect of application of Stroby, a commercially available fungicide consisting of 50% (w/w) kresoxim-methyl as active strobilurin compound, on Arabidopsis plant growth. Treatment of seeds and seedlings with Stroby resulted in larger rosettes and leaves due to an increase in the number of pavement cells. Transcriptomic analysis of Stroby-treated rosettes demonstrated an increased expression of genes involved in redox homeostasis and iron- and sugar transport-related genes. However, iron and sucrose content was not affected by Stroby application. This suggests that the Stroby-induced growth promotion is possibly not linked to increased accumulation of iron or sugars, but is rather a result of the optimized use of the available pools. Furthermore, Stroby treatment strongly induced the expression of the subgroup Ib basic helix-loop-helix transcription factors which are normally involved in iron homeostasis under iron-deficiency conditions. Single loss-of-function mutants of three *bHLH*s and their triple *bhlh039 bhlh100 bhlh101* mutant did not respond to Stroby treatment. We further focused on bHLH039 to unravel its role as a plant growth regulator downstream of Stroby treatment. Together, our data demonstrate that Stroby promotes Arabidopsis leaf growth by stimulating cell proliferation and that the iron-deficiency responsive bHLH transcription factors play a critical role in the Stroby-mediated positive effects on plant growth.

INTRODUCTION

With the rapidly growing population, global demand for food and bioenergy sources is continuously increasing. As a consequence, crop productivity and yield have to improve on the existing agricultural lands, minimizing deforestation with multiple negative effects on the ecosystem. It is therefore essential to understand how plants regulate their growth in order to improve crop yield.

Plant growth is a complex process integrating genetic and environmental factors. Leaves are the main energy producing organs of plants providing the metabolic energy and chemical building blocks. Although leaf size and architecture have a profound effect on productivity, the mechanisms that regulate leaf growth are still not completely understood. Final leaf size is spatiotemporally regulated by two main processes that drive growth: cell division and cell expansion (Gonzalez *et al*, 2012). In

dicotyledonous plants, leaves first grow exclusively by cell proliferation. Subsequently, in the transition phase, cells stop dividing at the tip of the leaf and all cells exit the mitotic cell cycle and start to expand in a basipetal or tip-to-base direction (Andriankaja *et al*, 2012; Donnelly *et al*, 1999). In the final stage, leaf growth is mainly driven by cell expansion resulting in a large increase in cell size. Ultimately, cell expansion slows down and the leaf reaches its mature size. The transition phase from cell proliferation to expansion has been studied in detail at cellular and transcriptomic level in the third leaf of *Arabidopsis thaliana* (Andriankaja *et al*, 2012). This study showed that the cell cycle arrest front abruptly disappears and coincides with the differentiation of the photosynthetic machinery. Interestingly, all four members of the subgroup Ib of the basic helix-loop-helix transcription factors, *bHLH038*, *bHLH039*, *bHLH100* and *bHLH101*, are strongly up-regulated during the transition. These transcription factors are well known regulators of iron homeostasis in roots in response to iron limitation (Wang *et al*, 2007). bHLH038 and bHLH039 physically interact with the FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1 (FIT1) to induce the expression of the iron-uptake genes *FERRIC REDUCTASE OXIDASE2*, *FRO2* and *IRON TRANSPORTER*, *IRT1* (Yuan *et al*, 2008). Recently, a role of *bHLH038*, *bHLH039, bHLH100* and *bHLH101* in the regulation of iron metabolism during leaf development was suggested (Andriankaja *et al*, 2014). As the onset of cell expansion coincides with chloroplast differentiation, the newly differentiated chloroplasts could act as iron sinks resulting in iron-deficiency in other parts of the leaf, leading to the up-regulation of the subgroup Ib bHLH transcription factors. However, no differences were observed in iron content and the expression of iron response genes during early leaf development (Andriankaja *et al*, 2014).

Strobilurins are natural substrates that were isolated from the fungus *Strobilurus tenacellus* which produces a fungicide compound, called Strobilurin-A, to eliminate other fungi and yeasts growing in the same area (Anke *et al*, 1977). After their identification, a variety of synthetic strobilurins were produced by improving chemical features, such as stability and activity (Balba, 2007). Nowadays, many different strobilurins are commercially available (e.g. kresoxim-methyl, pyraclostrobin, azoxystrobin and trifloxystrobin) and used as fungicides on a wide range of crops throughout the world (Bartlett et al, 2002). The fungicidal mode of action of strobilurins is the inhibition of the fungal mitochondrial respiration by binding to a specific site of

cytochrome b (the ubiquinol site), which blocks the electron transfer between cytochrome b and cytochrome c (Ammermann, 1992; Earley *et al*, 2012). As a result, mitochondria cease production of energy and the fungus eventually dies. In addition to their protective role against pathogens, experimental findings suggest that strobilurins also exert positive effects on plant growth. Several studies have been reporting different physiological effects in wheat, barley and corn, as a result of strobilurin treatment. These include higher yield, increased photosynthetic activity, delayed senescence and shifting hormone balance by decreasing ethylene and increasing abscisic acid levels (Beck *et al*, 2002; Bertelsen *et al*, 2001; Grossmann *et al*, 1999; Ruske *et al*, 2003; Wu & von Tiedemann, 2001). In addition, strobilurin treatment results in increased antioxidative activity and tolerance to abiotic stress in *Medicago* (Filippou *et al*, 2015; Zhang *et al*, 2010). Contrarily, Mahoney and Gillard (2014) recently showed no differences in the final yield of dry bean after the application of azoxystrobin and pyraclostrobin (Mahoney & Gillard, 2014). Another study tested the effect of pyraclostrobin treatment on four soybean varieties and found no improvement in plant physiology or yield (Swoboda & Pedersen, 2009). Based on the available literature it is clear that positive effects of strobilurins strongly depend on the crop and environmental conditions. To date, the underlying mechanisms responsible for the strobilurin-dependent growth promoting effects still remain largely unclear. Furthermore, the use of these growth promoting compounds, could be another approach to identify genes involved biomass production and crop yield.

This study aimed to decipher the effect on growth of Stroby® WG (BASF), a commercially available strobilurin consisting of 50 % (w/w) kresoxim-methyl (KM; active ingredient) and 50 % surfactants, in Arabidopsis plants. Stroby is used as a protectant fungicide on apples for the control of apple scab, strawberries and blackcurrants for powdery mildew control and roses for the control of mildew and blackspot. We found that application of Stroby by watering twice a week led to an increase in final rosette and leaf size. Cellular analysis of the third leaf showed that Stroby promotes leaf growth by enhancing cell proliferation. Furthermore, transcriptome analysis of 17-day-old rosettes showed, amongst other genes, a significant up-regulation of the bHLH subgroup Ib transcription factors (*bHLH038*, *bHLH039*, *bHLH100*), 24 hours after Stroby treatment. In addition, several iron and sugar-related genes were overrepresented in the differentially expressed genes of

rosettes treated with Stroby but no differences were found in total iron and sucrose content. By analyzing the growth response of single loss-of-function mutants of the four *bHLH*s and the triple *bhlh039 bhlh100 bhlh101* mutant to Stroby application, we showed that bHLH039 is an essential player in regulating Stroby-induced growth promotion.

RESULTS

Application of Stroby at Low Concentrations Enhances Plant Growth

To investigate whether strobilurins can exert a positive effect on the growth of *Arabidopsis thaliana* plants, Col-0 Arabidopsis plants were grown in soil for 22 days and watered twice a week with or without Stroby® WG (BASF). A scheme of the treatment protocol is presented in Supplemental Fig. S1A. Stroby was applied in a concentration gradient from 10^{-6} M to 10^{-11} M for KM and final rosette sizes were measured by making leaf series (Fig. 1A). Three from the six KM concentrations tested, 10^{-8} M, 10^{-9} M and 10^{-10} M, resulted in an increase in rosette area of 16%, 16% and 18% ($P > 0.05$), respectively, compared with untreated control plants at 22 days after stratification (DAS). Subsequently, these three concentrations (10^{-8}, 10^{-9} and 10^{-10} M) were chosen to further confirm the Stroby-induced growth promotion using the same treatment protocol (Fig. 1B). Concentrations of 10^{-9} and 10^{-10} M KM, but not the concentration of 10^{-8} M, resulted in a significant average increase in rosette area of 73% and 69%, respectively ($P < 0.05$; Fig. 1B). It was therefore concluded that a low concentration of Stroby is able to promote Arabidopsis rosette growth but that the range of increase is variable probably due to differences in environmental growth conditions. The concentration of 10^{-9} M KM in Stroby, hereafter simply called Stroby, was selected for all following experiments aiming at unravelling the mode of action of strobilurins during leaf growth and development.

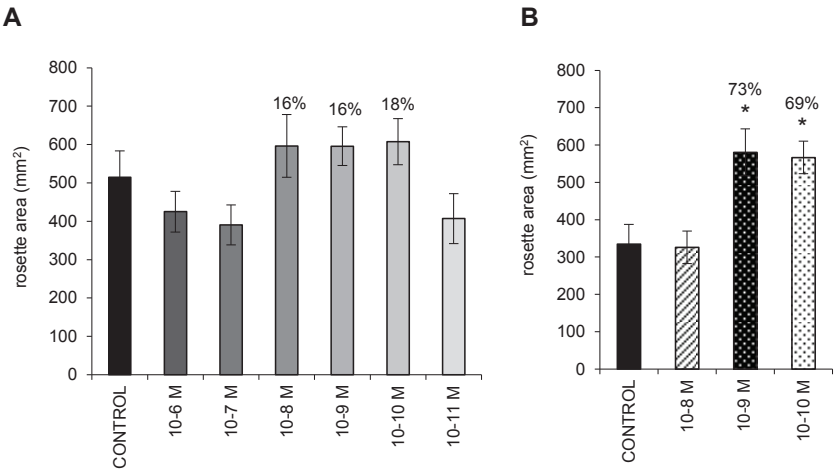

Figure 1. Effect of a concentration gradient of KM in Stroby on rosette area. Average rosette area of 22-days old plants (A) treated with Stroby in a range of KM concentrations (10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M and 10^{-11} M) and (B) treated with three KM concentrations (10^{-8} M, 10^{-9} M and 10^{-10} M) and compared with control plants treated with water. Values are the averages of 5 to 12 plants of one biological repeat with their SE. *, $P < 0.05$, Students t-test.

Stroby Promotes Leaf Growth by Stimulating Cell Proliferation

To explore the growth promoting effect of Stroby in detail, rosette and leaf size as well as cell number and size were determined in Col-0 plants treated with a Stroby solution containing 10^{-9} M KM using the same protocol as described above. Plants treated with Stroby produced significantly larger rosettes, with an average increase of 36% ($P < 0.0001$), and had more biomass, as measured by fresh weight (32 %, $P < 0.0001$), compared with control plants (Fig. 2A). The measurements of individual leaf area at 22 DAS showed that all leaves up to the tenth leaf, with the exception of the cotyledons and the two first leaves, of Stroby-treated plants were significantly larger ($P < 0.05$; Fig. 2B). Remarkably, relative differences in leaf size between Stroby-treated plants and control plants were more pronounced for younger leaves, with a significant increase of 87% and 112% ($P < 0.0001$) for the eighth and ninth leaf respectively, and 132% for the tenth leaf ($P < 0.05$). Considering that treatment of plants with strobilurins is frequently reported to be accompanied by increased greening of the plant (Beck *et al*, 2002), photosynthetic pigments were determined in Stroby-treated and control rosettes of 22-day-old plants. However, no differences in chlorophyll a (Chla), Chlb and carotenoid levels per mg fresh tissue were observed (Supplemental Fig. S2).

To further investigate the effect of Stroby during leaf development, the growth of the third true leaf was followed over time. Seedlings were harvested daily from 10 until 22 DAS, the third leaf was dissected and its area was measured (Fig. 2C). Third leaves of Stroby-treated plants were significantly larger as early as 10 DAS (80%, $P < 0.0001$; Fig. 2D) and remained larger compared with control plants throughout the time-course analysis (Fig. 2C). At 22 DAS, final size of the third leaf was significantly increased by 23% after Stroby treatment ($P < 0.05$; Fig. 2D). To investigate the underlying cellular processes responsible for the Stroby-enhanced leaf growth, epidermal cell number and cell size were measured both at 10 and 22 DAS. At both time points, the larger leaf size of Stroby-treated plants was due to a significant increase in epidermal cell number by 71% and 26% ($P < 0.05$; Fig. 2D), at 10 and 22 DAS, respectively. Cell size was unaffected by Stroby ($P = 0.86$ for 10 DAS and $P = 0.92$ for 22 DAS; Fig. 2D). Furthermore, stomatal index was calculated at 10 and 22 DAS and no difference was found between Stroby-treated plants and control plants at both time points (Supplemental Fig. S3). Taken together, the above described results provide clear evidence that Stroby promotes Arabidopsis plant growth by stimulating cell proliferation.

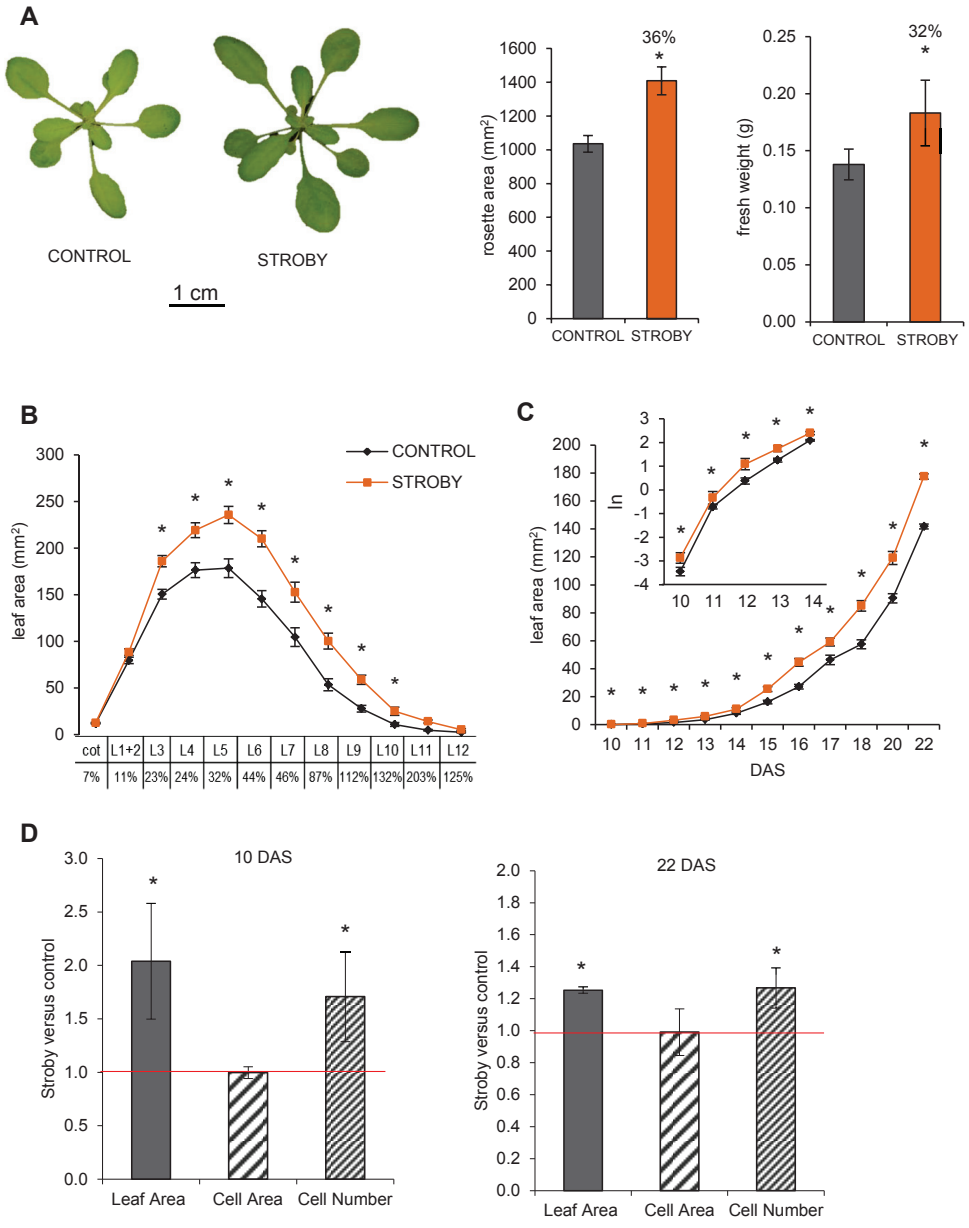

Figure 2. Rosette, leaf and cellular parameters of plants treated with Stroby. Plants were grown in soil, stratified in the presence of Stroby in a concentration of 10^{-9} M KM, and subsequently watered twice a week with Stroby or water (control). A, Average rosette area and fresh weight measured at 22 days after stratification (DAS). At the right, representative images of rosettes of Stroby-treated and control plants at 22 DAS. B, Average individual leaf areas of rosettes in (A). X-axis represents the cotyledons (cot) and leaves in the order of appearance on the rosette (L1+2- L12). C, Third leaf area measured over time from 10 until 22 DAS. The inset is a close-up of 10 until 15 DAS. D, Ratio of average third leaf areas, pavement cell area and cell number of wild type plants treated with Stroby compared with control plants at 10 DAS (left) and at 22 DAS (right). Values are averages of three biological repeats with their SE. Rosette and leaf area data are from 8 to 9 plants in each repeat. Cellular data are from three to five leaves in each repeat. *, $P < 0.05$, mixed

Transcriptome Analysis in whole rosettes upon Stroby treatment

Transcriptome analysis of Stroby-treated and control plants was performed in order to identify the molecular mechanisms underlying Stroby-mediated plant growth enhancement. RNA-sequencing analysis was carried out on RNA extracted from a pool of four rosettes of control or Stroby-treated plants at 17 DAS, 24 hours after the fourth Stroby treatment (Supplemented Fig. S1A). Three biological repeats were analyzed using the general linear model (see Materials and Methods) and 111 genes were found to be differentially expressed after Stroby treatment: 81 genes were up-regulated and 30 genes were down-regulated (FDR < 0.05; Supplemental Table 1).

Gene Ontology enrichment analysis of the 111 differentially expressed genes using PageMan (Usadel *et al*, 2006) showed genes encoding proteins related to 'development' and 'redox homeostasis' were significantly overrepresented (Supplemental Fig. S4). From the 12 enriched redox-related genes, five encode dismutase proteins, six encode thioredoxin superfamily proteins and one gene encodes a protein with a sequence similar to a 2-oxoglutarate and Fe-dependent oxygenase superfamily protein (AT1G06640). Four of the five genes encoding dismutases were suppressed after Stroby treatment: *SUPEROXIDE DISMUTASE1* (SOD1; AT1G08830) and *SOD2* (AT2G28190), *COPPER CHAPERONE FOR SOD1* (CCS1; AT1G12520) and *FE SUPEROXIDE DISMUTASE 2* (*FSD2*; AT5G51100). Contrastingly, *FSD1* (AT4G25100) was slightly up-regulated (Log_2FC of 0.47). The functional category 'development' includes a mixture of genes assigned to proteins involved in diverse developmental processes. Two genes belong to the so-called *DARK-INDUCED* class of genes repressed after sugar treatment, *SEN1* and *DIN11* (Fujiki *et al*, 2000), one gene encodes an aspartyl protease family protein (AT5G19120) and one encodes a late embryogenesis abundant family protein (AT1G02520; Supplemental Table 1). Another developmental-related gene encodes a SQUAMOSA-PROMOTER BINDING protein-like (SPL) transcription factor, SPL5 (AT3G15270). SPLs play a role in different plant developmental programs, such as vegetative phase change and flowering (Wang *et al*, 2009; Wu & Poethig, 2006). Besides *SPL5*, ten genes also encoding for (putative) transcription factors were differentially expressed after Stroby treatment (Table 1). Half of these genes was suppressed and the other half was induced after Stroby treatment. Three of the five Stroby-induced genes were basic helix-loop-helix (bHLH) DNA-binding superfamily

proteins, bHLH039 (AT3G56980), bHLH038 (AT3G56970) and bHLH100 (AT2G41240). These bHLH transcription factors belong to the same subgroup Ib, together with a fourth member, bHLH101 (AT5G04150), and are well known regulators of genes implicated in iron-uptake under iron-deficient conditions (Yuan *et al*, 2008). The transcripts of *bHLH038, bHLH039* and *bHLH100*, were significantly up-regulated in Stroby-treated plants compared with control plants with log_2FC of 3.22, 3.39 and 3.13, respectively. *bHLH101* was not significantly up-regulated in the RNA-seq analysis (Log_2FC of 1.27 and FDR of 0.80). Quantitative reverse transcription (qRT)-PCR analysis confirmed its induction after Stroby treatment (Log_2FC of 3.14 and *P*-value of 0.22; Supplemental Fig. S5).

Table 1. Transcription factors down- or up-regulated after Stroby treatment.

	Gene ID	Log_2FC	FDR	Gene Name	Description
1	AT2G21650	-0.94	0	**MEE3**	Homeodomain-like and MYB-related superfamily protein
2	AT3G05690	-0.64	0.02	**HAP2B**	CCAAT box binding factor family / NUCLEAR FACTOR Y, SUBUNIT A2
3	AT1G68840	-0.47	0	**EDF2**	APETALA2/ETHYLENE RESPONSE DNA BINDING FACTOR 2
4	AT1G22590	-0.39	0	**AGL87**	MADS box transcription factor family AGAMOUS-like 87
5	AT1G20693	-0.36	0	**HMGB2/NFD2**	High mobility group B; NUCLEOSOME/CHROMATIN ASSEMBLY FACTOR GROUP
6	AT3G48100	0.36	0.02	**ARR5**	Transcription repressor in cytokinin signaling / RESPONSE REGULATOR 5
7	AT3G15270	1.06	0.02	**SPL5**	SQUAMOSA-PROMOTER BINDING protein-like (SPL) transcription factor
8	AT2G41240	3.13	0	**bHLH100**	basic helix-loop-helix (bHLH) DNA-binding superfamily protein; subgroup Ib
9	AT3G56970	3.22	0	**bHLH38**	basic helix-loop-helix (bHLH) DNA-binding superfamily protein; subgroup Ib
10	AT3G56980	3.39	0	**bHLH39**	basic helix-loop-helix (bHLH) DNA-binding superfamily protein; subgroup Ib
11	AT1G03790	5.43	0.04	**SOM**	C3H zinc finger protein

Besides the bHLHs transcription factors, another gene known to be regulated under iron limiting conditions was found to be slightly up-regulated after Stroby treatment, the *NICOTIANAMINE SYNTHASE* gene, *NAS1* (Log_2FC of 0.42). NAS proteins are implicated in remobilization and redistribution of NA-Fe2+ complexes through the vascular system (Briat *et al*, 2007).

Several genes encoding proteins involved in sugar transport were differentially expressed: genes encoding three SWEET sucrose transporters (SWEET2 and SWEET11/12; Eom *et al*, 2015), a sugar-porter family protein (SFP1; AT5G27350) which is induced upon senescense and and has a possible role in sugar transport

(Quirino et al 2001) and a sucrose-proton-like symporter, SUC7. The latter gene was one of the strongest up-regulated transcripts with a Log_2FC of 4.82. The *SUC7* gene is a member of a multigene family and has high similarity with *SUC6* and *SUC8* (Sauer *et al*, 2004), which were not differentially expressed after Stroby treatment. *SWEET2*, involved in glucose secretion in roots (Chen *et al*, 2015), was induced and SWEET 11 and 12, two homologous proteins involved in phloem loading of sucrose (Eom *et al*, 2015), were suppressed in Stroby-treated rosettes. Other transport-related genes were also up-regulated in Stroby-treated plants, such as one transcript encoding nitrate transporter NRT1.8 (AT4G21680).

Nine genes were selected for gene expression verification analysis by qRT-PCR (Supplemental Fig.S5). Higher transcript levels were found for the subgroup Ib bHLH transcription factors (*bHLH038*, *bHLH039*, *bHLH100* and *bHLH101*), *OXIDATIVE SIGNAL INDUCIBLE1* (*OXI1*), *UGT74E2* and *NRT1.8* in Stroby-treated rosettes, whereas the transcripts of *SUC7* and *SPL5* were not changed.

Because several genes related to iron absorption and remobilization were significantly induced after Stroby treatment, it was hypothesized that the Stroby-induced plant growth may be linked with a difference in iron accumulation. Therefore, total iron was measured in rosettes and individual rosette leaves of 22 days-old Stroby-treated and control plants grown in soil (Fig. 3A and B). No significant differences were found in total iron contents between Stroby-treated and control rosettes (Fig. 3B). In addition, the abundance of ferritin, the iron storage form in the cell, was estimated by western blot, but no obvious changes in abundance were found (Fig. 3C). Furthermore, Stroby treatment resulted in the differential expression of sugar transport-related genes. Therefore, total sucrose content was analyzed in 22-days old rosettes but no difference in sucrose levels was found between control and Stroby-treated plants (Fig. 3D).

In conclusion, transcriptome profiling of 17-day-old rosettes demonstrated a significant induction of three members of the subgroup Ib bHLH transcription factors, as well as differential expression of eight sugar transporters.

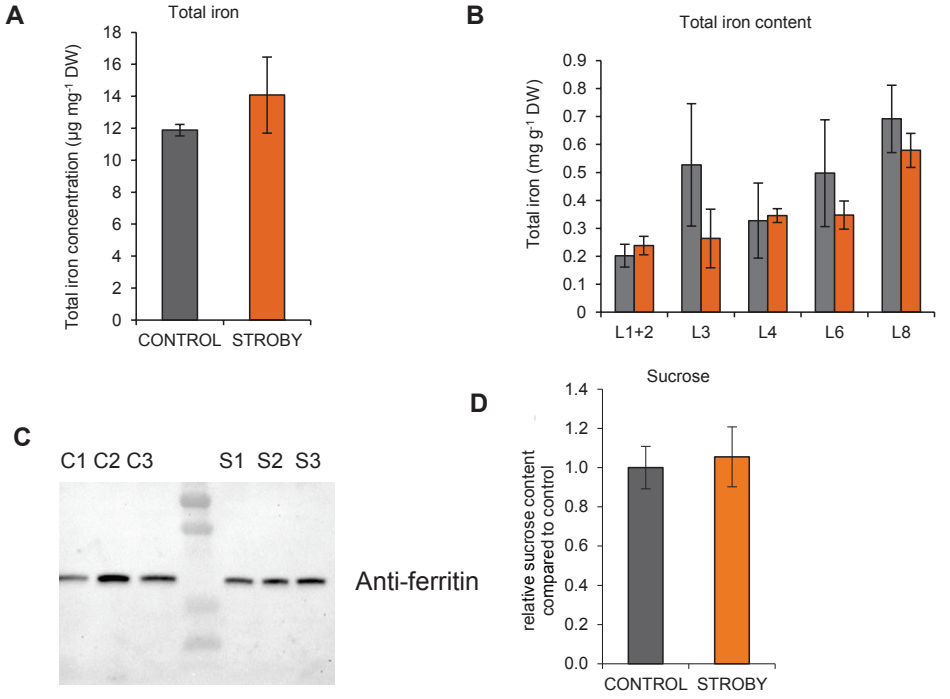

Figure 3. Iron and sucrose levels in Stroby-treated and control rosettes. Plants were grown in soil and watered twice a week with water (control) or Stroby at a concentration of 10^{-9} M KM (Protocol B). At 22 DAS total iron in rosettes (A) and individual leaves (B) was measured from six rosettes for (A) and 10-20 leaves for (B) in each biological repeat. DW = dry weight. Lx= leaf position x in the order of appearance on the rosette. C, Western blot of 22-day-old rosettes of control (C1-3) or Stroby-treated plants (S1-3) using primary antibody against ferritin. D, Sucrose content was measured in three rosettes of control or Stroby-treated plants for each biological repeat. Values in the y-axis are normalized fold-changes relative to the sucrose content in control plants. Values in (A) and (D) are averages of three biological repeats with their SE. Values in (B) are averages of two biological repeats with their SE.

Stroby treatment Enhances Leaf Growth in Wild Type Plants but Not in *bhlh* mutants

In order to decipher the positive effect of Stroby on growth in a leaf developmental context, a modified application protocol was used. In this setup, seedlings were grown without Stroby for eight days. Plants were then watered twice a week with Stroby at a concentration of 10^{-9} M for KM from 8 DAS, time point at which the third leaf is still fully proliferating (Andriankaja *et al*, 2012), until 22 DAS. Control plants were watered twice

a week with water during the complete growth period. A schematic representation of this application protocol is presented in Supplemental Fig. S1B.

To evaluate the effect on growth of Stroby using the modified protocol, average rosette and leaf areas were measured at 22 DAS by making leaf series. Stroby treatment, starting at 8 DAS, resulted in significantly larger rosettes by 19% ($P < 0.0001$) as well as significantly larger third leaves (10%; $P < 0.0001$) compared with control plants (Fig. 4A). To investigate whether Stroby already exerts its effect early during leaf growth, the third leaf was micro-dissected and its area measured 24 hours (at 9 DAS) and 48 hours (at 10 DAS) after treatment. Interestingly, the third leaf area was increased by 32% ($P = 0.25$) and significantly increased by 65% ($P < 0.05$), respectively, 24 and 48 hours after Stroby treatment (Fig. 4B). Cellular analysis at 10 DAS confirmed that the increased leaf area was mainly due to more pavement cells (54%, $P < 0.05$; Fig. 4C).

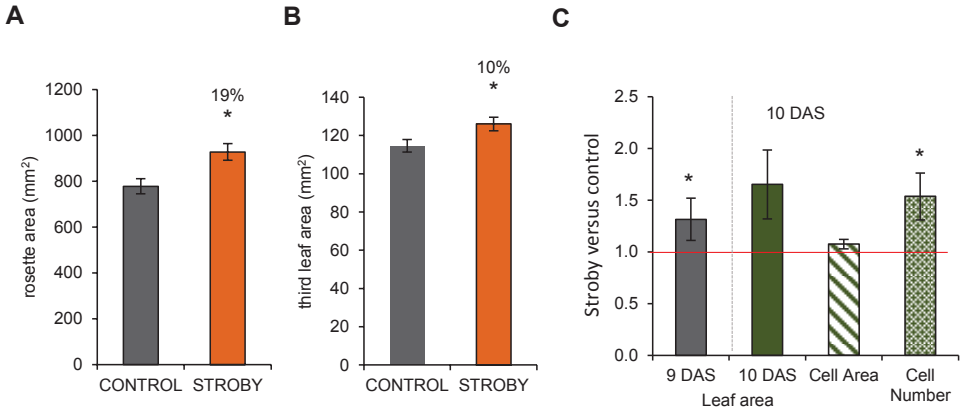

Figure 4. Rosette, leaf and cellular parameters of plants treated with Stroby from 8 DAS. Plants were grown in soil without Stroby for 8 days. At 8 DAS, plants were watered with Stroby or water (control) for the first time after which they were treated twice a week. At 22 DAS, average rosette area (A) and average third leaf area (B) were measured for Stroby-treated and control plants. C, Ratio of the average leaf areas 24 hours (9 DAS) and 48 hours (10 DAS) after first Stroby application and cellular measurements at 10 DAS compared with control plants. Values are averages of three biological repeats with their SE. Rosette and leaf area data are from 8 to 9 plants in each repeat. Cellular data are from three to four leaves in each repeat. *, $P < 0.05$, mixed models.

In order to further unravel the importance of the subgroup Ib bHLH transcription factors in Stroby-induced growth promotion, the effect of Stroby treatment on the rosette sizes of *bhlh* loss-of-function mutants was determined by making leaf series at 22 DAS. For this, single SALK T-DNA insertion mutants of *bHLH039*, *bHLH100* and *bHLH101* and a triple *bhlh039 bhlh100 bhlh101* mutant were used (Andriankaja *et al*, 2014; Wang *et al*, 2013). Transcript levels of *bHLH039*, *bHLH100* and *bHLH101* were determined in all

lines by qRT-PCR analysis and were found to be strongly down-regulated in the respective single and triple mutant (Supplemental Fig. S5).

Wild type and the single and triple *bhlh* mutants were grown in the absence of Stroby for 8 days, treated with Stroby for the first time at 8 DAS and subsequently treated twice a week until 22 DAS. Under control conditions, rosettes of the *bhlh039* and the triple mutant were significantly larger than wild type plants, with an average increase of 23% and 24%, respectively ($P < 0.05$; Fig. 5A). Rosette sizes of *bhlh100* and *bhlh101* single mutants were similar to those of wild type plants. Stroby treatment significantly increased wild type rosette area by 20 % ($P < 0.05$) compared with control plants (Fig. 5C). The *bhlh100* and *bhlh101* single mutants did not show significant increase in the final rosette size when treated with Stroby, whereas average rosette size of the *blhlh039* single mutant and triple mutant was reduced by 12% ($P = 0.18$) and 6% ($P = 0.08$), respectively (Fig. 5B). Because single *bhlh039* and triple *bhlh* mutants were not responsive to Stroby treatment and this seemed to be more pronounced for the single *bhlh039* mutant, we further focused on this mutant. To determine whether the effect of Stroby was already absent at early time points in the *bhlh039* mutant, the area of the third leaf and total pavement cell number was calculated 48 hours (10 DAS) after the first Stroby treatment (Fig. 5C). At this time point, the leaves of the *bhlh039* mutant were already larger than wild type leaves and Stroby only positively affected the growth of wild type leaves ($P = 0.07$). The increased leaf size in wild type Stroby-treated plants was due to a significant increase in pavement cell number (77%, $P < 0.05$) which was not seen in the *bhlh039* mutant (12%, $P = 0.08$; Fig. 5D).

Treatment of Stroby starting at 8 DAS significantly enhanced leaf growth already after 48 hours by increasing total pavement cell number. Loss-of-function of only *bHLH039* or of all three genes *bHLH039, bHLH100 and bHLH101,* in the triple mutant, resulted in larger plants, suggesting their involvement in the regulation of growth independently of Stroby. Furthermore, the positive effect of Stroby on leaf growth was abolished in all single and triple mutants, which suggests a central role of these transcription factors in Stroby-dependent regulation of growth.

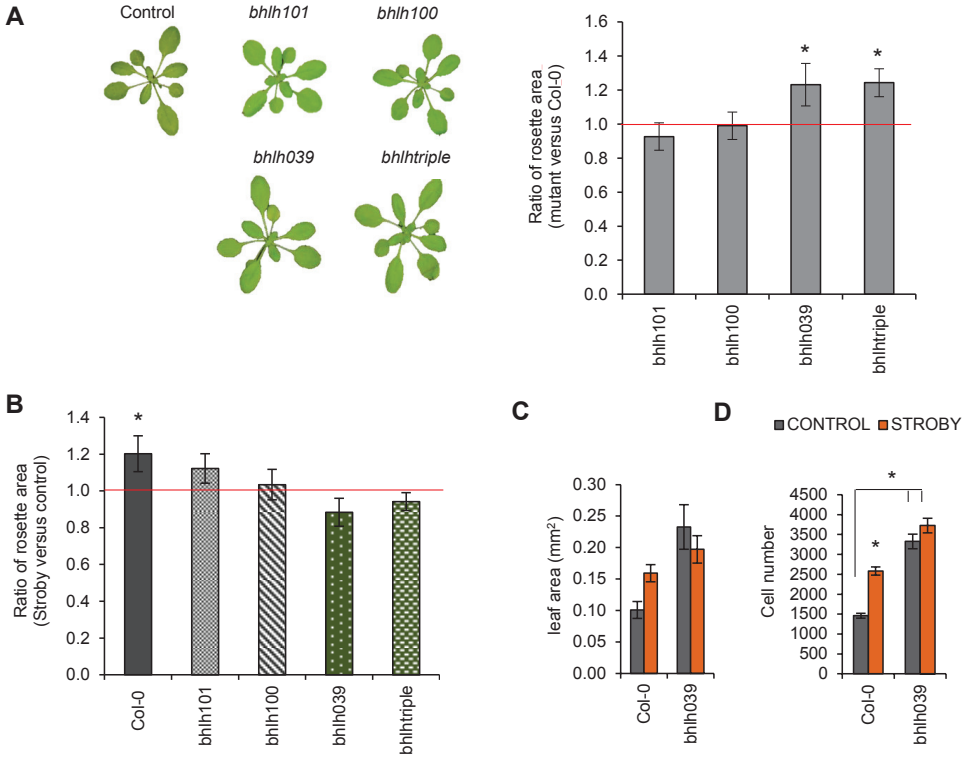

Figure 5. Rosette size, leaf size and cellular parameters of wild types and *bhlh039, bhlh100*, *bhlh101* single and the triple mutant. Plants were first grown in soil without Stroby for 8 days and treated with Stroby for the first time at 8 DAS. Subsequently plants were watered twice a week with or without Stroby (control plants). A, Pictures of rosettes at 22 DAS and ratio of average rosette sizes at 22 DAS of *bhlh101, bhlh100, bhlh039* single mutants and the triple mutant *bhlh039 bhlh100 bhlh101* compared to wild type plants grown under control conditions. B, Ratio of the rosette area of Stroby-treated plants compared with control plants. C, Total pavement cell number of third leaves at 10 DAS. Values in (A) and (B) are averages of three biological repeats with their SE. Rosette sizes are measured for 8 to 9 plants in each repeat. *, $P < 0.05$, mixed models. Leaf area in (C) are averages of 9-12 leaves. Cellular data are from three leaves of each line and each condition. *, $P < 0.05$, ANOVA.

DISCUSSION

Strobilurins are one of the most important classes of agricultural fungicides used worldwide because of its broad-spectrum disease control and reported positive effects on plant yield and physiology (Bartlett *et al*, 2002; Dias, 2012). In the last decades, several studies aimed at unraveling how strobilurins influence plant physiology in the absence of pathogens but the mode-of-action remains unclear (Diaz-Espejo *et al*, 2012; Glaab & Kaiser, 1999; Grossmann *et al*, 1999). In this

study, we used the commercially available strobilurin Stroby consisting of 50% KM (Ypema & Gold, 1999), to test its effect on Arabidopsis plant growth and to study the underlying cellular and molecular mechanisms. Stroby was externally applied by watering and both long-term and short-term consequences of its application on developing leaves and final leaf size were determined. Interestingly, both germination on Stroby and treatment of Stroby later during leaf development resulted in a profound positive effect on Arabidopsis plant growth, which was consistent with the previously reported positive effects of KM on grain yield and dry biomass of wheat plants (Gold and Leinhos 1995). In both cases, cellular analysis revealed a Stroby-induced promotion of cell proliferation.

KM has been suggested to mimic the effect of auxin by stimulating shoot formation in different plant species and influencing other hormone levels, such as decreasing ethylene and cytokinin levels (Grossmann *et al*, 1999). Current findings showed that the tested concentration of KM in Stroby followed a hormone-like dose response curve (Purohit, 2012) with an optimum concentration resulting in the stimulation of growth and lower or higher concentrations acting as growth inhibitors. It is well-known that auxin can promote cell proliferation (Perrot-Rechenmann, 2010). However, no major transcriptional changes in hormone biosynthesis and signaling pathways were observed in Stroby-treated rosettes, with the exception of a down-regulation of *EDF2*, suggested to be involved in the repression of ethylene signaling (Licausi *et al*, 2013), and an up-regulation of ARR5 involved in cytokinin signaling (Kieber & Schaller, 2014). Strobilurin-induced physiological effects have also been linked with nitric oxide (NO) signaling (Köhle, 2002) and NO is an important messenger that regulates growth by stimulating cell proliferation (Shen *et al*, 2013; Takahashi *et al*, 2014). KM increases nitrate reductase activity in spinach leaf discs (Glaab & Kaiser, 1999) and pyraclostrobin treatment induces NO-release in soybean and tobacco (Conrath *et al*, 2004). Remarkably, in our transcriptomic results of Stroby-treated rosettes, two genes linked with NO were also up-regulated, a transcript encoding a nitrate transporter (*NRT1.8*) and to a lesser extent a transcript encoding a nitrate reductase (*NR2*). NR2 is responsible for the vast majority of NR activity in plants and several studies suggested a role for NR2 in the production of NO (Yu *et al*, 2014). NRT1.8 is induced by nitrate and probably involved in unloading nitrate from the xylem (Li *et al*, 2010). Interestingly, NO signaling was found to induce many Fe-regulated genes,

such as those found in Stroby-treated rosettes: *bHLH038*, *bHLH039*, *bHLH100*, *bHLH101* and *NAS1*, amongst others (Garcia *et al*, 2010), further suggesting that Stroby treatment might affect NO levels which elicit downstream transcriptional responses.

A significant up-regulation was shown of all four members of the subgroup Ib bHLH transcription factors (*bHLH038*, *bHLH039, bHLH100* and *bHLH101*) upon Stroby treatment. In addition, *bhlh039* single and triple mutants were larger under control conditions. These four bHLHs are strongly induced in roots and leaves upon iron-deficiency and act redundantly to promote iron uptake in roots (Wang *et al*, 2013; Yuan *et al*, 2008). The phenotypes of the single, double and triple *bhlh* mutants are highly dependent on the environment and contrasting phenotypes have been reported when grown on iron-limiting and normal conditions. Ling and colleagues (2007, 2008) reported no obvious growth effects of the single mutants when grown under optimal iron conditions (Wang *et al*, 2007; Yuan *et al*, 2008), whereas reduced rosette sizes and no effect on growth were observed when grown in soil under standard conditions and *in vitro,* respectively (Andriankaja *et al*, 2014). Furthermore, in iron-limiting and optimal conditions, the double mutants *bhlh039 bhlh100* and *bhlh039 bhlh101* show stunted growth and chlorosis linked with a reduced iron content of the shoots (Wang *et al*, 2013). Overexpressing bHLH039 leads to normal growth under optimal conditions and reduced growth with chlorosis under iron-limiting conditions (Yuan *et al*, 2008). Here, *bhlh039* single and *bhlh039 bhlh100 bhlh101* triple mutants were larger compared with wild type plants under control growth conditions. In addition, young growing leaves of the *bhlh039* mutant were larger than wild type because of an increase in cell number. These findings were rather surprising because of the previously reported reduced growth of all single mutants as well as the redundant role of the four bHLHs in the regulation of iron-deficiency responses (Wang *et al*, 2013), suggesting that bHLH039 plays a pivotal role in leaf growth. Indeed, *bHLHs* expression is up-regulated between 9 and 10 DAS, when cells start to expand during leaf development (Andriankaja *et al*, 2012). The increased number of cells in the *bhlh039* mutant at 10 DAS also suggests a role of bHLH039 in restricting the duration of cell proliferation and/or allowing cells to start expanding and, thus, regulating the onset of the transition phase between cell proliferation and expansion. Moreover, Stroby did not affect growth of the *bhlh039*

mutant and triple mutant. Growth of the *bhlh100* and *bhlh101* was not or only slightly promoted after Stroby application. Different hypotheses could explain the absence of effect of Stroby on mutants without functional *bHLHs* transcription factors. On the one hand, a combination of a mutation in *bHLH039* and Stroby treatment result in an increase in cell proliferation by which cell proliferation cannot be positively affected anymore, while on the other hand, a functional bHLH039 may be needed for Stroby to exert its growth effect by inducing downstream growth-regulatory responses. To further explore this, additional experiments are necessary, such as unraveling the transcriptional responses in growing leaves of *bhlh039* mutant and wild type plants with and without Stroby treatment.

Several transcripts involved in sugar transport were differentially expressed in Stroby-treated plants pointing to a possible effect on sugar transport or accumulation. It was recently shown that sucrose promotes cell proliferation early during leaf development (Van Dingenen *et al*, 2016), and, thus, the positive effect of Stroby on epidermal cell number could also indirectly result from an accumulation of sucrose in growing leaves. SWEET11 and 12 that are normally involved in exporting sucrose in the apoplast of the mesophyll cells prior to phloem loading (Chen *et al*, 2012) were both repressed in Stroby-treated plants. SWEET2, which is localized at the vacuolar tonoplast and possibly plays a role in reducing the loss of sugars in root tissue (Chen *et al*, 2015), was induced after Stroby application. Double *sweet11/12* mutants show increased endogenous levels of sucrose and hexoses and, oppositely, the *sweet2* mutants exhibit lower glucose accumulation. These observations suggest that application of Stroby could result in differences in sugar transport or sugar accumulation, although, no difference in sucrose content was found between rosettes of Stroby-treated and control plants. Additionally, Stroby treatment did not result in an increase in photosynthetic pigments which has been described in wheat (Beck *et al*, 2002). These data suggest that Stroby does not affect photosynthetic activity, and, thus, sucrose production. The effect of Stroby was found to be more pronounced on the younger leaves that did not reach maturity yet and are probably still proliferating (8^{th} until 12^{th} leaf, Fig. 2B), suggesting that sugars might accumulate in actively growing leaves. Sucrose quantification experiments in these young leaves should shed light on the potential differences in sugar accumulation in the different leaves of Stroby-treated rosettes.

Another iron-related gene, *NAS1*, was up-regulated in Stroby-treated rosettes. NAS proteins are involved in the transport of iron from the phloem to sink organs (Schuler *et al*, 2012). Iron is important in many different processes during plant growth, especially because it acts as an essential cofactor in the major complexes of the photosynthetic electron transport chain. In addition, a rise in iron content can increase plant biomass (Briat *et al*, 2007). Treating plants with Stroby did not result in changes in rosette iron content, although a small decrease in iron levels was found in younger leaves (Supplemental Fig. 3B). Notwithstanding that this observation needs further experimental evidence, it is tempting to speculate that Stroby treatment stimulates cell proliferation, in a yet unidentified way, by which the cells are depleted in the necessary resources resulting in nutrient-deficiency conditions. This might lead to insufficient iron and sugars in young proliferating leaves, by which bHLH transcription factors are up-regulated as well as NAS1 to promote iron transport to these organs. In accordance, these cells will prevent export of the necessary sugars, by which the sugar transporters probably involved in phloem loading (SWEET11/12, SUC7, SFP1) are repressed and the sugar transporters involved in maintaining sugar levels high inside the vacuole (SWEET2) are induced.

In conclusion, we clearly demonstrate that watering plants with low concentrations of kresoxim-methyl, in its commercial formulation Stroby, can promote Arabidopsis plant growth. Independently of the time of Stroby treatment, leaf growth was profoundly stimulated by an induction in cell proliferation. Transcriptome profiling of complete rosettes treated with Stroby demonstrated an interesting link with iron and sugar transport but no differences in iron or sugar content were found. Moreover, *bhlh* single and triple mutant plants were less sensitive or insensitive to Stroby suggesting a pivotal role of the subgroup Ib bHLH transcription factors in Stroby-induced growth. Further experiments are necessary to unravel the exact Stroby-induced downstream mechanisms specifically occurring in actively growing leaves.

MATERIALS AND METHODS

Growth conditions and plant material

Plants were grown in soil for 22 days at 21℃ under a 16-h day (80-100 µmol m^{-2} s^{-1}) and 8-h night regime. Four seeds per pot were sown on soil. After one week, the

seedling with a projected rosette area closest to median area of that genotype was selected per pot. In the first application protocol, seeds were stratified and germinated in presence or absence of Stroby® WG (BASF) after which they were watered twice a week until 22 days. In the second application protocol, plants were grown without Stroby for eight days. Plants were then watered twice a week with Stroby from 8 DAS until 22 DAS. Control plants were watered twice a week with water during the complete growth period. Stroby is used at a concentration of 10^{-9} M for KM, unless specified differently, dissolved in water. All experiments were performed on *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0). Homozygous seeds of the *bhlh039*, *bhlh100*, *bhlh101* mutant lines as well as the triple *bhlh39 bhlh100 bhlh101* were described in (Andriankaja *et al*., 2014).

Growth Measurements

Leaf series were made by cutting each individual leaf of the rosette and ranking them from old to young on a square agar plate. Plates were photographed and pictures were subsequently analyzed using ImageJ software (http://rsb.info.nih.gov/ij/) to measure the size of each individual leaf. Represented rosette sizes are calculated by taking the sum of the average individual leaf areas.

For the leaf area analysis, leaves were cleared in 100% ethanol, mounted in lactic acid on microscope slides, and photographed. Leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov//ij/). Abaxial epidermal cells of leaves were drawn with a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective. Drawings were scanned and analyzed using automated image analysis algorithms (Andriankaja *et al*, 2012). Subsequently, drawings were used to measure average cell area, from which the total pavement cell number was calculated. The stomatal index was defined as the percentage of stomata compared with all cells.

RNA Extraction and expression analysis by qRT-PCR

Seedlings were frozen in liquid nitrogen and RNA was extracted using Trizol (Invitrogen) and the RNeasy Plant Mini Kit (Qiagen). DNase treatment was done on columns with RNase-free DNase I (Promega). The iScript cDNA synthesis kit (Bio-

Rad) was used to prepare cDNA from 500 ng-1 µg RNA and qRT-PCR was done on the LightCycler 480 with SYBR Green I Master (Roche) according to the manufacturer's instructions. Normalization was done against the average of three housekeeping genes *AT1G13320*, *AT2G32170*, *AT2G28390*. Primer sequences are listed in Supplemental Table S2.

Chlorophyll and carotenoid measurements

Chlorophyll a (Chla), Chlb and total carotenoid levels were measured per mg fresh tissue of whole rosettes from six independent experiments. Pigments were determined spectrophotometrically in acetone extracts and quantification was done based on the equations described in (Porra, 2002).

RNA-Sequencing Analysis

Library preparation was done using the TruSeq RNA Sample Preparation Kit v2 (Illumina). Briefly, polyA-containing mRNA molecules were reverse transcribed, double-stranded cDNA was generated and adapters were ligated. After quality control using 2100 Bioanalyzer (Agilent), clusters were generated through amplification using the TruSeq PE Cluster Kit v3-cBot-HS kit (Illumina), followed by sequencing on an Illumina HiSeq2000 with the TruSeq SBS Kit v3-HS (Illumina). Sequencing was performed in Paired-End mode with a read length of 50 nt. The quality of the raw data was verified with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, version 0.9.1). Next, quality filtering was performed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/, version 0.0.13): reads where globally filtered, in which for at least 75% of the reads, the quality exceeded Q20 and 3' trimming was performed to remove bases with a quality below Q10. Re-pairing was performed using a custom Perl script. Reads were subsequently mapped to the Arabidopsis reference genome (TAIR10) using GSNAP (Wu et al, 2010, version 2012-07-20), allowing maximum two mismatches. These steps were performed through Galaxy (Goecks *et al*, 2010). The concordantly paired reads that uniquely mapped to the genome were used for quantification on the gene level with HTSeq-count from the HTSeq.py python package (Anders *et al*, 2015). The statistical analysis was

performed with the R software package edgeR (Robinson et al, 2010, R core team (2014), R version 3.1.2). TMM normalization (Robinson and Oshlack, 2010) was applied using the calcNormFactors function. Variability in the dataset was assessed with a MDSplot. Trended negative binomial dispersion parameters were estimated with the default Cox-Reid method based on a model with a fixed treatment and batch effect using the estimateGLMTrendedDisp function, followed by the estimation of the empirical bayes dispersion for each transcript. A negative binomial regression model was then used to model the overdispersed counts for each gene separately (27421 genes) with fixed values for the dispersion parameter as outlined in McCarthy et al (2012) and as implemented in the function glmFit using the above described model. A likelihood ratio test (LRT) was performed for each gene to test for a treatment effect with the glmLRT function. False discovery rate adjustments of the p values were done with the method described by Benjamini and Hochberg (1995). All edgeRfunctions were applied with default values.

Iron and sucrose measurements

Whole rosettes or individual leaves were harvested for total iron measurements. Plant iron measurements were conducted as previously described (Li *et al*, 2011). Iron concentration was determined using 0.05 % 1,10-phenanthroline and measuring the absorbance at 510 nm against an iron standard curve that was established with defined amounts of $FeSO_4 \cdot 7H_2O$. The analysis was conducted for two to three biological repeats, per sample six rosettes or 10 to 20 leaves were pooled. Sucrose was measured with GC-MS analysis in three rosettes of Stroby-treated or control plants.

Western blotting

Protein extraction was done as described before (Van Leene *et al*, 2007). Western-blot was performed with primary rabbit anti-ferritin antibodies (Santa Cruz; diluted 1:10000) for 1 hours and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibodies (1:10000) for 1 hours. Proteins were detected by chemiluminescence (NEN Life Science Products).

SUPPLEMENTAL DATA

The following supplemental figures and table are available at the end of this chapter.

Supplemental Figure S1. Schematic representation of the Stroby application protocols.

Supplemental Figure S2. Chlorophyll and total carotenoid measurements.

Supplemental Figure S3. Stomatal index of Stroby-treated and control plants at 10 and 22 DAS.

Supplemental Figure S4. Gene ontology enrichment analysis of Stroby-responsive genes using PageMan.

Supplemental Figure S5. qRT-PCR analysis of selected Stroby-responsive genes

Supplemental Figure S6. Transcript levels of *bHLH039*, *bHLH100* and *bHLH101* in the single mutants and triple *bhlh* mutant.

Supplemental Table S1. Differentially expressed genes after Stroby treatment in 17 day-old rosettes.

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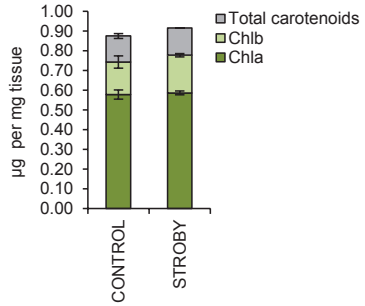
SUPPLEMENTAL DATA

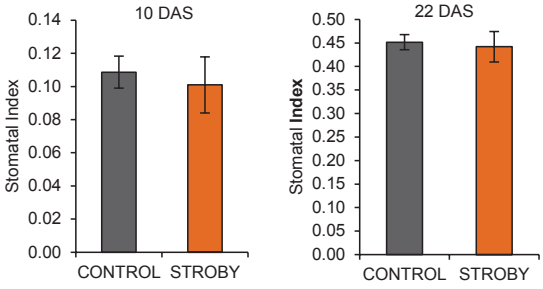
Supplemental Figure S1. Schematic representation of the Stroby treatment protocols. (A) Seeds were stratified and germinated in soil soaked with water (control) or Stroby. Subsequently, plants were treated twice a week with either water or Stroby until 22 days after stratification (DAS). RNA sequencing analysis was performed on rosettes harvested at 17 DAS. (B) Modified protocol to allow a more detailed study of the effects of Stroby on leaf growth. Seeds were stratified and germinated in soil soaked with tap water. The first Stroby treatment started at 8 DAS and from then plants were watered twice a week with water or Stroby.

Protocol	Treatment	Stratification for 3days	1	2	3	4	5	8	9	11	12	15	16	17	18	19	22
A	Control	Water					Water		Water		Water		Water	RNA-seq rosettes		Water	
	Stroby	Stroby					Stroby		Stroby		Stroby		Stroby			Stroby	
B	Control	Water						Water		Water		Water			Water		
	Stroby	Water						Stroby		Stroby		Stroby			Stroby		

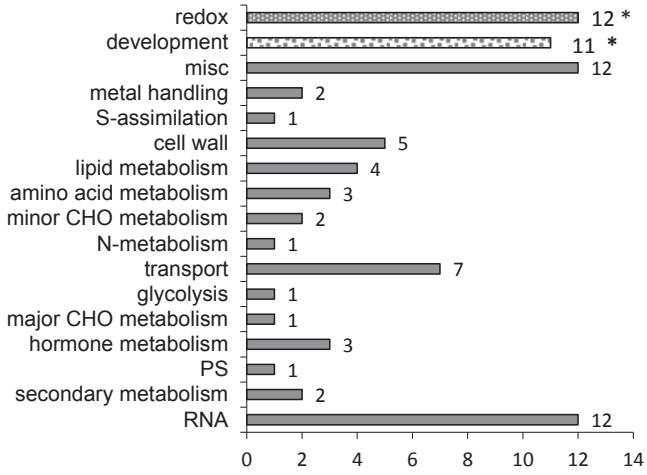
Supplemental Figure S2. Chlorophyll and total carotenoid measurements. Plants were grown in soil in the presence of Stroby from germination onwards for 22 days (Protocol A). Pigments were measured from the whole rosette at 22 DAS. Values are means of six independent repeats with their SE.



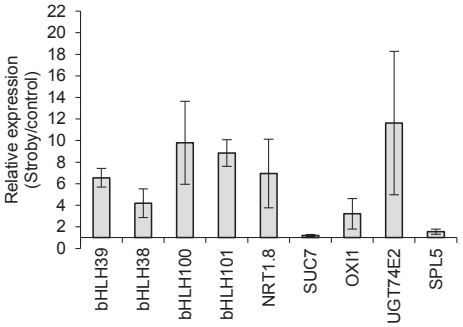
Supplemental Figure S3. Stomatal index of Stroby-treated and control plants at 10 and 22 DAS. Plants were grown in soil and watered twice a week with water (control) or Stroby at a concentration of 10^{-9} M KM. Values are means of three biological repeats with their SE. Cellular data are from three to five plants.



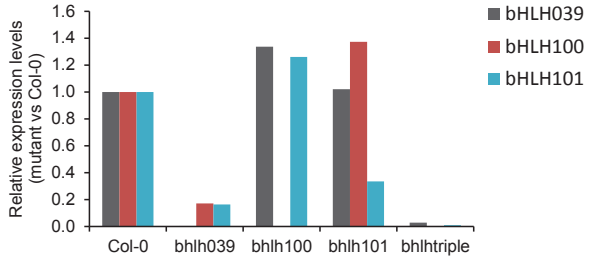
Supplemental Figure S4. **Gene ontology enrichment analysis of Stroby-responsive genes using PageMan**. GO terms are summarized and represented in functional categories in Y-axis. X-axis represents the number of genes in each category. Misc = large enzyme families, PS = photosynthesis-related. *, *P* < Bonferoni corrected P-value of 0.0004.



Supplemental Figure S5. qRT-PCR analysis of selected Stroby-responsive genes. Relative expression of nine Stroby-responsive genes selected from the 111 differentially expressed genes found by RNA-sequencing analysis of 17 day-old rosettes, 24 h after Stroby treatment. Values are the means of the ratios (Stroby/control) of three biological repeats with their SE.



Supplemental Figure S6. Transcript levels of *bHLH039, bHLH100* and *bHLH101* in the single mutants and triple *bhlh* mutant. Expression levels were measured by qRT-PCR in 10-day-old seedlings and are presented relative to expression in wild type seedlings.



Supplemental Table S1. Differentially expressed genes after Stroby treatment in 17 day-old rosettes.

Gene ID	Name	Description	Log_2FC	FDR
UP-REGULATED TRANSCRIPTS				
AT2G29480	**GST2**	glutathione S-transferase tau 2	5.57	0.01
AT1G03790	**SOM**	Encodes SOMNUS (SOM), a nucleus-localized CCCH-type zinc finger protein.	5.43	0.04
AT1G66570	**SUC7**	sucrose-proton symporter 7	4.82	0.00
AT4G37990	**CAD8**	Encodes an aromatic alcohol:NADP+ oxidoreductase whose mRNA levels are increased in response to treatment with a variety of phytopathogenic bacteria	4.48	0.00
AT1G52060		Mannose-binding lectin superfamily protein	3.95	0.03
AT3G56980	**bHLH39**	Encodes a member of the basic helix-loop-helix transcription factor protein	3.39	0.00
AT3G56970	**bHLH38**	Encodes a member of the basic helix-loop-helix transcription factor family protein.;basic helix-loop-helix protein 100	3.22	0.00
AT2G41240	**bHLH100**	Encodes a member of the basic helix-loop-helix transcription factor family protein	3.13	0.00
AT1G66700	**PXMT1**	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	2.85	0.02
AT3G25250	**OXI1**	Arabidopsis protein kinase;AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein	2.81	0.04
AT1G66280	**BGLU22**	Glycosyl hydrolase superfamily protein	2.80	0.00
AT4G22470		protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.64	0.02
AT1G05680	**UGT74E2**	Encodes a UDP-glucosyltransferase, UGT74E2, that acts on IBA (indole-3-butyric acid) and affects auxin homeostasis	2.44	0.02
AT3G49620	**DIN11**	dark-inducible; encodes a protein similar to 2-oxoacid-dependent dioxygenase	2.38	0.00
AT2G14247		unknown	2.20	0.00
AT4G21680	**NRT1.8**	Encodes a nitrate transporter (NRT1.8). Functions in nitrate removal from the xylem sap	2.15	0.02
AT5G44620	**CYP706A**	Encodes a member of CYP706A	2.15	0.00
AT5G48000	**CYP708A**	Encodes a member of the CYP708A	2.14	0.01
AT1G13609	**DEFL**	Defensin-like (DEFL) family protein	1.91	0.04
AT4G15210	**BAM5**	beta-amylase 5	1.63	0.00
AT4G15660		Thioredoxin superfamily protein	1.47	0.00
AT3G28500		60S acidic ribosomal protein family	1.38	0.03
AT4G15680		Thioredoxin superfamily protein	1.29	0.00
AT2G30766		unknown protein	1.29	0.01
AT2G38380		Peroxidase superfamily protein	1.28	0.02
AT3G49580	**LSU1**	response to low sulfur 1	1.26	0.01
AT1G47395		unknown protein	1.25	0.01
AT3G22600		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	1.20	0.02
AT5G26260		TRAF-like family protein	1.14	0.01
AT4G15670		Thioredoxin superfamily protein	1.14	0.01
AT5G05250		unknown protein	1.12	0.00
AT1G43800		Ferritin/ribonucleotide reductase-like	1.12	0.00
AT4G15690		Thioredoxin superfamily protein	1.07	0.00
AT4G15700		Thioredoxin superfamily protein	1.07	0.00
AT3G15270	**SPL5**	Encodes a member of the SPL (squamosa-promoter binding protein-like)gene family	1.06	0.02
AT5G35480		unknown protein	0.92	0.00
AT5G37990		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	0.84	0.04

AT4G14130	**XTR7**	xyloglucan endotransglycosylase-related protein	0.78	0.05
AT5G05960		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.77	0.00
AT1G06830		Glutaredoxin family protein	0.74	0.00
AT4G34950		Major facilitator superfamily protein	0.73	0.00
AT3G26200	**CYP71B22**	putative cytochrome P450	0.73	0.01
AT1G07610	**MT 1C**	one of the five metallothioneins (MTs) genes identified in Arabidopsis	0.62	0.00
AT2G47180	**GOLS1**	galactinol synthase 1	0.59	0.05
AT3G20100	**CYP705A**	member of CYP705A;cytochrome P450	0.59	0.00
AT1G08650		Encodes a phosphoenolpyruvate carboxylase kinase 1	0.57	0.00
AT2G16660		Major facilitator superfamily protein	0.55	0.00
AT1G06080		Encodes a protein homologous to delta 9 acyl-lipid desaturases of cyanobacteria and acyl-CoA desaturases of yeast and mammals	0.49	0.02
AT3G19030		unknown protein	0.48	0.02
AT3G56360		unknown protein	0.48	0.02
AT3G54260		Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain.	0.48	0.01
AT5G24030		Encodes a protein with ten predicted transmembrane helices. The SLAH3 protein has similarity to the SLAC1 protein involved in ion homeostasis in guard cells.	0.47	0.00
AT2G42610	**LSH10**	LIGHT SENSITIVE HYPOCOTYLS 10	0.47	0.00
AT1G67865		unknown protein	0.47	0.00
AT4G25100	**FeSOD1**	Fe-superoxide dismutase	0.47	0.00
AT3G26960		Pollen Ole e 1 allergen and extensin family protein	0.47	0.00
AT1G02820	**LEA3**	Late embryogenesis abundant 3 family protein	0.47	0.02
AT1G76930	**MLP28**	MLP-like protein 28	0.46	0.05
AT5G49480		Ca2+-binding protein 1	0.44	0.00
AT2G34070		Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain	0.44	0.00
AT5G65010	**ASN2**	asparagine synthetase 2	0.43	0.01
AT5G04950	**NAS1**	nicotianamine synthase 1	0.42	0.00
AT4G30110		encodes a protein similar to Zn-ATPase, a P1B-type ATPases transport zinc	0.41	0.00
AT1G63090		phloem protein 2-A11 (PP2-A11)	0.40	0.01
AT2G24762		Encodes a member of the GDU (glutamine dumper) family proteins involved in amino acid export	0.39	0.04
AT5G27350	**SFP1**	Encodes a sugar-porter family protein that is induced during leaf senescence	0.38	0.01
AT1G37130	**NR2**	nitrate reductase 2	0.38	0.02
AT3G48100	**ARR5**	cytokinin response regulator 5	0.36	0.02
AT2G37460		nodulin MtN21 /EamA-like transporter family protein	0.36	0.00
AT5G24660	**LSU2**	RESPONSE TO LOW SULFUR 2	0.35	0.02
AT4G04830	**MSRB5**	methionine sulfoxide reductase B5	0.35	0.01
AT3G14770	**SWEET2**	Nodulin MtN3 family protein	0.34	0.01
AT4G30270		xyloglucan endotransglucosylase/hydrolase 24	0.34	0.00
AT5G53450		OBP3-responsive gene 1	0.34	0.01
AT1G07590		Tetratricopeptide repeat -like superfamily protein	0.34	0.00
AT1G06640		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.33	0.01

AT1G49500		unknown protein	0.32	0.02
AT5G65660		hydroxyproline-rich glycoprotein family protein	0.29	0.04
AT4G37800	**XTH7**	xyloglucan endotransglucosylase/hydrolase 7	0.28	0.02
AT5G11790	**NDL-2**	N-MYC downregulated-like 2	0.28	0.02
AT2G38170		cation exchanger 1	0.24	0.04

DOWNREGULATED TRANSCRIPTS				
AT3G47340	**DIN6/ASN1**	glutamine-dependent asparagine synthase 1	-1.18	0.00
AT2G21650	**MEE3**	Homeodomain-like superfamily protein	-0.94	0.00
AT1G77960		unknown protein	-0.86	0.00
AT4G35770	**SEN1**	Senescence-associated gene that is strongly induced by phosphate starvation	-0.84	0.01
AT5G23660	**SWEET12**	homolog of the Medicago nodulin MTN3	-0.78	0.00
AT3G55240		unknown function	-0.73	0.00
AT3G05690	**HAP2B**	Encodes a subunit of CCAAT-binding complex;nuclear factor Y, subunit A2	-0.64	0.02
AT2G38530		Involved in lipid transfer between membranes	-0.48	0.00
AT2G28190	**SOD2**	Encodes a chloroplastic copper/zinc superoxide dismutase 2	-0.48	0.00
AT1G68840	**EDF2**	Rav2 is part of a comAPETALA2/ETHYLENE RESPONSE DNA BINDING FACTOR 2	-0.47	0.00
AT1G12520	**CCS1**	COPPER CHAPERONE FOR SOD1	-0.47	0.00
AT4G22513		Encodes a Protease inhibitor/seed storage/LTP family protein	-0.46	0.02
AT5G55930		oligopeptide transporter	-0.46	0.01
AT5G43780		sulfate adenylyltransferase	-0.46	0.00
AT5G10170		myo-inositol-1-phosphate synthase isoform 3	-0.42	0.03
AT1G22590	**AGL87**	AGAMOUS-like 87	-0.39	0.00
AT2G40610	**EXP8**	member of Alpha-Expansin Gene Family	-0.37	0.01
AT1G20693	**HMGB2**	Encodes a protein belonging to the subgroup of HMGB (high mobility group B) proteins t	-0.36	0.00
AT5G36910		Encodes a thionin that is expressed at a low basal level in seedlings and shows circadian variation	-0.35	0.04
AT3G48740	**SWEET11**	Nodulin MtN3 family protein	-0.35	0.00
AT1G64370		unknown protein	-0.34	0.03
AT4G26530		Aldolase superfamily protein	-0.33	0.01
AT4G34250	**KCS16**	3-ketoacyl-CoA synthase 16	-0.33	0.01
AT5G51100	**FSD2**	FE SUPEROXIDE DISMUTASE 2	-0.32	0.01
AT2G20670		Protein of unknown function	-0.32	0.01
AT2G33770	**UBC24**	Encodes a ubiquitin-conjugating E2 enzyme	-0.31	0.05
AT2G46600		Calcium-binding EF-hand family protein	-0.31	0.02
AT1G08830	**SOD1**	SUPEROXIDE DISMUTASE 1	-0.31	0.04
AT2G29290		NAD(P)-binding Rossmann-fold superfamily protein	-0.30	0.02
AT5G19120		Eukaryotic aspartyl protease family protein	-0.30	0.02

Supplemental Table S2. qRT-PCR primer sequences of selected Stroby-induced transcripts

Gene ID	Name	Forward Primer	Reverse Primer
AT3G56970	*bHLH038*	TGAGCTTTACGATAAGCAGCAACC	AGCCTAGTGGCAGAAACCGTTG
AT3G56980	*bHLH039*	TGCCTCTGGCCAATCGAAGAAG	TGTACTTCAAGCTTCGAGAAACCG
AT2G41240	*bHLH100*	TTCCTCCCACCAATCAAACGAAG	TGACCCGAAATTTGAAACGAGAGC
AT5G04150	*bHLH101*	TCACAACGCAAGCGAACGAGAC	AGAGGCAAGAGAGCACGAAGTG
AT4G21680	*NTR1.8*	AGCAAGTTTCGTTGCAGGGTTG	ACTCCACAACCACTTGGTTCAAGC
AT1G66570	*SUC7*	ACAACAAACACAACCTCTAA	CTATCCACAGTCGTCTCA
AT3G25250	*OXI1*	ACCGTCACTGTCTAAACCATCGC	TGCAGAAACTGGTGAAGCGGAAG
AT1G05680	*UGT74E2*	CGATGCTGACTGCAAATGAT	CACAAGCTTTGGACCCATTT
AT3G15270	*SPL5*	AAGGCATCTGCTGCGACTGTTG	TCTGGTAGCTCATGAAACCTGCTG

Part 3:
Concluding remarks

CHAPTER 8

General Discussion, Conclusions and Perspectives

In this chapter, I discuss the major findings of this PhD. In the first part, focus is given to sucrose-induced early leaf growth and I present a model by which I integrate the sucrose effects mediated by the *GLUCOSE-6-PHOSPHATE/PHOSPATE 2* translocator (*GPT2*) and the glucose-signaling protein HEXOKINASE1 (HXK1). *GPT2* expression was found to be essential for the sucrose-induced stimulation of cell proliferation as well as repression of chloroplast DNA transcription during the growth of young leaves that act as sugar-importing sinks. Furthermore, upon transfer to sucrose, chloroplast differentiation was restricted in these leaves which probably led to a delay in cell expansion. In addition, we found that HXK1 plays a role in regulating cell proliferation during early leaf growth and is less sensitive to sucrose-induced promotion of cell proliferation. Our findings suggest that HXK1-signaling might act downstream and independent of GPT2. As described in Chapter 6, several novel protein partners of HXK1 were isolated via tandem affinity purification experiments and some of these are further discussed possibly opening the path for future research. In the second part of this chapter, I highlight several considerations about the use of strobilurins as growth-promoting compounds as well as future experiments which will be essential to further unravel the Stroby-induced positive effects on leaf growth.

Sugars, Sugar Signaling and Leaf Growth

As illustrated in Chapter 1, sugars play essential and diverse roles during plant growth and development. Leaves are the primary organs performing photosynthesis to fixate carbon into sugars that are used as major building blocks for the cell. Sugars are the essential backbones of DNA and RNA, are necessary for the formation of cell wall polysaccharides and are used as cellular energy. Some proteins also need the attachment of often complex sugar-trees for their functionality. The primary photosynthetic products are sugar phosphates that are used to form sucrose to facilitate long-distance transport or to form starch as an important storage compound to sustain growth at night (Stitt & Zeeman, 2012; Wind *et al*, 2010). Furthermore, sugars are able to directly influence the transcriptional and translational machinery of the cell, which highlights the important role of sugars as signaling molecules able to link development with genetic factors and environmental signals (Baena-Gonzalez *et al*, 2007; Lastdrager *et al*, 2014; Rolland *et al*, 2006; Xiong & Sheen, 2014). In the last decades, there has been large interest to unravel the downstream sugar signaling pathways but because of the high complexity and interconnection with many other molecular, hormonal, and environmental signals, their functions during growth are still far from resolved.

Preliminary Considerations: working with tiny leaves…

One of the main objectives of my PhD was to gain more insight into the sucrose-induced cellular and molecular changes during early leaf growth. For this, we developed an experimental setup in which the availability of sucrose can be altered during leaf development, as described in Chapter 4. Seedlings were grown at a low light intensity of 50±5 µmol m^{-2} s^{-1} on sugar-free medium for 9 days. At 9 DAS, the time point at which the third leaf is fully proliferating (Andriankaja *et al*, 2012), seedlings were transferred to medium with or without sucrose. We found that sucrose solely affects cell proliferation so the third leaf had to be micro-dissected very early during development for further characterization, which brought some preliminary considerations that are briefly discussed below.

During leaf development, growing leaves first act as sugar-importing sinks until they become photosynthetically active and therefore act as source leaves. Although this sink-to-source transition of leaves is a well-known and old stated fact (Roitsch, 1999), it is still not completely understood when it occurs and how sugars can influence it. When enough sugars are available and photosynthesis is not needed to support growth, sugars are involved in feedback regulation to repress photosynthesis-related transcripts (Paul & Foyer, 2001; Paul & Pellny, 2003). These sugar-mediated transcriptional responses are well described and were investigated by many research groups as discussed in Chapter 1. Surprisingly, although it is obvious that sugars have different responses during organ development, most transcriptional studies performed so far, were using complete seedlings, mature leaves or cell cultures (Muller *et al*, 2007; Osuna *et al*, 2007; Price *et al*, 2004; Usadel *et al*, 2008). The use of entire seedlings for transcriptional experiments mainly reveals the expression of genes active in expanding and mature (but not dividing) cells (Skirycz *et al*, 2010). To understand how sugars affect specific developmental programs, such as the growth of young sink leaves, the different developmental stages of the organ have to be analyzed separately. At the start of my PhD, Andriankaja and colleagues (2012) characterized the development of the third leaf of Arabidopsis in detail at the cellular and transcriptional level and uncovered a close interaction between the establishment of the photosynthetic machinery and, thus, chloroplast differentiation and subsequent sugar production, and the transition to cell expansion. After

originating from the shoot apical meristem (SAM), the third leaf first exclusively grows by cell proliferation until around 9 days after stratification (DAS). Before this time point, the third leaf is not-photosynthetically active and to elucidate the underlying molecular and cellular mechanisms responsible for growth, it has to be micro-dissected to separate it from the cotyledons and the first true leaves, which are already in expansion phase. However, at this stage, the leaf only measures around 0.04 mm^2 making it rather challenging to obtain enough input material currently needed to perform experiments such as metabolic profiling, hormone quantifications or protein-protein interaction studies (Dedecker *et al*, 2015; Kojima & Sakakibara, 2012; Lisec *et al*, 2006). Nevertheless, techniques are evolving fast and becoming more and more sensitive. Currently, for RNA-sequencing (RNA-seq) methodologies less than 100 ng RNA is required (Wang *et al*, 2009). Obtaining this amount of RNA is feasible for young proliferating leaves and provided us with new insights in the sugar-mediated and developmental stage-specific molecular mechanisms as described in Chapter 4 (Van Dingenen *et al*, 2016).

Sucrose-induced *GPT2* expression in young proliferating leaves

Studying the regulation of the sink-to-source transition in leaves was one of the first objectives of my PhD. For this, we developed an experimental setup in which the availability of sucrose can be altered during early leaf development in a condition that corresponds as much as possible to a physiological relevant situation. To do so, seedlings were grown on meshes, making that exogenously supplied sugars mainly enter the plant via the roots and are transported to the leaves via the phloem.

Using this setup, we tested different concentrations of both sucrose and glucose and found that only sucrose was able to stimulate leaf growth. Sucrose is the main transported sugar that is translocated through the phloem from source leaves to sink tissues (Rennie & Turgeon, 2009), besides other carbon skeletons, such as polyols and raffinose oligosaccharides (Noiraud *et al*, 2001). Many research groups studied the effect of glucose on plant development (Gibson, 2005), mostly using high concentrations (2-6%) and during different developmental programs, such as seed germination (Osuna *et al*, 2015; Price *et al*, 2003), root growth direction (Singh *et al*, 2014) and lateral root development (Gupta *et al*, 2015). Furthermore, Heinrichs and colleagues (2012) also demonstrated an increase in rosette growth using 50 mM of

glucose. Based on the above described findings, the absence of effect of glucose in our setup was rather surprising but could be easily explained by the fact that we used low concentrations (compared with the other studies) and because glucose is not transported via the phloem (Liu *et al*, 2012). Nevertheless, the effects of sucrose and glucose are tightly linked and it is not easy to elucidate their individual effects. When sucrose arrives at the sink tissue, it might enter the cell symplast via plasmodesmata, via apoplastic transport and transporters or it can be first converted into its hexose products, glucose and fructose, and then imported via hexose transporters (Williams *et al*, 2000). Inside the cytosol, sucrose can be cleaved to UDP-glucose and fructose by sucrose synthase or to glucose and fructose by cytosolic invertases. Glucose will then be converted to glucose-6-phospate (G6P) by hexokinases for further metabolic processing (Granot *et al*, 2014). To unravel the underlying molecular mechanisms responsible for the sucrose-induced stimulation of leaf growth, short-term transcriptional responses were analysed using RNA-seq which allowed us to discover rapid upregulation of *GPT2* upon transfer to sucrose-containing medium. Induction of *GPT2* expression by sugars has been reported in many different studies, using mature leaves and whole seedlings grown in liquid cultures, as well as with both sucrose and glucose treatments (Osuna *et al*, 2007; Price *et al*, 2004). It remains elusive whether sucrose, or its conversion to glucose and G6P are involved in *GPT2* induction. One approach to tackle this could be the use of a non-metabolizable sucrose analogue, turanose, which is still able to be transported through the phloem via the active sucrose/H+ symporter SUC2 (Chandran et al, 2003) to elicit extracellular sucrose-signaling (Sinha et al, 2002). However, Gonzali et al. (2006) did not report induction of *GPT2* expression by turanose possibly because it cannot be translocated inside the cell (Loreti *et al*, 2000).

In our setup, sucrose was unable to stimulate cell proliferation or to repress chloroplast transcription in the *gpt2* mutant 24 hours after transfer to sucrose (10 DAS). These findings clearly suggest an essential role of the expression of *GPT2* in both processes. However, at the final time point, leaf sizes of the *gpt2* mutant were still slightly, but not significantly, increased by sucrose as described in Chapter 5. This could suggest that early during development *GPT2* expression has a predominant function in the sucrose-induced effects on cell proliferation but that during leaf development sucrose might stimulate leaf growth through an independent

pathway like HXK1-mediated glucose signaling or other signaling pathways. Based on our findings, I propose a model of the sucrose-mediated responses in young proliferating leaves. The different paths are discussed below and schematically represented in Figure 1.

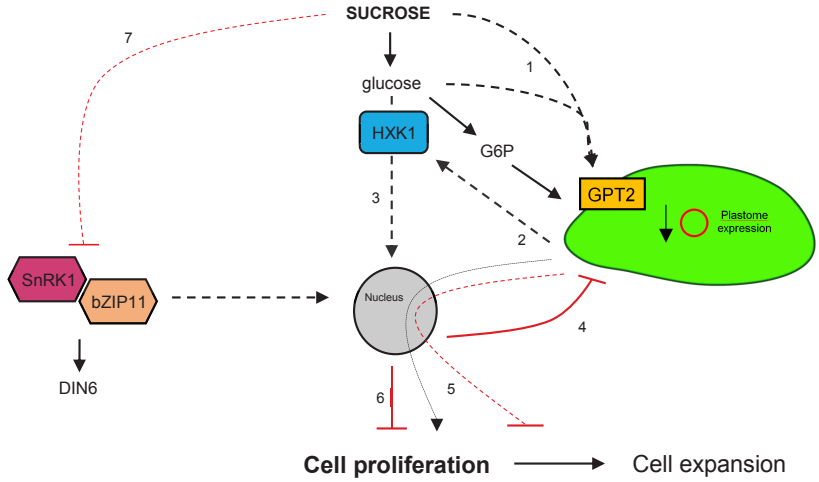


Figure 1. Model of sucrose-mediated responses in young proliferating leaves. When sucrose enters the sink cell it is converted to glucose and both sugars might rapidly trigger the expression of GPT2 (1) resulting in repression of chloroplast transcription that might result in less or more retrograde signals (2) that stimulate cell proliferation and/or repress the onset of cell expansion (5). In addition, part of the GPT2-induced retrograde signaling might also go through HXK1 (2). Also a GPT2-independent pathway exists via direct HXK1-signaling (3), which inhibits nuclear-encoded photosynthesis genes (4), also resulting in less chloroplast differentiation and retrograde signaling. HXK1 can inhibit cell proliferation early during development, when leaves are not yet performing photosynthesis (6). Sucrose can also directly inactivate SnRK1/bZIP signaling which also affects transcription, such as induction of their common target gene DIN6 (7).

It was previously reported that *gpt2* seedlings show a delay in development which could not be rescued by exogenously supply of glucose (Dyson *et al*, 2014), which is consistent with our cellular data, i.e. less cells compared with wild type leaves. A kinematic analysis of cell size and cell number, similarly as described for the wild type and the *hxk1* mutant in Chapter 4 and 6, should shed more light on the underlying cellular mechanisms resulting in the increase in the *gpt2* mutant at the final stage. To further explore the downstream molecular players involved, it would be interesting to transcriptionally profile *gpt2* mutant leaves. Furthermore, making double mutants between *hxk1* and *gpt2* might help in further unraveling their interconnection during sucrose-induced leaf growth. To provide further evidence for

the link between the repression of chloroplast transcription, the stimulation of cell proliferation and the inhibition of chloroplast differentiation upon transfer to sucrose, we are currently performing transmission electron microscopy (TEM) on the *gpt2* mutant. Chloroplast morphology, area and number of *gpt2* mutant leaves, 24 hours after transfer to control or sucrose-supplemented media, as described for wild type seedlings in Chapter 4, will be analyzed. Transfer of wild type seedlings to sucrose-containing medium resulted in less and smaller plastids which were irregular in shape and less differentiated, which possible led to less retrograde signals resulting in a delay in cell expansion. If GPT2 also plays a critical role during this process, chloroplasts of *gpt2* seedlings are expected to not be affected by sucrose.

Alternative Sugar-Responsive Pathways

Diverse and conserved sugar-signaling pathways are involved in the regulation of transcription and translation during plant growth. However, it remains unclear which signaling path underlies the repression of chloroplast transcription by sucrose through *GPT2* expression. GPT2 has been described to act as a buffer during acclimation to high light in the exchange of metabolites between the cytosol and the chloroplast (Dyson *et al*, 2015). The main substrate of GPT2 is G6P, although, it also has affinity for other sugar phosphates (Kammerer *et al*, 1998). In Chapter 4, we proposed one hypothesis in which high sucrose levels result in higher G6P levels which might be transported in the chloroplast resulting in a repression of chloroplast transcription (Van Dingenen *et al*, 2016). However, also other secondary and downstream signaling pathways might be involved in this inhibition. High sucrose levels positively correlate with G6P and trehalose-6-phosphate levels (Lunn et al, 2006), and can inhibit the SNF1-related protein kinase (SnRK1; Nunes *et al*, 2013; Zhang *et al*, 2009), resulting in stimulation of general cellular biosynthetic pathways and repression of energy-consuming catabolic reactions (Baena-Gonzalez & Sheen, 2008). Three hours after transfer to sucrose, we found that PV42a, a ɣ-like protein based on its structure similarity with the regulatory subunits of SNF1/AMPK/SnRK1, was strongly repressed (Chapter 4). *kin10* plants mutated in the catalytic α-subunit of SnRK1, have higher transcripts of chloroplast biogenesis-related genes under phosphate starvation (Fragoso *et al*, 2009), suggesting a possible role of SnRK1-signaling in the inhibition of chloroplast transcription during early leaf growth.

I performed some preliminary experiments with a *kin10* T-DNA mutant which revealed a potential additional role of KIN10 in the sucrose-induced stimulation of leaf growth when seedlings were subjected to our experimental setup (data not shown). SnRK1 is inactivated by high sugar levels and, thus, sucrose might affect transcription via SnRK1-mediated signaling in young leaves to regulate growth. Interestingly, one of the highly repressed genes upon transfer to sucrose at 3 and 24 hours was *DIN6/ ASN1,* encoding asparagine synthetase, which is a known target of SnRK1 (Baena-Gonzalez & Sheen, 2008). Furthermore, SnRK1-signaling is partially mediated through the S-group bZIP transcription factors (Baena-Gonzalez *et al*, 2007). Sucrose is able to repress *bZIP* translation directly (Wiese *et al*, 2005) and *DIN6* expression is also rapidly activated by the *bZIP11* transcription factor (Fig. 1) (Hanson *et al*, 2008).

The Mysterious Role of HEXOKINASE1 in Sink and Source Leaves

In Chapter 6, we studied the role of HXK1 during early leaf development. For this, we used *hxk1* mutant seedlings in the Col-0 background subjected to the experimental setup described in Chapter 4. By this, the role of HXK1 in sink tissue as well as in the transition from sink-to-source could be examined.

HXK1 has been suggested to mainly act in photosynthetically active tissues as it is responsible for the repression of photosynthesis-related genes (Moore *et al*, 2003; Xiao *et al*, 2000), serving as a sensor to fine-tune the investment of the cell in photosynthesis with intracellular sugar levels. However, HXK1 is ubiquitously expressed in the plant, both in sink and source tissues (Karve *et al*, 2008). This expression reflects its main metabolic role as a central enzyme during glycolysis responsible for the phosphorylation of glucose to G6P but it can also reflect a possible signaling role in sink leaves. A role of HXK1 as a sink-tissue-specific glucose sensor has not been explored, although it is likely because of the defects in hypocotyl elongation and root growth in the *gin2* mutant, the *hxk1* mutant in the Landsberg *erecta* background (Moore *et al*, 2003; Xiao *et al*, 2000).

In Chapter 6, we found that young proliferating leaves of *hxk1* seedlings contained more pavement cells without exogenously supplied sucrose in the medium. One possibility that we discussed is that *hxk1* leaves might already have more cells when

initiating from the shoot apical meristem (SAM) or have increased cell cycle progression. Further experiments are needed to quantify the leaf sizes prior to 9 DAS to have a clue on which of the two processes, leaf initiation or cell proliferation, are affected by HXK1 impairment. For this, we could make use of a technique developed in our lab, which is based on histological sections and 3D reconstruction to easily measure the size of small leaves initiating from the SAM (Vanhaeren *et al*, 2010). Leaf primordia are known to form at regions of high auxin and low cytokinin accumulation (Bar & Ori, 2014) and the HXK1-mediated pathway is tightly interconnected with different hormone signaling pathways such as auxin, cytokinin and abscisic acid (ABA) signaling. The *gin2* mutant, is insensitive to auxin and hypersensitive to cytokinin (Moore *et al*, 2003). In addition, several *aba* deficient and *aba insensitive* (*abi*) mutants are also insensitive to high glucose concentrations similarly as the *gin2* mutant (Arenas-Huertero *et al*, 2000; Rolland *et al*, 2006). These hormones are also involved in the regulation of cell division (Gutierrez, 2009) supporting a potential close interplay between HXK1 and hormonal signaling during leaf initiation and/or early leaf proliferation phases.

Later during leaf development, *hxk1* cells showed a faster onset of cell differentiation compared with wild type, while at the final time point *hxk1* and wild type leaves did not differ in cell number or size. This observation could be explained by the fact that wild type plants undergo a longer cell proliferation phase by which the differences in cell number and size between wild type and *hxk1* are compensated at the final stage. This developmental effect in the *hxk1* mutant on the cellular processes further highlights the importance to characterize mutants not only at the macroscopic level but that a thorough analysis at the cellular level during development is necessary for a better and more correct understanding of the functions of the genes in growth regulation (Chapter 2).

Upon transfer to sucrose, cell proliferation of *hxk1* leaves was not stimulated as much as in wild type plants and this positive effect was not maintained throughout leaf development and therefore did not result in a significant increase in final leaf size. The remaining positive effect at the final stage might rather be due to an increase in total cell number at later time points. Meristemoids are stomatal precursor cells which are dispersed in the leaf and divide asymmetrically resulting in the

formation of additional pavement cells at later time points during leaf development (Andriankaja *et al*, 2012; Geisler *et al*, 2000). So, the late increase in cell number in *hxk1* mutants might reflect a positive effect of sucrose on asymmetric division of meristemoid cells. *GPT2* expression was still induced by sucrose in the *hxk1* shoots and this induction might be responsible for the short-term positive effect on cell proliferation at 10 DAS. When entering the sink cell, higher sucrose levels might rapidly result in the induction of *GPT2* both in wild type and *hxk1* seedlings. In the cell, sucrose can be cleaved in its hexose products, glucose and fructose (Ruan *et al*, 2010). The increased intracellular glucose levels might not be sensed by HXK1 in the *hxk1* mutant seedlings and therefore probably not result in further inhibition of photosynthesis explaining the absence of the stimulation of cell proliferation at later time points (Fig. 1). Accordingly, this effect was also verified at the chloroplast level in the third leaf of *hxk1* mutants at 11 DAS via TEM analysis. Chloroplasts of sucrose-transferred *hxk1* seedlings were larger at the tip of the leaf and seem to have more grana and thylakoids but these observations need further quantifications. More differentiated chloroplasts in the *hxk1* mutant probably result in more or less chloroplast retrograde signals, still unknown, that trigger the onset of cell expansion (Andriankaja *et al*, 2012). This effect might then overrule the sucrose effect on chloroplast transcription mediated through *GPT2*. At 10 DAS, sucrose-induced cell proliferation is completely abolished in *gpt2* seedlings, whereas sucrose still partially increased cell number in *hxk1* seedlings. These findings suggest that GPT2 acts upstream of HXK1 and that these two proteins independently affect chloroplast differentiation to regulate early leaf development.

Furthermore, transfer to sucrose resulted in less and smaller chloroplasts which are less differentiated in young leaves. This restriction in chloroplast development might also result in the formation of less sugar phosphates, and, consequently, glucose during photosynthesis. By this, some of the sucrose-induced GPT2-mediated effects on chloroplast transcription and differentiation might also be sensed by the glucose-sensor HXK1 later during development. Transcript levels are generally more rapid than changes in enzyme activities as studied during the diurnal cycle in Arabidopsis rosettes (Gibon *et al*, 2006). This observation might explain why sucrose is still able to shortly induce cell proliferation in *hxk1* seedlings by the rapid induction of *GPT2* expression. However, previous reports demonstrate no clear role of GPT2 and HXK1

in retrograde signaling during acclimation to high light in mature leaves (Hausler *et al*, 2014).

Next to the signaling effects, we cannot ignore that the sucrose-mediated effects that we observe might be due to general changes in metabolite levels and it remains difficult to make a distinction between such dual effects. Several findings suggest that the *hxk1* growth phenotype is mainly regulated through signaling as catalytically inactive alleles of HXK1 can rescue the reduction in leaf size of the *gin2* mutant (Frommer *et al*, 2003; Harrington & Bush, 2003; Moore *et al*, 2003). In addition, in the *in vitro* conditions that we used, no clear differences could be found in rosette growth between wild type and *hxk1* mutant plants, except at early time points during leaf development as discussed above. The similar growth phenotypes suggest that primary metabolism is possibly not heavily affected. Furthermore, GPT2 and HXK1 are acting via a common metabolite G6P, by which there might be a link between both proteins. A metabolite-based hypothesis to explain the effects of sucrose on leaf growth is that a mutation in HXK1 might result in less formation of G6P, which is probably necessary for the inhibition of chloroplast transcription through GPT2. However, it has been demonstrated that the *gin2* mutant still has half of the amount of glucose phosphorylation capacity as wild type plants and even increased glucose-6-phosphate (G6P) levels (Moore *et al*, 2003). G6P can be produced by other HXKs, such as HXK2 and HXK3, which are highly expressed in sink tissues such as flowers and roots, probably resulting in no major differences in G6P levels between *hxk1* and wild type leaves (Jang *et al*, 1997; Karve *et al*, 2008). Metabolomics would shed some more light on the changes in metabolite levels in young leaves of *hxk1, gpt2* and wild type seedlings but this technique is unfortunately not feasible because of the lack of enough input material due the fact that the we are studying the responses in small leaves that need to be micro-dissected to distinguish between expanding and proliferating tissues.

The “Complex” HXK1 protein-protein Complex

We performed several tandem affinity purification experiments (TAP) on cell cultures grown under a 16-h day and 8-h night regime to further unravel the protein complexes around HXK1 as well as to identify other HXK1-dependent players. TAP

has been shown to be a powerful technique to elucidate important protein complexes involved in diverse developmental pathways (Dedecker *et al*, 2015; Gadeyne *et al*, 2014; Vercruyssen *et al*, 2014;), but it remains challenging to uncover transient and weak protein interactions in signaling pathways. We used the C-terminal HXK1 TAP-tagged fusion protein and Arabidopsis cell suspension cultures subjected to three different sugar treatments to distinguish between glucose-triggered and non-triggered HXK1 interactions. In Chapter 6 we only focused our discussion on the protein partners that were independently confirmed in two biological repeats. Here, I want to discuss on some of the other proteins that I think should be considered to be subjected to further research since they present some interesting features. Of course, additional experiments, such as reverse-TAP, bimolecular fluorescence complementation and yeast-two-hybrid assays, are needed to confirm that these are real interactors.

In the first growth condition, cells were grown in the presence of sucrose and were photoautotrophic. Under these conditions, two nucleosome assembly proteins, NAP1;1 and NAP1;2, co-purified with HXK1. A role of these proteins in leaf development has been reported (Galichet & Gruissem, 2006), and the *nap1;1* mutant line demonstrates a similar phenotype as the *hxk1* seedlings that we observed. NAP1;1 also promotes cell proliferation and expansion depending on the developmental stage of the leaf. Galichet and Gruissem (2006) demonstrate a nuclear-localization of the protein during the cell proliferation phase to promote cell division and a subsequent cytosolic localization during cell expansion. Interestingly, recently an interaction between NAP1;1 and the ribosomal protein S6 (RPS6) was discovered (Son *et al*, 2015). RPS6 is phosphorylated by S6 kinase, a downstream effector of the TOR-signaling pathway (Ruvinsky & Meyuhas, 2006). TOR or target of rapamycin is one of the conserved glucose-signaling pathways that is activated by hormones, nutrients and energy to coordinate growth with environmental signals. Hence, these findings could suggest an interesting and yet unreported link between the HXK1 and TOR glucose-signaling pathway.

Protein purifications were also done on cell cultures which were sucrose-starved and re-supplemented with sucrose for several minutes before protein extraction. This sucrose starvation probably induced a general stress response and many proteins that co-purified with the HXK1-GS^{green} fusion protein had functions in primary

metabolism (Table 1, Chapter 6). Sucrose starvation commonly affects the expression of sugar metabolizing genes (Koch, 1996). Surprising, although, was the isolation of two other proteins the cytosolic KINγ1 and the H2A histone variant HTA6 when cells were sucrose-starved. Glucose-free HXK1 might be translocated to the nucleus to bind HTA2 to release its repression as discussed in Chapter 6. The other isolated protein was KINγ1, which might also interact with HXK1 under certain stress conditions to further regulate growth.

Finally, a preliminary *in planta* TAP experiment resulted in the additional identification of many more potential HXK1-interactors. Complete seedlings were harvested for 1 hour after transfer to sucrose-added or control medium at 9 DAS. Again, harvesting complete seedlings reflects different cell types and tissues in diverse developmental stages making it difficult to link the unraveled protein complexes with developmental status of a particular leaf. Similarly as in cell cultures, many primary metabolism related proteins were identified (Supplemental Table S1, Chapter 6).

Concluding remark

In conclusion, during my PhD, we were able to develop an *in vitro* experimental setup in which the effect of sugars as signaling molecules can be further explored in a developmental context. The sugar-mediated effects during leaf growth as well as growth of the plant itself are very complex due to their integrated nature of regulation at the cellular, organ and whole organism level as well as the influence of diverse environmental, hormonal and developmental signals. Understanding the sugar-mediated effects during growth regulation will clearly benefit from research objectives taking into account the development stage of the plant.

Strobilurins as Growth-promoting Compounds

In the second project of my PhD, I focused on the potential growth promoting effects of strobilurins. Kresoxim-methyl (KM), azoxystrobin and pyraclostrobin have been previously reported to positively affect growth, greening and yield in the absence of fungal diseases. These strobilurin-induced physiological effects have been described in diverse plant species such as wheat, barley, duckweed, tobacco and corn (Kiersten & Daren; Klaus & Günter, 1997; Köhle, 2002; Ypema & Gold, 1999). However, these effects were not always consistent and it seems that treatment with strobilurins not always resulted in positive effects, especially in soybean, as discussed in Chapter 3. In this study, we demonstrated that watering Arabidopsis plants with Stroby, consisting of 50% KM, routinely used in the field, results in an increase in rosette and leaf size. Similarly as reported before, also in our hands, Stroby treatment did not always increase growth for a yet inexplicable reason. KM degrades very rapidly and has a very short half-life of only one day (PubChem Compound Database) which might explain some of the inconsistency that was observed. Another aspect which might explain this inconsistency is the fact that strobilurins are normally fungicides applied by spraying on the leaves. In addition, because of their independent structural modifications, different strobilurin compounds have different physical properties (Bartlett *et al*, 2002). In contrast to azoxystrobin, KM is non-systemic and is mainly distributed locally on the plant surface via the air or it moves translaminar into the leaves (Ypema & Gold, 1999). In our setup, Stroby is applied by watering the soil where Arabidopsis plants are growing. Both seeds and seedlings grown on soil soaked with Stroby resulted in a rapid and final increase in leaf area. These observations together with the above described characteristics of KM indicate that the main effect of Stroby would be on the roots triggering an unknown signal to stimulate shoot growth. In addition, because strobilurins have a highly specific mode-of-action in fungi and yeasts, i.e. blocking the electron transport chain of the mitochondrial respiration, which is a highly conserved process, it is possible that the positive effect on plants would also originate from an interaction with plant mitochondria (Diaz-Espejo *et al*, 2012). One attractive explanation as discussed in Chapter 7 is that production of NO signaling that might act as a secondary Stroby-induced signal to stimulate the positive effects on growth. It has been reported that NO can be formed inside plant mitochondria through nitrite reduction mediated by

enzyme complexes of the mitochondrial electron transport chain downstream of quinone pool (Gupta *et al*, 2011; Moreau *et al*, 2010). Interestingly, we found that a nitrate transporter NRT1.8, upregulated by nitrate, and NR2 were induced in Stroby-treated rosettes. The Stroby-induced positive effects on growth could also result from the general negative effect of Stroby on fungi in the rhizosphere allowing the plant to not spend energy into its defense response. However, this indirect Stroby response is rather unlikely because we previously demonstrated that Stroby treatment is still able to induce growth when using sterilized soil (data not shown).

The transcriptome analysis of whole rosettes at 17 DAS provided us with a general view of the molecular pathways affected by Stroby. However, these transcriptional responses are probably reflecting a general steady-state response to the repeated Stroby treatments and are, thus, rather indirect long-term effects. These molecular responses do not provide further knowledge on how Stroby directly influences early leaf development. As demonstrated in Chapter 7, Stroby-treated seedlings at 8 DAS resulted in the stimulation of cell proliferation in young growing leaves already at 48 hours. To understand the specific molecular pathways regulated by Stroby during the growth of a young leaf, we are planning to perform RNA sequencing on micro-dissected third leaves harvested after Stroby treatment. Furthermore, including the *bhlh039* mutant, which is insensitive to the Stroby-induced growth promotion, will further elucidate downstream bHLH039 regulators involved in the growth effect.

Recently, another example of a plant growth-promoting compound, melatonin, which is a well-known animal hormone, was also found to improve growth of soybean plants (Wei *et al*, 2015). The existence of plant growth-promoting compounds opens the door for an exciting novel and direct approach to identify genes involved in leaf growth regulation. Chemical screens for compounds that are able to increase plant growth will be an interesting research field to further explore.

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------ THE END ------

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Publications

- Van Dingenen J, De Milde L, Vermeersch M, Maleux K, De Rycke R, De Bruyne M, Storme V, Gonzalez N, Dhondt S, Inzé D (2016) Chloroplasts Are Central Players in Sugar-Induced Leaf Growth. *Plant Physiol,* 171: 590-605.

- Vercruyssen L, Tognetti VB, Gonzalez N, Van Dingenen J, De Milde L, Bielach A, De Rycke R, Van Breusegem F, Inze D. (2015) GROWTH REGULATING FACTOR5 stimulates Arabidopsis chloroplast division, photosynthesis, and leaf longevity. *Plant Physiol*, 167:817-832.

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- Van Dingenen J*,* Antoniou C*, Filippou P, Gonzalez N, Dhondt S, Fotopoulos V, Inzé D. Strobilurins as yield-enhancing compounds: How Stroby regulates Arabidopsis plant growth. *Research article in preparation.*

- Blomme J*, Van Dingenen J*, Gonzalez N, Dhondt S, Inzé D. Plants Grow with a Little Help of their Organelle Friends. *Review in preparation*.

* Shared first authorship

Patent

"Means and methods to increase plant yield" by Dirk Inzé, Judith Van Dingenen, Chrystalla Antoniou and Vasileios Fotopoulos filled on 22/09/2015. EP 15186304.0.

Posters and presentations

- FEBS Plant Organellar Signaling workshop, September 16-20th, 2015, Primosten, Croatia. Selected presentation: "Sucrose-induced stimulation of early leaf growth"

- Plant Organ Growth Symposium, March 10-12th , 2015, Ghent, Belgium. *Poster*: "Sucrose promotes early leaf growth by stimulating cell proliferation, delaying chloroplast differentiation and restricting chloroplast division"

- 5th European Plant Science Retreat (EPSR) Conference, July 2013, Ghent, Belgium. *Poster:* "Leaf Growth and Sugars: With or Without You"

Teaching and supervising

Guiding Master project of Martha Elena Ibarra-Vaele “Sucrose-induced leaf growth stimulation”, 2015

Guiding Master dissertation of Isa Cools “Study of growth promoting effects of strobilurins in Arabidopsis”, 2014-2015

Guiding Master project of Gaëlle Houthaeve “Characterization of putative sugar-dependent leaf growth regulators”, 2015

Guiding Practicum ‘Plant Physiology’ , October 2014

Guiding Bachelor project of Vanheule Greet, Mortier Cindy and Descamps Elke, 2013