The sucrose storage metabolism regulates carbohydrate supply and growth independently from the circadian clock in barley

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Tag der mündlichen Prüfung:

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist.

Die Dissertation habe ich in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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1 Zusammenfassung

Die Zielsetzung dieser Arbeit ist zu verstehen, wie die Kohlenhydratversorgung in Gerste während der Nacht gesteuert wird und wie diese Steuerung das vegetative Wachstum beeinflusst. Die Arbeit war von der Tatsache motiviert, dass die Kohlenhydratversorung für das Wachstum in Arabidopsis während der Nacht aus Stärke stammt und streng durch die circadiane Uhr gesteuert ist. Da Gerste überwiegend Saccharose als Kohlenhydratquelle für die Nacht verwendet aber keine Stärke, war die Regulation der Kohlenhydratversorgung in Gerste für das Wachstum in der Nacht unbekannt. Ich konnte zeigen, dass die Kohlenhydratversorgung aus Saccharose, im Gegensatz zur Versorgung aus Stärke, nicht durch die circadiane Uhr gesteuert ist. Das bedeutet, dass eine schwerwiegende Fehlfunktion der circadianen Uhr in der "*early flowering 3*" (*elf3*)-Mutante keine Verminderung der Biomasse in Gerste aber in Arabidopsis hervorruft. Das zeigt, dass die circadiane Uhr wichtig für die Wuchsleistung in Stärke speichernden Arabidopsispflanzen aber nicht in Saccharose speichernden Gerstenpflanzen ist. Stattdessen wird die Abnahme an Saccharose in der Nacht durch das Transportenzym „SUCROSE TRANSPORTER 2" (SUT2) katalysiert und folgt einer konzentrationsabhängigen Kinetik, welche die Abnahme an Saccharose an die Länge der Nacht anpasst ohne dass die circadiane Uhr für die Regulation notwendig ist. Meine Ergebnisse zeigen die physiologischen Unterschiede im nächtlichen Metabolismus zwischen Arten auf, die Saccharose oder Stärke als Kohlenhydratquelle für die Nacht speichern. Diese Ergebnisse erklären, warum die Kohlenhydratversorgung von Saccharose für Wachstum in der Nacht nicht von der circadianen Uhr kontrolliert wird und deswegen nicht gegen kühle Nachttemperaturen kompensiert wird. Das ist gegensätzlich zur Kohlenhydratversorgung aus Stärke. Folglich ist die Kohlenhydratversorgung für

Wachstum in Gerste, jedoch nicht in Arabidopsis, von der Nachttemperatur aber nicht der circadianen Uhr abhängig, obwohl die circadiane Uhr in beiden Arten konserviert ist.

2 Preface: Review paper

2.1 Connections between circadian clocks and carbon metabolism reveal species-specific effects on growth control

This preface was published as review article as: Müller, L.M., von Korff, M. & Davis, S.J., 2014. Connections between circadian clocks and carbon metabolism reveal species-specific effects on growth control. *Journal of Experimental Botany*.

Abstract

The plant circadian system exists in a framework of rhythmic metabolism. Much has been learned about the transcriptional machinery that generates the clock rhythm. Interestingly, these components are largely conserved between monocots and dicots, but key differences in physiological and developmental output processes have been found. How the clock coordinates carbon metabolism to drive plant growth performance is described with a focus on starch breakdown in Arabidopsis. It is proposed that clock effects on plant growth and fitness are more complex than just matching internal with external rhythms. Interesting recent findings support that the products of photosynthesis, probably sucrose, in turn feed back to the clock to set its rhythm. In this way, the clock both controls

and is controlled by carbon fluxes. This has an interesting connection to stress signaling and water-use efficiency, and it is now known that the clock and abscisic acid pathways are reciprocally coordinated. These processes converge to drive growth in a species-specific context such that predictions from the Arabidopsis model to other species can be restricted. This has been seen from phenotypic growth studies that revealed that dicot shoot growth is rhythmic whereas monocot shoot growth is continuous. Taken together, emerging evidence suggests reciprocal interactions between metabolism, the circadian clock and stress signaling to control growth and fitness in Arabidopsis, but transferability to other species is not always possible due to species-specific effects.

Introduction

The rotation of the earth causes repetitive changes between day and night that are reflected in diurnal cycles of temperature and light. Plants have to adapt to these consistent and predictable environmental conditions. The circadian clock, an endogenous timing mechanism with a periodicity of about 24h, is a key regulator in this adaptive process. It allows measurement of time independently from day–night phases and enables the plant to trigger metabolism and stress responses at particular time points of the day. In this manner, plants can regulate physiology in an anticipatory manner. This exists as a process that is thought to maximize fitness and growth performance, as well as yield and reproductive success. This review discusses the reciprocal regulatory interaction between the circadian clock and carbon metabolism and its impact on stress signaling and water use. This collectively creates plant performance.

The circadian clock of plants

The transcriptional-translational clock of plants is a set of proteins that form an interconnected feedback system with multiple loops. These provide temporal information to organisms to coordinate developmental and metabolic responses in coincidence with the environment (Sanchez et al. 2011; Farré & Weise 2012; Bujdoso & Davis 2013; Kinmonth-Schultz et al. 2013; Staiger et al. 2013). In a process called entrainment, external cues like temperature and light are used as inputs to set the

circadian clock every morning and create synchrony between internal rhythmicity of the oscillator and external rhythmicity of the environment. Under conditions of continuous light and temperature, such constant environmental inputs reveal the internal rhythmicity of the oscillator. This internal rhythm is subject to extensive natural variation both within and between species, and this is reported to influence fitness and performance of the plant (Dodd et al. 2005; Boikoglou et al. 2011; Edwards et al. 2011; Edwards et al. 2012; Izawa et al. 2011; Yerushalmi et al. 2011; Farré 2012; Farré & Weise 2012; Faure et al. 2012; Matsubara et al. 2012; Weller et al. 2012; Anwer & Davis 2013; Habte et al. 2014; Kinmonth-Schultz et al. 2013; Sulpice et al. 2014). As a consequence, the circadian clock is considered a key regulator of plant physiology and adaptation to different geographic environments.

The Arabidopsis model

In the dicotyledonous model plant *Arabidopsis thaliana*, the shoot circadian clock consists of multiple, interlocking feedback loops with predominant elements of negative regulation. The central loop consists of two partially redundant MYB transcription factors CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) as well as the PSEUDO RESPONSE REGULATOR PRR1 (also known as TIMING OF CAB EXPRESSION 1, TOC1). The morning-expressed proteins CCA1 and LHY repress TOC1 by direct binding to its promoter, leading to TOC1 accumulation in the evening and, in turn, transcriptional repression of CCA1/LHY by TOC1 (Pokhilko et al. 2012; Pokhilko et al. 2013). This core clock is considered crucial for rhythmic maintenance as the *cca1/lhy/toc1* triple mutant is described to be arrhythmic (Ding et al. 2007).

Associated to this central loop, a morning-phased loop comprising the dawn-phased pseudo-response regulators (PRRs) PRR7 and PRR9 that repress CCA1/LHY expression during daylight (Pokhilko et al. 2012; Bujdoso & Davis 2013). Feedback to the PRRs is established by the evening complex, which is composed of EARLY FLOWERING 3 (ELF3) (Dixon et al. 2011; Herrero et al. 2012), a light-signal-mediator required for the oscillator to cycle (McWatters et al. 2000; Kolmos et al. 2011; Herrero et al. 2012), the ligand ELF4 and the DNA-binding protein LUX ARRYTHMO (LUX) (Dixon et al. 2011; Helfer et al. 2011; Herrero & Davis 2012). Together, this evening complex represses PRR9 and PRR7 to indirectly activate CCA1/LHY (Kolmos & Davis 2007; Pokhilko et al. 2012; Bujdoso & Davis 2013;

Herrero & Davis 2012). They are themselves evening expressed because of repression by CCA1 and LHY (Lu et al. 2012). Another associated loop is evening phased; here, TOC1 protein is in autoregulative feedback with GIGANTEA (GI) and ZEITLUPE (ZTL). The manner of this regulation includes post-translational modifications and proteolysis that results in transcriptional regulation within this feedback loop (Pokhilko et al. 2012). The specific details of how GI fits into this regulatory loop are not fully established. Based on this multiple interlocked feedback system, the oscillator generates continuous rhythms even under rapidly fluctuating conditions typical of weather patterns (Troein et al. 2009).

Transcripts and protein products of clock components cycle in repetitive, diurnal patterns. Most clock components are transcription factors that not only regulate each other in an interactive manner but also regulate other genes outside the clock loops themselves. These are called clock-output genes. Output genes often sit at core internodes of physiology and development and regulate biological processes like growth, metabolism, hormone, and stress signaling (Lu et al. 2005; Covington et al. 2008; Hanano et al. 2008; Dalchau et al. 2010; Sanchez et al. 2011; Farré & Weise 2012; Kinmonth-Schultz et al. 2013; Stitt & Zeeman 2012; Seaton et al. 2014). It has been estimated that around 30% of the whole Arabidopsis transcriptome follows anticipatory rhythms generated by the clock (Covington et al. 2008), which underpins the central importance of the circadian system (Davis & Millar 2001; Staiger et al. 2013).

Inference from cycling transcripts to diurnal physiological patterns is not necessarily straightforward. For example, the gene encoding glucan water dikinase (GWD, also termed SEX1), a key enzyme regulating nocturnal starch breakdown in leaves, revealed cycling transcription during the day, but constant protein abundance under free-running conditions (Lu et al. 2005). Interestingly, starch breakdown, the physiological trait regulated by GWD, cycled under constant conditions. Thus, post-translational modification of proteins can hamper direct conclusiveness from diurnal transcription patterns to physiological effects.

Orthology of clock components in higher plants

The angiosperm oscillator seems to be largely conserved in higher plants and between eudicot and monocot species. For example, Arabidopsis clock homologues with comparable patterns of transcript accumulation, with respect to peaking time and amplitude, were also identified in other eudicot and monocots species (Song et al. 2010; McClung 2013). Consequently, ectopic overexpression of rice OsTOC1 and OsZTL in Arabidopsis showed clock-related phenotypes comparable to overexpression of their Arabidopsis orthologs AtTOC1 and AtZTL (Murakami et al. 2007). Furthermore, OsTOC1 was able to partly rescue the Arabidopsis *toc1* null mutant (Murakami et al. 2007), implying a level of commonality within the circadian clockwork of higher plants. Work on the barley orthologous set of clock genes came to a similar conclusion (Campoli et al. 2012). It appears that there is significant overlap in the repertoire of clock genes between species (McClung 2013).

Reports describe a high degree of conserved genomic sequences and functional protein domains in barley and rice in comparison to Arabidopsis (Murakami et al. 2007; Campoli et al. 2012). In those two monocots, however, LHY constitutes the only ortholog to the Arabidopsis paralogs AtCCA1/AtLHY (Murakami et al. 2007; Campoli et al. 2012). In addition, the gene family of PRRs appears to present a paralogous relationship, suggesting independent duplication and evolution of the three ancestral PRRs in dicots and monocots (Takata et al. 2010). For example, Arabidopsis PRR9 and PRR5 are phylogenetically separate, and the phylogenetically associated monocot orthologs PRR95/59 do not clade with these dicot counterparts (Murakami et al. 2003; Campoli et al. 2012). The same holds true for AtPRR7 and AtPRR3, where both resemble the monocot genes PRR73/37 (Murakami et al. 2003; Campoli et al. 2012). Further, whereas the Arabidopsis PRRs display transcript peaks in the sequential order AtPRR9, 7, 5, 3 and TOC1 (Matsushika 2000), the PRRs of barley and rice display broad peaks over the day in the sequence PRR37/73, PRR59/95 and TOC1 (PRR1) (Murakami et al. 2007; Campoli et al. 2012). This finding raises a complex question: What are the roles of these paralogous genes in the monocotyledonous clock system? In addition, and in contrast to Arabidopsis, GI and TOC1 have been hypothesized as being in positive feedback in rice (Izawa et al. 2011). Nevertheless, remarkable similarities in architecture and function of circadian clocks in higher plants exist.

Plant performance at the physiological level

Circadian clock and photosynthesis

Plants benefit from circadian control of photosynthesis and physiology to achieve higher fitness (Dodd et al. 2005). In that work, higher chlorophyll content, higher carbon fixation and increased water-use efficiency was associated with synchrony between circadian clock period and day length. This correlated with both a doubling of plant biomass and higher survival rates in a competitive environment. Under a T-cycle of 20h with a fake day of 10h light/10h dark, the *toc1* mutant with a 20h period grew with higher performance than the *ztl* mutant with a 28h period. Conversely, the *ztl* mutant grew better than *toc1* under T-cycles of 28h. Thus, the authors observed that a match between the internal and the external period of plants increases plant performance, whereas a rhythmic mismatch reduces growth and survival (Dodd et al. 2005).

As a consequence, the study by Dodd et al. (2005) implies that circadian clocks properly timed to day/night cycles can reliably anticipate dusk and dawn to prepare photosynthesis and physiology in an anticipatory manner for the course of the upcoming day. However, this study is missing the 24h control to draw a general conclusion. Later work by Graf et al. (2010) demonstrated that *toc1* and *ztl* mutants grow best under 24h T-cycles, irrespective of a mismatch of the free-running period with the length of the day. They report that biomass of *toc1* and *ztl* mutants was highest under 24h growth conditions but not under conditions where the internal period of the oscillator matched the period of the T-cycle. Thus, effects of the circadian clock on plant performance are more complex than just matching the internal period of the oscillator with the length of the day. Instead, the authors suggested that the circadian control of starch degradation must not be overlooked in this context (Graf et al. 2010; Graf & Smith 2011).

It is plausible that the cooperative circadian control of anabolism and catabolism are decisive for high plant-growth performance. Indeed, an extensive transcriptional network was uncovered that linked clock and metabolic intersections in diurnally regulated gene expression that is seen for thousands of genes (Blasing et al. 2005). Extending this, Ni et al. (2009) reported in allopolyploids that

photosynthesis and starch metabolism were differentially regulated by the circadian clock, in comparison to their diploid parents. This led to superior growth in allopolyploids. In these allopolyploids, daytime expression of TOC1, GI and additional clock-output genes containing cis-elements associated to clock regulation were elevated relative to both parental diploids. This led to an alteration in circadian rhythmicity. This could be attributed to a reduction of transcription-activating methylation marks at the CCA1 and LHY promoters of the allopolyploids, leading to lower CCA1/LHY protein levels at noon (Ni et al. 2009). Thus, clock-output genes causative for higher chlorophyll content, as well as for starch metabolism and sugar transport, were expressed higher in the allotetraploids than their diploid parents. All this was associated with higher biomass in the polyploidy lines, which is one form of hybrid vigour (Ni et al. 2009). However, the extent of circadian effects on photosynthesis to drive growth performance has not been clearly resolved. For example, it has been reported that Arabidopsis wild-type plants grown under 28-h period cycles with mismatch to the internal clock period fixed more CO_2 as compared to 24-h cycles, but showed reduced biomass (Graf et al. 2010). CO_2 uptake alone does not appear as the single major factor to increase fitness and performance. It has been proposed that alterations of chlorophyll content happen in a scale that might be insignificant for CO_2 uptake (Jenkins et al. 1989; Sperling et al. 1997; Andersson et al. 2003; Graf et al. 2010). Nevertheless, rhythmicity and expression levels of CCA1/LHY and PRR7/PRR9 are capable of influencing plant growth performance by inducing and entraining primary metabolism (Fukushima et al. 2009; Graf et al. 2010; Lai et al. 2012).

Circadian clock and carbon supply at night

Apart from photosynthesis, mobilization of storage compounds is another factor known to contribute to plant growth performance (Sulpice et al. 2009). Graf et al. (2010) reported that in Arabidopsis starch degradation at night is controlled by CCA1/LHY. They concluded that this regulation is necessary to prevent sucrose starvation and growth penalties at night. Specifically, *cca1/lhy* double-mutant plants of Arabidopsis in 24-h T-cycles and wild-type plants in 28-h T-cycles depleted starch reserves prematurely and showed significantly reduced growth. This premature depletion of nocturnal depots led to activation of sucrose starvation-induced genes before dawn. As sucrose addition could

complement for the observed biomass reduction in otherwise starving plants, carbon shortage during the night was causative for reduced growth (Graf et al. 2010). Thus, the circadian clock via CCA1/LHY sets a maximum rate of carbon supply during the night that is adjusted in such a way that starch reserves last until the next morning.

It appears that a fixed rate of sucrose supply from starch cannot be overcome by increased demand. Consequently, in well-nourished plants, where carbon supply limits growth, CCA1/LHY directly regulates plant growth at night (Graf et al. 2010; Yazdanbakhsh et al. 2011). Short-period mutant phenotypes do not necessarily impose premature starch depot depletion. For example, the Arabidopsis *toc1* mutant is, like *cca1/lhy*, a short-period clock mutant, but it was not found to deplete starch reserves prematurely in 24-h T-cycles (Graf et al. 2010). Additionally the *ztl* long-period mutant did not delay starch exhaustion, as would have been predicted by its delayed-periodicity phenotype. This mutant was found to deplete starch levels in a similar manner as the wild type (Graf et al. 2010). Thus, avoidance of premature starch depletion at night appeared as direct effect of CCA1/LHY on respective clock output targets and not to the short-period phenotype itself. This provided further evidence that a match of circadian period with day length is not sufficient to generally explain higher growth performance in plants.

When Arabidopsis wild-type plants were measured for starch levels over 24-h T-cycles, it became clear that the linear rate of starch degradation varied dependent on the length of the night. This was true even when an unexpected early or late onset of the dark phase was encountered. Interestingly, in these experiments, depletion of starch depots was always timed to the onset of the next day (Graf et al. 2010). Even under skeleton days and nights, where a normal day or night is interrupted by a short dark/light phase that partly depletes or regenerates starch depots, starch levels were reliably depleted at the next dawn (Graf et al. 2010; Scialdone et al. 2013; Sulpice et al. 2014). This means that the starch-degradation rate, calculated as the negative slope of starch content loss over time, must be tailored to the starch content present at the onset of night. This strongly indicates that both temporal information and information about starch content are integrated to ensure proper regulation (Graf &

Smith 2011). Obviously, the circadian clock could provide temporal estimation to predict the next dawn, but how plants determine starch content is less obvious.

Several recent modeling studies tackled the question how starch content could be integrated over time and how plants might be able to adjust nocturnal starch-degradation rate to fit experimental data of starch depletion under various conditions and treatments (Scialdone et al. 2013; Seaton et al. 2014). Based on chemical kinetic models, Scialdone et al. (2013) proposed that phosphoglucan water dikinase (PWD, also called GWD3) is a key player in this process. It is an enzyme that acts as a focal point to modulate flux through the starch-degradation pathway (Scialdone et al. 2013). PWD and its related enzyme GWD1 (glucan water dikinase) work as initial enzymes in the pathway to phosphorylate starch molecules in the chloroplastic granule and trigger its degradation (Smith et al. 2005). Phosphorylation on the granule surface is expected to open up the compacted starch molecules for easier access for further hydrolysis by β-amylases and isoamylase 3. Loss of PWD function as well as mutations in major genes involved in the starch-degradation pathway such as *lsf1* (*like sex4 1*)and *sex4* (*starch excess 4*) in Arabidopsis led to reduced overall starch-degradation rate and caused these mutant plants to retain higher amounts of starch at the end of the night than wild-type plants (Scialdone et al. 2013). Nevertheless, after a sudden shift from a 12h/12h to an 8h/16h light/dark cycle to impose an early unexpected night, all tested mutant plants impaired in starch degradation were, with an exception of *pwd*, able to adjust and lower starch-degradation rate to meet conditions of an early onset of night. The *pwd* mutant retained a higher starch-degradation rate not different to the entrained 12h light conditions (Scialdone et al. 2013). Thus, PWD function appears to be required to adapt nocturnal starch-degradation rate to unexpected onset of the night. As PWD is involved in initiating the phosphorylation status of the starch granules to trigger starch degradation, the starch phosphorylation status was considered a promising candidate to store starch-content information (Scialdone et al. 2013). Indeed, an experiment following starch phosphorylation status over the day found diurnally cycling phosphorylation that could follow starch content during the day (Scialdone et al. 2013). Thus, PWD appears as the hub to control starch-content-dependent flux through the starch-degradation pathway.

Entrainment and gating

The interplay between the circadian clock and metabolism is bidirectional. Several indications exist that metabolites feedback to the oscillator to adjust the circadian clock. Based on computational modeling, GIGANTEA (GI) was identified as a mediator of sucrose-dependent changes on rhythmicity of the shoot clock (Dalchau et al. 2011). Further experiments confirmed that sucrose application acted as a Zeitgeber (time giver) to generate and set circadian rhythms in continuous darkness. A different study reported that the circadian clock in Arabidopsis roots is a slave of the shoot clock and is set by a photosynthesis-related signal from the shoot, which was proposed to be sucrose (James et al. 2008). Consequently, sucrose feeding to the root altered clock rhythmicity. Related to that, Haydon et al. (2013) reported that photosynthesis-derived sucrose entrains the circadian clock of Arabidopsis seedlings (Haydon et al. 2013). Peaking of sucrose levels from photosynthesis in the morning defined a 'metabolic dawn', which could be related to decreased PRR7 expression. Thus, peaking of leaf sucrose in the morning repressed PRR7 expression, which in turn, mitigates repression of CCA1 transcription to set the clock. As a consequence, expression of the clock component CCA1 advanced in dependance on the metabolic status. Taken together, metabolic cues through sucrose direct the resetting of the clock at dawn dependent on metabolic status. This means that sucrose is signal and metabolite at the same time.

In addition to carbon metabolism, water status is a further determinant for plant growth. The plant hormone abscisic acid (ABA) is involved in responses to water shortage, and the ABA-signaling pathway is controlled by the circadian clock (Hanano et al. 2006; Covington et al. 2008; Sanchez et al. 2011). This causality generates a phenomenon termed gating, where physiological responses differ to comparable stimuli during the course of the day. For example, application of ABA in the morning revealed a higher affect on stomatal closure than application in the afternoon (Correia et al. 1995). This indicated that responses to ABA are gated by the circadian clock.

A prominent example of ABA entrainment on the oscillator and gating was given by Legnaioli et al. (2009). This study showed that TOC1 can directly bind to the promoter of one putative ABA receptor ABAR/CHL5/GUN5, where TOC1 negatively regulated ABAR expression in a periodic manner.

Conversely, ABAR was found to positively regulate TOC1 transcription, linking ABA perception to the circadian clock. Notable here is that hormone synthesis and perception mutants in the ABA pathway have clock periodicity phenotypes (Hanano et al. 2006). Taken together, sensitivity to ABA is described as a clock-gated process, which, in turn, acts to fine tune the speed of the generated oscillations.

Administration of ABA during the day, but not during the night, led to immediate induction of TOC1 transcription (Legnaioli et al. 2009). In addition, lower levels of ABAR transcripts were linked to higher stomatal conductance and water loss via the leaf surface area, indicating that repression of ABAR reduces ABA-mediated stomata closure and savings of water to reduce wilting (Legnaioli et al. 2009). Thus, reciprocal regulation of TOC1 and ABAR might function as a fine-tuned switch to modulate plant sensitivity to ABA, which is likely to affect water-use efficiency and plant performance. Interestingly, a recent study in barley has shown that osmotic stress applied to the roots altered the expression of clock genes in the shoot, suggesting that plant water status can feed back into the clock (Habte et al. 2014). In addition, mutations in the barley clock orthologues Ppd-H1 (HvPRR37) and HvELF3 affected the expression of stress-genes, demonstrating that the clock also controls stress responses in monocotyledonous plants. Thus, reciprocal feedback between stress signaling and the clock exists in both monocot and eudicot species, which apparently adapts the clock to acute stress to better regulate future physiological responses. This is likely to increase plant fitness.

Circadian control of ecophysiological traits has been shown to impact on plant growth performance. Edwards et al. (2011) reported that natural genetic variation in the Brassica rapa circadian clock is associated to phenotypic variation in traits related to photosynthesis. Photosynthesis rate, stomatal conductance and transpiration rate were significantly correlated with circadian period, indicating that the circadian clock might regulate photosynthesis and related traits in a way to increase resource use efficiency of water and light. The authors then showed that genetic variation exists for these correlative events (Edwards et al. 2011). Together, one can wonder if the extensive variation seen in circadian periodicity is in part physiologically selected based on water-use traits.

Plant performance at the phenotypical level

Rhythmicity of expansion growth and clock effects on carbon allocation and plant architecture

Even though structure and function of the circadian system might be widely conserved in higher plants, regulation of clock-output traits is not necessarily conserved. For example, the circadian clock has been shown to be one of the major signaling pathways regulating growth rates in plants (Walter et al. 2009). In dicotyledonous species, two different shoot growth types were found and they differ in peaking of growth rate, which is either at dawn or dusk (Walter et al. 2009). Furthermore, shoot growth of several dicot species remained rhythmic under conditions of continuous temperature and light, proving the circadian clock to control growth processes in the dicot shoot (Walter et al. 2009). In contrast, however, examined monocots displayed constant growth rates under continuous temperature and light (Walter et al. 2009; Poire et al. 2010) (Illustration 1). These discrepancies between dicot and monocot studies indicate that growth in the monocot shoot is driven by temperature cycles instead of the circadian clock (Walter et al. 2009; Poire et al. 2010). Such a difference in growth control questions if the clock oscillator has the same importance to regulate physiology, metabolism and growth in monocots as it does in dicots.

In Arabidopsis, shoot growth peaks towards the end of the night in a clock-controlled manner. In other dicot species, shoot growth is also rhythmic, but a second growth type exists where growth peaks at the beginning of the night (Walter et al. 2009). Monocot shoot growth largely follows diurnal temperature cycles over endogenous rhythms (Poire et al. 2010). There is no obvious relationship here when considering root growth. For example, rice roots grew, like Arabidopsis roots, rhythmically under constant darkness (Iijima & Matsushita 2011). Conversely, roots of the dicot plant tobacco grew in a linear pattern under constant light conditions while the shoot extends rhythmically (Nagel et al. 2006; Poire et al. 2010). As a consequence, these findings indicate that circadian regulation of growth in shoot and root appears to be species and/or organ specific.

Differences in morphology between monocot and dicot plants have been hypothesized as one evolutionary reason to explain the basis for such contrasting growth phenotypes (Walter et al. 2009; Ruts et al. 2012b). The monocot shoot meristem is located in the inner bundle sheath and can be more protected from the environment. This is in contrast to dicot species where the shoot meristem is placed in the leaf and directly exposed to the environment (Ruts et al. 2012b). Thus, clock effects on growth physiology might be questionable for knowledge transfer from Arabidopsis to other dicot and monocot species. It appears that species-specific investigation has to be undertaken. In addition, differences in root and shoot growth within the same species underline the importance to separately consider organ specific clocks.

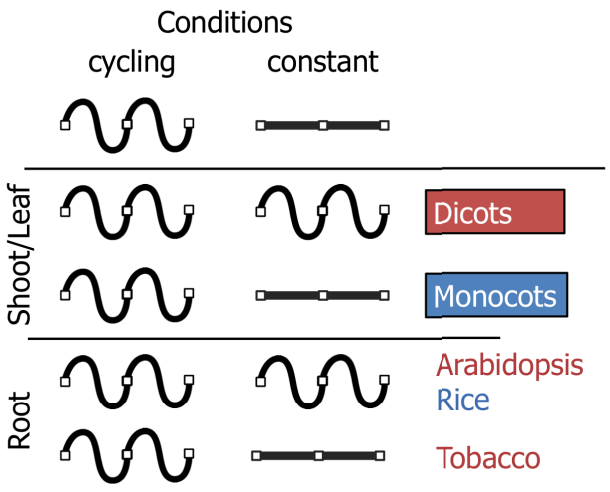
Apart from diurnal control of growth rates, the circadian clock has also been implicated to shape plant morphology. Ruts et al. (2012) reported that Arabidopsis clock mutants reveal aberrant shoot and root phenotypes. Disruption of the clock in the *CCA1-overexpressor* and the *prr5,7,9*-triple mutant significantly reduced rosette leaf size and suppressed lateral root formation. The authors attributed their observation to altered carbon allocation between shoot/root and growth/storage in clock mutants, compared to wild-type plants (Ruts et al. 2012a).

Circadian clock and direction of growth

Yazdanbakhsh et al. (2010) reported that growth can be shifted from the night into the day when clock genes are mutated. This study analyzed rhythmicity of root growth in Arabidopsis clock mutants

Illustration 1: Species-specific growth control by circadian oscillations.

For explanations and references see text. Cycling conditions refer to cycles in temperature and light, constant conditions refer to constant temperature and light.



and found that *elf3*, which is arrhythmic and displays an oscillator arrested at dusk (McWatters et al. 2000; Kolmos et al. 2011), lacked the ability to repress root growth during the day nor to promote it at the end of a 24-h cycle (Yazdanbakhsh et al. 2011). This shift of root growth from the night into the day appeared independently of carbohydrate metabolism as it occurred under absence and presence of sucrose fed to the roots. Instead, rhythmic root growth in the *elf3*-mutant under light/dark cycles was rapidly lost under constant light conditions (Yazdanbakhsh et al. 2011). These findings are reminiscent to Nozue et al. (2007), who reported that functional ELF3 was required to inhibit hypocotyl extension of Arabidopsis seedlings during the light and to release it at night. Likewise, ELF3 appears to repress Arabidopsis root growth during the day and direct it towards the end of the night. Consequently, light signaling, instead of carbon metabolism, appears to drive ELF3 action in respect to growth regulation in Arabidopsis root and shoot (Nozue et al. 2007; Nusinow et al. 2011; Yazdanbakhsh et al. 2011). This is potentially through a cascade of clock regulation on the transcription factors PIF4 and PIF5 and the subsequent role of these factors in controlling auxin-driven growth (Nusinow et al. 2011; Hornitschek et al. 2012). Such conclusions for ELF3 as a major hub controlling hormone driven growth are consistent with it acting as a mediator between light signaling and the circadian clock (McWatters et al. 2000; Kolmos et al. 2011).

It appears that regulation of growth by the circadian clock acts through at least two different layers, with one comprising carbon metabolism and the other light signaling (Nozue et al. 2007; Yazdanbakhsh et al. 2011). Interestingly here, the clock coordinates both processes. For the first, the clock components CCA1/LHY have been shown to define the rate of starch breakdown to ensure that carbon supply lasts until the end of the night (Graf et al. 2010). This avoids adverse effects on growth by sucrose starvation (Graf & Smith 2011). For the latter, ELF3, as a clock component mediating rhythmic light signaling, acts to repress growth during light and promote growth at the end of dark (Nozue et al. 2007; Nusinow et al. 2011). This regulatory pattern would be consistent with physiological and ecological benefits of coordinating growth control at several developmental checkpoints.

Repression of growth during the day favors the accumulation of carbohydrate reserves for the night. Direction of growth towards the end of the night would reduce the risk of excessive growth during the first half of the night that would leave the plant without carbon resources before the next sunrise. Furthermore, growth at the end of the night would coincide growth with maximum water availability. Thus, clock regulation of growth appears to coordinate signaling and metabolic pathways to ensure high growth performance during darkness, when the plant has to properly manage metabolic resources, as they cannot be refilled by light-driven carbon fixation until the next morning. However, as two different rhythmic growth types exist in dicot plants and as examined monocot plants do not show rhythms for shoot growth (Walter et al. 2009), one has to be very careful with generalization of the Arabidopsis model.

The Arabidopsis example shows that the circadian clock is interlinked with both light signaling and carbon metabolism to regulate growth performance. This does not necessarily apply to other species, and might not be general. What is curious here is that mutations in the evening clock components EAM8 and EAM10 in barley, which are respective orthologs of Arabidopsis ELF3 and LUX (Faure et al. 2012; Campoli et al. 2013), do not display dramatic growth phenotypes in the shoot. Taken together, it appears that the role of the clock in driving growth depends on the species under consideration. As such, much is to be done to translate Arabidopsis circadian findings to improve agricultural gains.

Summary and outlook

The Arabidopsis circadian transcriptome displays a particular overrepresentation of genes involved in key physiological pathways of hormone and stress signaling, growth, and development (reviewed in Davis and Millar, 2001; Sanchez-Villarreal et al., 2013; Staiger et al., 2013). Conclusions from circadian transcription to metabolic outputs and growth are not generally straightforward due to post-transcriptional and post-translational modifications (Baerenfaller et al. 2012). In addition, species-specific layers of regulation are noted. Related to that, it appears that clock benefits for growth and fitness are more complex than just a simple match between the internal periodicity of a plant with the

day/night cycle. It has been suggested that anticipated preparation of photosynthesis is causative for higher growth performance of Arabidopsis plants (Dodd et al. 2005), but this was only true for unnatural day-length conditions of 20- or 28-h T cycles but not the 24-h T-cycle (Graf et al. 2010). It appears likely that the 'dark side' of the day has to be considered to explain differences in plant growth performance between wild-type plants and respective clock mutants (Graf & Smith 2011).

Growth rates in Arabidopsis peak at the end of the night when the plants are not photosynthetically capable of capturing photons to fix carbon. Thus, mobilization and allocation of carbohydrate reserves under night must be considered in the explanation if a simple matching of day/night cycles with internal period of the clock mutant is sufficient to explain increased growth performance in Arabidopsis. In addition, *elf3*-mutant plants that show a severe disruption of clock oscillations of transcription were, in the case of barley, pea, and lentil, kept in breeding. All of these crops were selected and bred as high-yielding cultivars during the migration away from the equator to more northern and temperate latitudes (Faure et al. 2012; Weller et al. 2012). Quantitative variation at ELF3 has similarly been reported in rice (Matsubara et al. 2012). Understanding the selection pressures that led to clock variation in crops is undoubtedly to include roles for the clock in control of water use, temperature response, and processing stresses to create yield stability.

The circadian clock appears to be both master and a slave in regulation of carbon metabolism. Metabolic status is integrated into the clock during the day, leading to close coherence of oscillations with carbon metabolism. Later at night, this precise entrainment to metabolic status from the day appears beneficial for regulation of carbohydrate catabolism at night, where the plant has to feed from resources gained over the day. As a consequence, the circadian clock is both controlling and being controlled by carbon metabolism (Sanchez et al. 2011; Farré & Weise 2012; Haydon et al. 2013). This bimodal interaction leads to the phenomenon of entrainment by and gating of environment-related stimuli. This is likely to ensure coherence between environmental signals and the clock to properly adjust clock output pathways that modulate plant performance.

Clock genes appear to be substantially conserved across species, but their regulatory effects on fitness and growth performance are not as obviously conserved. Dicotyledonous and monocotyledonous

species have contradicting patterns in clock control of growth rhythmicity. This is evident from the finding that the circadian clock controls dicot shoot growth whereas this does not appear to be true in monocots. Secondly, monocots store much of their carbohydrates in soluble forms instead of starch (Smith et al. 2005). This difference in nocturnal carbohydrate mobilization is another intrinsic metabolic distinction between species that might explain fundamental differences in regulation of growth performance by the circadian clock between monocot and eudicot species.

3 Introduction

Plants as photoautotrophic organisms harvest light energy during the day to drive growth, development and reproduction. During the night under the absence of light, where no photosynthesis is possible, plants need an alternative energy source. For this reason, Arabidopsis plants partition some of the assimilates gained by photosynthesis during the day for deposition and build-up starch stores that are gradually consumed during the following night. Arabidopsis mutants that do not use their starch depots grow dwarf (Smith & Stitt 2007). As a consequence, break-down of storage starch at night is an important trait for plant growth in Arabidopsis as it provides energy in the absence of light.

Besides the ability to partition and utilize storage starch, the temporal regulation of starch degradation during the night is important for growth in Arabidopsis. Several studies have demonstrated that the better the carbohydrate supply is adapted to the length of the night, the higher is the plant productivity (Graf et al. 2010; Stitt & Zeeman 2012; Graf & Smith 2011). Thus, the temporal control of starch degradation during the night is an important component of efficient growth. In Arabidopsis, the circadian clock programs the starch degradation rate at night in such a way that reserves do not exhaust before the end of the night but in coincidence with the onset of the next day (Graf et al. 2010). This tight regulation of starch degradation and its flexibility to adapt to unexpected early and late nights requires continuous quantification of starch reserves during the night and the use of the circadian clock to estimate the time left to the next morning (Scialdone et al. 2013; Seaton et al. 2014). Both factors, the time left to next morning and the current starch abundance, are proposed to be integrated by the Arabidopsis plant to set the degradation rate that times depletion of starch reserves to coincide with the end of the night (Scialdone et al. 2013). Consequently, the correct

anticipation of the onset of the next day by the circadian clock, the continuous quantification of starch content over the day and the capability to flexibly adjust starch degradation rates to the length of the night are key traits for efficient growth in Arabidopsis. However, it is unknown if the circadian control of starch degradation during the night is conserved in other plant species, particularly in important crop plants. In addition, relatively little is known about the significance of the starch storage metabolism for growth in plant species other than Arabidopsis. Not all plants store starch as carbohydrate source for the night (Smith et al. 2005). Especially grasses accumulate little or no starch in the leaf during the day (Pollock & Cairns 1991). For example, barley, rice and wheat predominantly store sucrose instead of starch during vegetative growth in the leaf (Sicher et al. 1984; Hirose et al. 2013; Trevanion 2002). However, the contribution of the starch and the sucrose metabolism to the carbohydrate supply for growth at night in these species is not understood. In addition, it is unknown how the sucrose storage metabolism is regulated during the night and if the circadian clock is involved in its temporal control.

Homologues of circadian clock genes from Arabidopsis were identified in the monocot species barley (Hv) and rice (Os) (Song et al. 2010; Murakami et al. 2007; Campoli et al. 2012). Despite small structural differences of the monocot oscillator (Campoli et al. 2012; Takata et al. 2010), the function of clock genes to maintain self-sustaining rhythmicity is conserved between Arabidopsis and the grasses. Functional studies in rice and barley have demonstrated that the monocot circadian clock is, as in Arabidopsis, comprised of multiple interconnected regulatory loops of transcriptional activators and repressors which coordinate transcription and physiological responses to the appropriate time of the day (Hsu & Harmer 2013; Hsu et al. 2013). Loss of the clock component EARLY FLOWERING 3 (ELF3) causes conditional dysfunction of the oscillator in barley and Arabidopsis as the *elf3*-mutant of both species is arrhythmic in constant light but cycling in constant dark (Hicks 1996; Faure et al. 2012; Deng et al. 2015). This causes advanced expression of clock genes in diel cycles and strong developmental phenotypes like early flowering in both species (Hicks et al. 1996; Faure et al. 2012). As a consequence, all of the current knowledge suggests that the monocot circadian clock in barley and rice is structurally (Campoli et al. 2012) and functionally (Filichkin et al. 2011) similar to the Arabidopsis oscillator.

4 Objectives and summary

The objective of my thesis is to understand how carbohydrate supply is regulated in barley during the night and how this control affects vegetative growth. The work was motivated by the fact that carbohydrate supply and growth in Arabidopsis at night is fueled by starch and is tightly controlled by the circadian clock. As barley predominantly uses sucrose as carbohydrate source for the night but not starch, the regulation of nocturnal carbohydrate supply for growth was unknown in barley. I could demonstrate that nocturnal carbohydrate supply from sucrose was not controlled by the circadian clock, in contrast to carbohydrate supply from starch. As a consequence, a severe dysfunction of the circadian clock in *elf3*-mutant plants did not reduce biomass in barley, but in Arabidopsis. This demonstrated that the circadian clock is important for growth in starch-storing Arabidopsis but not the sucrose storing specie barley. Instead, depletion of storage sucrose at night is catalyzed by SUCROSE TRANSPORTER 2 (SUT2) and follows a concentration dependent kinetics that adjusts the depletion rate to the length of the night independently from the circadian clock. My findings reveal the physiological differences in nocturnal metabolism between species that store sucrose or starch as carbohydrate source for the night. These findings explain why carbohydrate supply for growth from sucrose is not controlled by an internal program set by the circadian clock and, as a consequence, not compensated against cool night temperatures. This is opposite to carbohydrate supply from starch. Thus, carbohydrate supply for growth in barley but not in Arabidopsis is driven by night temperature but not the circadian clock, although the circadian clock is conserved between both species.

5 Results

5.1 The sucrose storage metabolism in barley

First, I analyzed the composition of storage carbohydrates in the leaf of barley seedlings during vegetative growth. At the end of an 8h light period, 60% of the total storage carbohydrates was stored as sucrose, 20% as fructans and 15% as starch while fructose and glucose were hardly accumulated (Illustration 2 top left). During the following night all stores of sucrose, fructan and starch were consumed (Illustration 2 top left). This indicated that the carbohydrate metabolism in barley at night is multifaceted but dominated by sucrose. Arabidopsis on the other hand almost exclusively stored and depleted starch as carbohydrate source for the night (Illustration 2 top center). This demonstrated that the carbohydrate metabolism in Arabidopsis is dominated by starch. These differences in carbohydrate storage between barley and Arabidopsis were also reflected in the ratio of sucrose and starch present at the end of the light period in both species (Illustration 2 top right). During an 8h light period, barley accumulated sucrose and starch simultaneously and depleted them close to exhaustion at the end of the night (Illustration 2 bottom left). Levels of sucrose and starch 24h after dawn were comparable to those at dawn (Illustration 2 bottom left). In contrast, Arabidopsis almost exclusively build up starch reserves during the day and depleted it at night (Illustration 2 bottom right).

The timing of sucrose and starch exhaustion precisely to the end of the dark period prompted me to investigate if the depletion of sucrose and starch was controlled by the circadian clock in barley. Therefore, I measured sucrose and starch levels during the night in barley *elf3*-mutant plants (*hvelf3*)

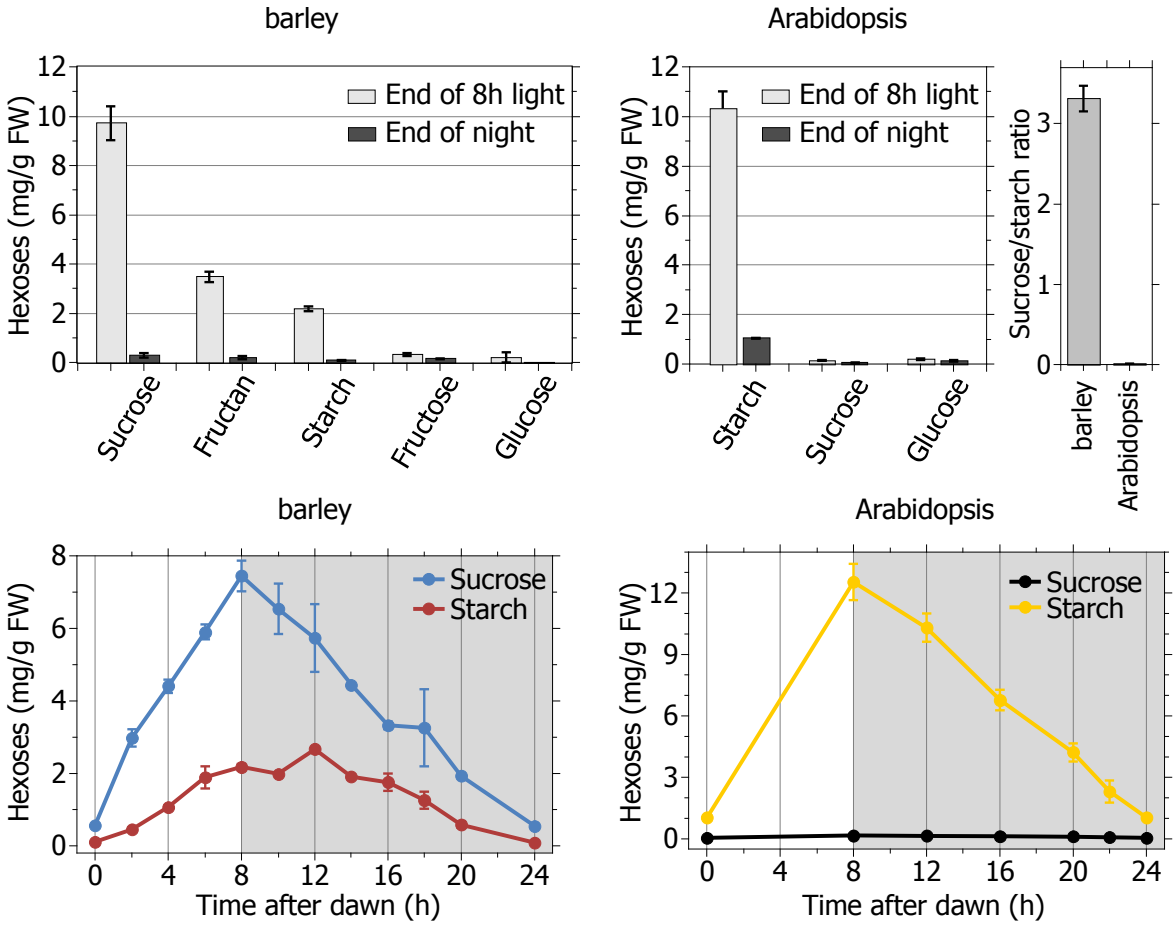


Illustration 2: Sucrose but not starch is the predominant storage carbohydrate in the barley leaf while starch is the almost exclusive storage carbohydrate in the Arabidopsis leaf.

Top left) Carbohydrate content in barley leaves and **(top center)** Arabidopsis leaves after an 8h light period and the end of the following night. **Top right)** Ratio of sucrose to starch in barley and Arabidopsis leaves at the end of the light period. **Bottom left)** Diel leaf pattern of sucrose and starch in 8h light/16h dark cycles in barley and **(bottom right)** Arabidopsis**.** *Error bars are standard deviation of the mean throughout. n=2-4 per time point.*

that are defective in the circadian clock (Faure et al. 2012). Depletion of transitory sucrose did not differ between *hvelf3* and wild type plants during the night (Illustration 3 top left, center left). This demonstrated that the dominant storage carbohydrate in barley, sucrose, depleted independently from the clock component ELF3 during the night. In contrast, the barley *elf3*-mutant degraded storage starch faster than wild type so that starch reserves depleted earlier (Illustration 3 top right, center right). This deviation of starch degradation from the wild type pattern did not alter the sucrose

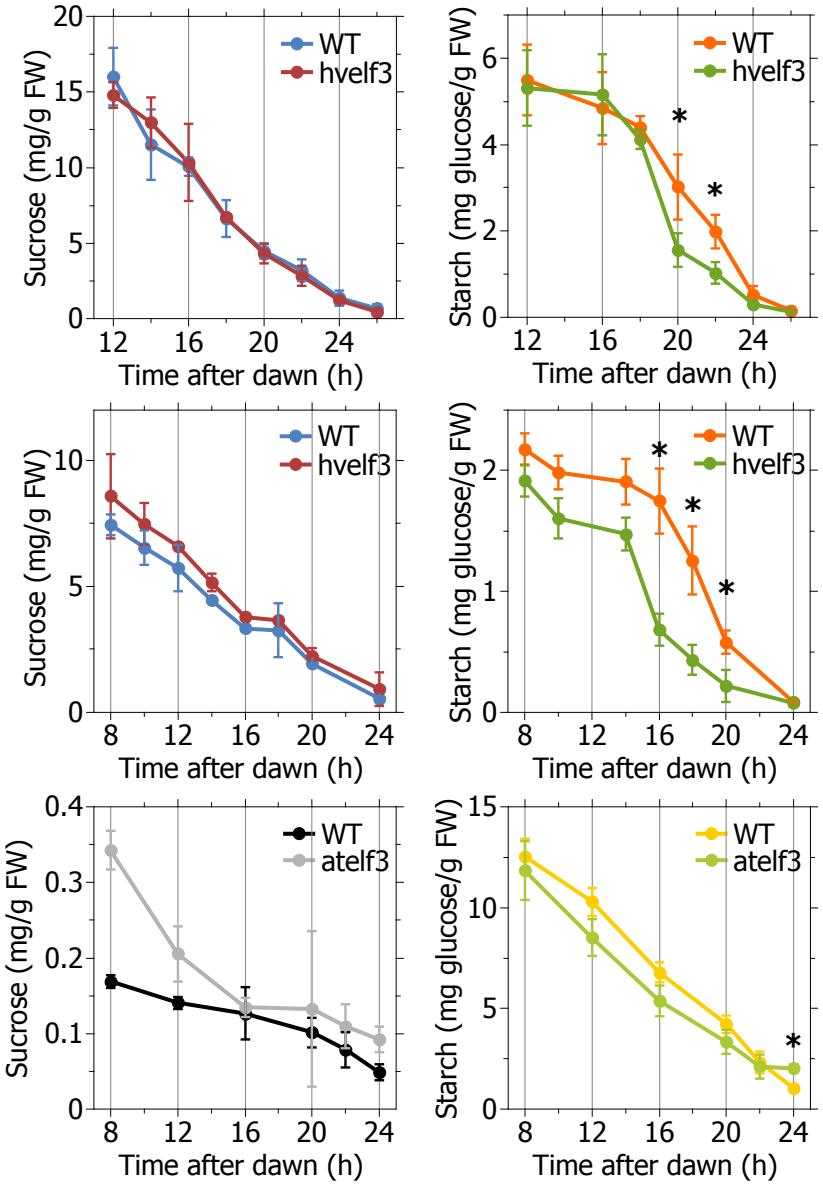


Illustration 3: The circadian clock controls carbohydrate supply from transitory starch but not transitory sucrose during the night.

Top left) Sucrose and **(top right)** starch levels in barley elf3-mutant and wild type plants during the night grown in 12h light/12h dark photoperiods. **Center left)** Sucrose and **(center right)** starch levels in barley elf3-mutant and wild type plants during the night grown in 8h light/16h dark photoperiods. **Bottom left)** Sucrose and **(bottom right)** starch levels in the Arabidopsis elf3-mutant and wild type plants during the night grown in 12h light/12h dark photoperiods. *Significant differences per time point (T-test, p≤0.05) are marked with *. Error bars are the standard deviation of the mean throughout. n=2-4 per time point.*

depletion in *hvelf3* at the end of the night (Illustration 3 top left, center left). This suggested that the premature starch degradation in *hvelf3* had negligible effects on the dominant sucrose metabolism. For comparison, I measured starch degradation in Arabidopsis wild type and *elf3*-mutant (*atelf3*) plants that almost exclusively store starch (Illustration 2 top center). Starch degradation in *atelf3* stopped prematurely relative to wild type before the night was over so that not all available starch was consumed during the night (Illustration 3 bottom right). This indicated that the dominant storage carbohydrate in Arabidopsis, starch, required the clock component ELF3 to maintain continuous degradation throughout the night to prevent a reduction of starch turnover in the last part of the night. Despite the differences in the starch degradation pattern between the *elf3*-mutant of barley and Arabidopsis, starch degradation in both species required temporal control by the clock component ELF3 for correct function. This suggested that the temporal control of starch degradation by the circadian clock is conserved between barley and Arabidopsis.

As the mutated ELF3 did not affect depletion of the dominant storage carbohydrate in barley but reduced turnover of the dominant storage carbohydrate in Arabidopsis, I hypothesized that the *elf3*-mutant of Arabidopsis but not of barley is short in carbohydrate supply at the end of the night and reduces metabolism and growth. In fact, Arabidopsis fresh weight and dry weight were significantly reduced in *elf3*-mutant plants compared to wild type (Illustration 4 left). By contrast, both fresh weight and dry weight were not significantly different between wild type and *elf3*-mutant plants in barley (Illustration 4 right). In addition, only the *elf3*-mutant of Arabidopsis but not of barley differed in growth morphology in comparison to wild type (Illustration 4). These results demonstrated that the circadian clock component ELF3 affected biomass accumulation and growth morphology in Arabidopsis but not in barley. Together, the carbohydrate depletion patterns and the biomass accumulation suggested that the *elf3*-mutation causes reduced biomass in Arabidopsis by reducing carbohydrate turnover during the night. In barley, however, carbohydrate supply at night is not affected by the *elf3*-mutation and biomass is not reduced.

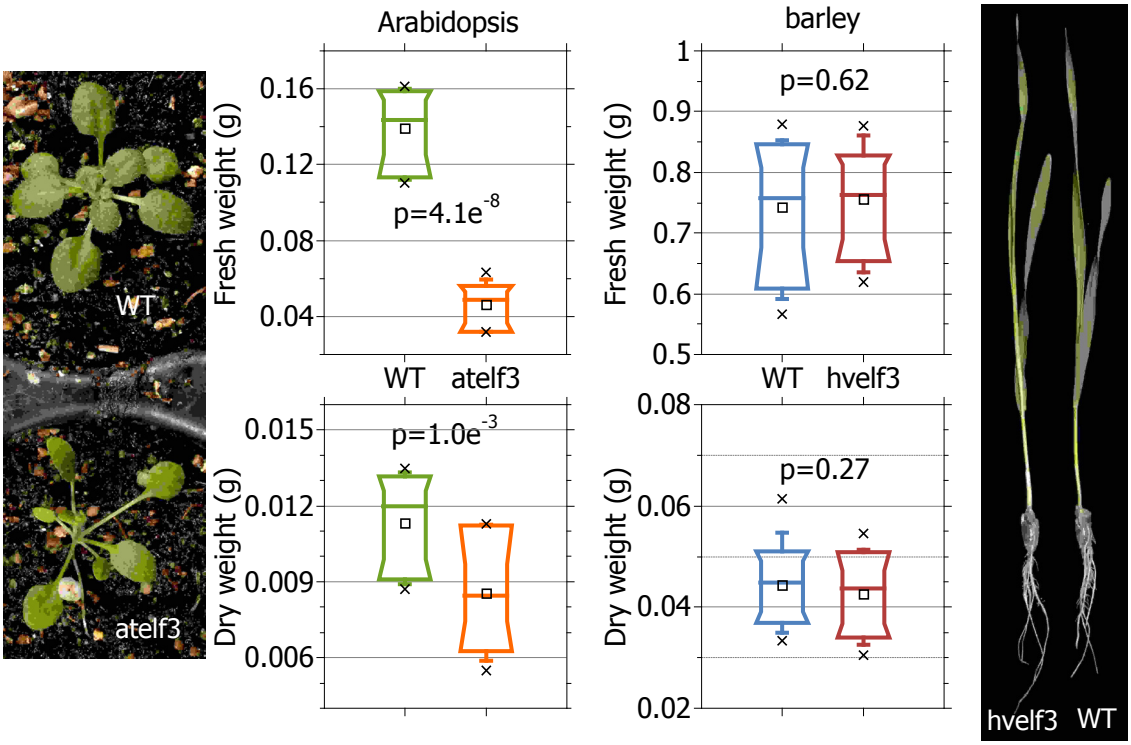


Illustration 4: The clock component ELF3 controls growth and biomass in Arabidopsis but not in barley.

Left) Phenotype of *atelf3* and **(right)** *hvelf3*. **Center)** Fresh (top) and dry weight (bottom) of Arabidopsis and barley *elf3*-mutant plants compared to wild-type. *Boxes show the 10, 25, 75 and 90% percentil, whiskers show the 5-95% percentil. Significant differences tested by T-test. Arabidopsis: n=15, barley: n=30.*

5.2 Temporal control of sucrose depletion during the night

Although the circadian clock did not control sucrose metabolism during the night, nocturnal sucrose depletion was under temporal control in barley (Illustration 2 bottom left, Illustration 3 top left, center left). In order to elucidate the temporal regulation of sucrose depletion at night, I investigated the depletion patterns under different photoperiods. Storage sucrose depletion at night was exponential and sucrose content in the barley leaf was identical each dawn at ZT0 and ZT24 under entrained conditions of 8h light and 16h dark (Illustration 5 left). After an unexpected extension of day length for 4h, the sucrose content was one-third higher after 12h compared to 8h of light but sucrose levels

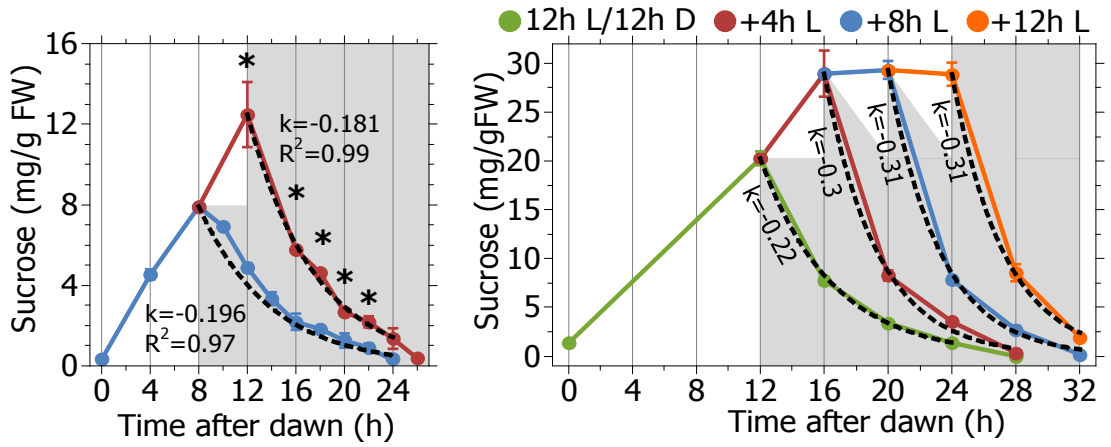


Illustration 5: A concentration-dependent kinetics controls sucrose depletion in the barley leaf and adjusts depletion rate to the length of the night.

Left) Sucrose levels in barley wild type plants entrained to 8h light/16h dark, then kept in 8h of light (blue) or unexpectedly shifted to 12h of light (red) and then released to constant darkness. *Significant differences per time point (T-test, p≤0.05) are marked with *)*. **Right)** Sucrose levels in barley wild type plants entrained to 12h light/12h dark, then kept in 12h light and released to constant darkness (green) or unexpectedly shifted to an extended light period of 16h (red), 20h (blue) or 24h (orange) before released to constant darkness. *The depletion constant k describes the first-order depletion kinetics. Error bars are standard deviation of the mean throughout. n=3 per time point.*

at the end of the diel cycle at ZT24 were comparable between both the entrained 8h/16h and the unexpected 12h/12h photoperiods (Illustration 5 left). This regulation was achieved even though the length of the night was unexpectedly shortened for 4h compared to entrained conditions (Illustration 5 left). This demonstrated that the barley plants were capable of instantly adjusting sucrose depletion to the length of the night even after an unexpected extension of day length for 4h. I further investigated the adaptability of the sucrose depletion mechanism to unexpected changes in photoperiod. Unexpected extension of the light period for 4h from 12h to 16h increased sucrose content in the leaf at dusk and led to sucrose levels at dawn (ZT24) that were comparable to the levels without the shift (Illustration 5 right). However, an unexpected extension of photoperiod to very long days of 20h and 24h of light did not lead to a further increase of sucrose levels as compared to 16h of light. This indicated that the sucrose storage capacity in the barley leaf reached its maximum

after 16h of light in the given conditions. Importantly, the respective depletion patterns from photoperiods of 16h, 20h and 24h of light were comparable so that identical amounts of sucrose were depleted in the dark under these different photoperiodic conditions. This showed that sucrose depletion in the dark was independent from the time of the day, demonstrating that this process was not gated by the circadian clock. Instead, sucrose depletion only depended on the sucrose concentration present at the onset of darkness and followed concentration-dependent kinetics.

Then, I investigated if storage sucrose depletion during the night follows biochemical kinetics of first-order reactions. First-order reactions describe chemical reactions where the reaction rate depends on the substrate concentration. As sucrose export from the leaf at night depended on its concentration, I applied the equation of first-order reaction kinetics $[suc]_t = [suc]_0 e^{-kt}$ to the measured data. In this equation $[suc]_t$ describes the sucrose concentration in the leaf at different time points during the night, $[suc]_0$ the sucrose concentration at the beginning of the night, t the duration of darkness and k the reaction constant which summarizes the reaction conditions like temperature and enzyme activity. The calculated $[suc]_t$ showed a high correlation with measured sucrose content during the night ($R^2 \geq 0.97$) (Illustration 5 left, right). Consistently, we found that k was increased after the unexpected shift to longer days where higher sucrose content had to be turned over in a shorter night (Illustration 5 right). Together, these findings suggest that sucrose depletion during the night is a concentration dependent process that is appropriately described by first-order enzyme kinetics. This mechanism works independently of the circadian clock but is capable to adjust nocturnal sucrose depletion to the length of the night even when the photoperiod is unexpectedly altered.

5.3 Physiology of the sucrose storage metabolism

The exponential depletion of first-order enzyme kinetics strongly suggested that a sucrose transporter catalyzed the depletion of sucrose over time during the night. As the vacuole is the main site of sucrose storage in plant leaves (Martinoia et al. 1987; Kaiser & Heber 1984; Kaiser et al. 1982; Neuhaus 2007; Hedrich et al. 2015), I investigated sucrose transporters at the vacuolar membrane that are involved in sucrose release from the vacuole. In literature studies I identified sucrose transporters of the SUCROSE TRANSPORTER 4 (SUT4)-clade (Kühn & Grof 2010; Eom et al. 2011) in barley, rice and Arabidopsis that specifically locate to the vacuolar membrane and exclusively control sucrose export from the vacuole into the cytoplasm (Endler et al. 2006; Eom et al. 2011; Schneider et al. 2012). These transporters are termed SUCROSE TRANSPORTER 2 (SUT2) in the monocot species barley and rice (HvSUT2, OsSUT2) and SUT4/SUC4 in Arabidopsis (AtSUT4/AtSUC4). I analyzed the *sut2*-mutant of rice, *ossut2*, as no barley *sut2*-mutant was available. Wild-type rice depleted storage sucrose exponentially in the dark while the *sut2*-mutant retained high sucrose levels during the night (Illustration 6 left). This demonstrated that OsSUT2 determined sucrose export from the vacuole to

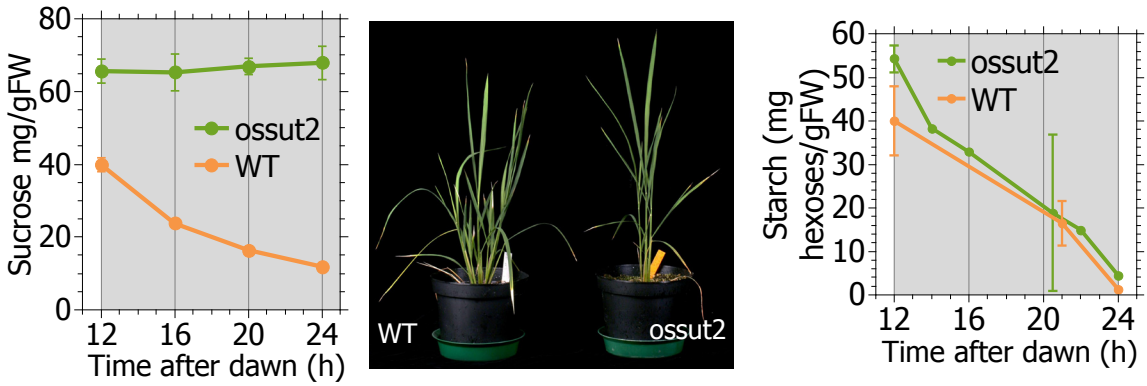


Illustration 6: SUT2 catalyzes the sucrose depletion from the leaf in rice and determines biomass.

Left) Sucrose and **(right)** starch levels in wild type (orange) and *sut2*-mutant rice (green) during a 12h night. Plants were 4 months old. **Center)** Growth phenotype of wild-type rice and *sut2*-mutant rice. *Error bars are the standard deviation of the mean throughout. n=2 per time point.*

the cytoplasm during the night and revealed that OsSUT2 catalyzes the first-order depletion kinetics of storage sucrose in the rice leaf. As a consequence, the vacuolar sucrose exporter SUT2 appears as indispensable regulator of metabolism in predominantly sucrose storing species.

I hypothesized that *ossut2*-mutant plants reveal reduced biomass in comparison to wild type as *ossut2* unproductively sequesters carbohydrate resources during the night that are, in contrast, utilized in wild type. In fact, rice *sut2*-mutant plants showed reduction by-half of biomass (Illustration 6 center). As starch degradation during the night (Illustration 6 right) was not different between *ossut2* and wild-type plants, this suggested that the impairment in sucrose supply caused the growth reduction in *ossut2*. Together, these results implied that the sucrose depletion in the leaf during the night is catalyzed by SUT4-clade sucrose transporters like OsSUT2 that export sucrose from the vacuole into the cytoplasm. In addition, this process appears important for carbohydrate supply and growth in predominantly sucrose storing species.

Next, I investigated transcriptional regulation of SUT2. I tested if HvSUT2 expression was under the control of the circadian clock. I did not find consistent periodic cycling of HvSUT2 expression in constant light conditions in barley wild-type plants and HvSUT2 cycled in *hvelf3* comparably to wild type in day/night cycles (Illustration 7 top left). This suggested that HvSUT2 transcription was not controlled by the circadian clock. In addition, the diel HvSUT2 expression pattern in light/dark cycles showed a peak at the end of the day and a decrease during the night in both wild-type and *elf3*-mutant plants alike (Illustration 7 top left), indicating that loss of ELF3 function in *hvelf3* did not interfere with HvSUT2 transcription in day/night cycles. These findings indicated that HvSUT2 followed light-related regulation. I observed an activation of HvSUT2 transcription over time in dissected, sucrose-depleted barley leaves fed with 30mM sucrose in the dark (Illustration 7 top right). Together, the findings indicated that HvSUT2 expression was driven by sucrose derived from photosynthesis during the day. Consequently, I hypothesized that transcription of HvSUT2 follows photosynthetic activity along the day and investigated if HvSUT2 protein in the leaf was regulated by day length. I measured HvSUT2 protein abundance during the night in plants grown in short day and then shifted to long day conditions five days after sampling for short-day (Illustration 7 bottom). I found that the

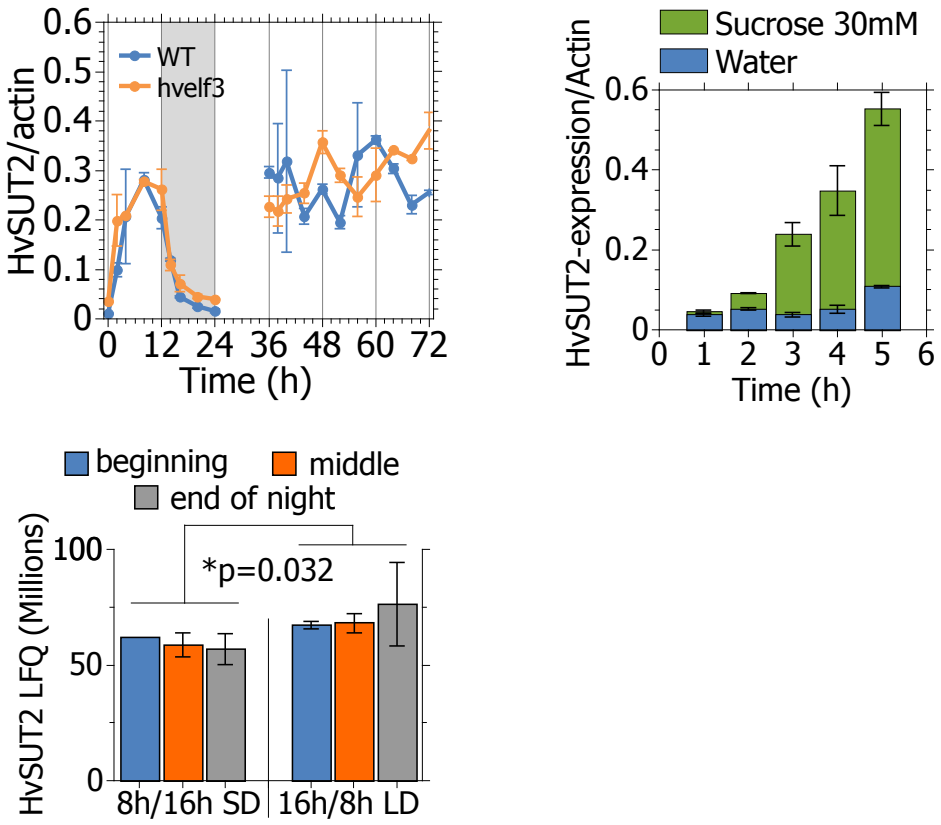


Illustration 7: Sucrose and day length but not the circadian clock control HvSUT2 expression.

Top left) Expression of HvSUT2 in barley wild type and *hvelf3* mutant plants during 12h light/12h dark (0-24h) and free running conditions in constant light (36-72h). *n=2 per time point.* **Top right)** HvSUT2 expression in detached barley wild type leaves in the dark fed with 30mM of sucrose (green) compared to water control (blue). Leaves were detached at the end of the night to deplete storage sucrose. *n=3 per time point.* **Bottom)** HvSUT2 protein abundance in barley wild type plants in the beginning (blue), middle (orange) and the end of the night (grey) in short and long day photoperiods. Plants were first grown in short day, then sampled for short day and shifted to long day where samples were taken five days after the shift. *Significant differences between short day (SD) and long day (LD) (T-test, p≤0.05) are marked with *. n=2 per time point. Error bars are the standard deviation of the mean throughout.*

HvSUT2 protein was relatively stable during the night in both short and long day, but long days significantly enriched SUT2 protein in the leaf. Thus, longer days induced higher amounts of HvSUT2 transporters in the leaf, which is consistent with a self-regulatory system that achieves higher sucrose turnover in shorter nights that followed a longer day.

5.4 Temperature sensitivity of sucrose depletion and growth in barley

Biochemical reactions of first-order kinetics are temperature sensitive as a temperature change instantaneously alters enzymatic activity. Thus, I hypothesized that SUT2-catalyzed sucrose depletion is reduced at lower temperatures. I exposed barley plants entrained to a night temperature of 18°C to an unexpected cool night at 10°C and measured sucrose and starch depletion during the night (Illustration 8). The unexpected temperature drop halted sucrose depletion for the first 2h of the night and the depletion was slower at 10°C compared to 18°C during the night as indicated by the lower reaction constant (Illustration 8 left). This demonstrated that carbohydrate supply from storage sucrose was reduced at cool night temperatures. Starch degradation on the other hand was not

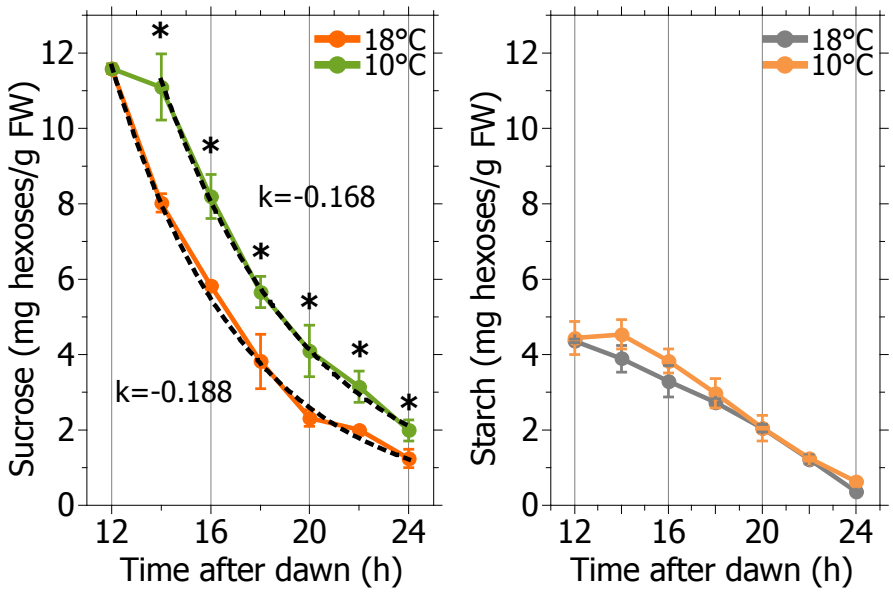


Illustration 8: Starch degradation, but not sucrose depletion is compensated against cool temperatures.

Left) Sucrose depletion and **(right)** starch degradation in barley wild type plants entrained to 12h light at 20°C and 12h dark at 18°C. Plants were either shifted to an unexpected 10°C cool night or kept in entrained conditions at 18°C. *Significant differences per time point (T-test, p≤0.05) are marked with *. n=3 per time point. Error bars are the standard deviation of the mean throughout.*

significantly different between the entrained night temperature at 18°C and the unexpected cool night at 10°C (Illustration 8 right). This finding showed that carbohydrate supply from starch was unaffected by low temperature and demonstrated that starch degradation is compensated against cool night temperatures. Considering that starch degradation but not sucrose depletion was controlled by the circadian clock, this suggested that an internal program driven by the circadian clock is responsible for the temperature compensation of starch degradation. As sucrose depletion was not controlled by the circadian clock and not compensated against low night temperatures, it appears that sucrose depletion is driven by temperature but not an internal program.

As carbohydrate supply from transitory sucrose but not transitory starch was reduced at cool temperatures, I investigated growth in cool nights between species that depend on starch or sucrose as carbohydrate source at night. I compared Arabidopsis with barley and measured differences in biomass after one week of growth at 20°C during the day and either 18°C or 10°C during the night. Barley reduced biomass during growth in cool nights while biomass of Arabidopsis remained

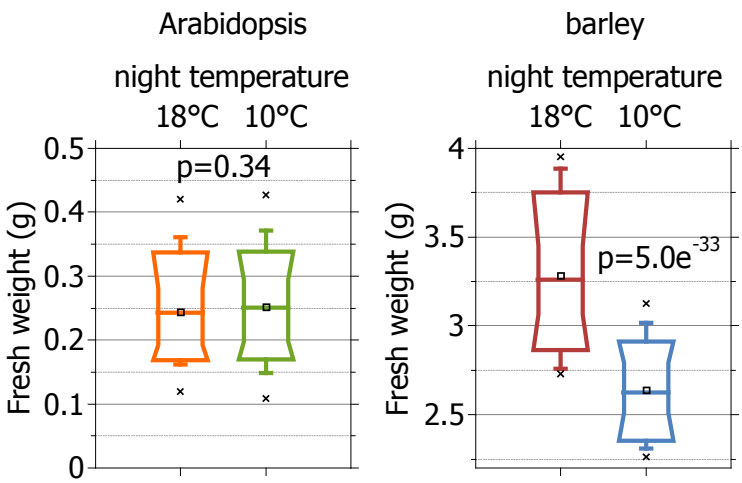


Illustration 9: Only barley, but not Arabidopsis, reduces growth in cool nights.

Fresh weight of Arabidopsis (Col-0) and barley (Bowman) wild type plants after 1 week of growth at different night temperatures. Plants were grown in 12h light at 20°C and 12h dark at 18°C for 3 weeks before night temperature was reduced to 10°C for a subset of plants for one further week before harvest. Day temperature remained at 20°C. *Boxes show the 10, 25, 75 and 90% percentil, whiskers show the 5-95% percentil. Significant differences tested by T-test. n=90.*

unaffected (Illustration 9). For Arabidopsis, the findings of this work suggested that the temperature compensation of starch degradation by a clock-driven program maintains carbohydrate supply for metabolism and growth in cool nights. For barley, the findings of this work suggested that carbohydrate supply from sucrose is driven by night temperature but not an internal program so that carbohydrate supply is reduced at low temperatures. As a consequence, cool night temperatures reduce carbohydrate supply, growth and biomass in barley but not in Arabidopsis.

6 Discussion

6.1 The sucrose storage metabolism is an alternative to the starch storage metabolism

It remains largely unknown how different species store carbohydrate resources during the day and use them during the night for metabolism and growth in the absence of photosynthesis. In the model plant Arabidopsis, starch is the major transitory storage carbohydrate in diel cycles (Graf & Smith 2011). However, the cereal grasses rice, barley and wheat primarily store sucrose instead of starch as carbohydrate source for the night (Hirose et al. 2013; Sicher et al. 1984; Trevanion 2002). As a consequence, at least two different forms of carbohydrate supply at night exist in plants: The turnover of transitory starch or transitory sucrose. That different species like Arabidopsis and rice depend on different carbohydrate sources at night was already shown in previous studies. For example, the incapability to degrade transitory starch at night due to a mutation in the α-glucan water dikinase (GWD1, also termed SEX1), an enzyme involved in the initiation of starch degradation, causes dwarf growth in Arabidopsis but not in rice (Hirose et al. 2013; Caspar et al. 1991). Here I could demonstrate that carbohydrate supply from starch and sucrose during the night is regulated by two different mechanisms. While carbohydrate supply from starch depends on the circadian oscillator through the clock component ELF3 for temporal control, carbohydrate supply from sucrose is independent from the circadian clock and is regulated by a concentration-dependent kinetics during the night. These findings clearly demonstrate that the starch and the sucrose storage metabolism are two separate mechanisms for carbohydrate supply. However, they are not mutually exclusive and the

cereal grasses rice, barley and wheat use both of them simultaneously for carbohydrate supply at night.

6.2 Storage sucrose depletes by concentration-dependent kinetics unrelated to the circadian clock

The clock control of carbohydrate supply from starch at night was conserved in barley, however, carbohydrate supply from storage sucrose was unaffected by a defective circadian clock. I identified a sucrose concentration-dependent regulation by first-order kinetics that depletes sucrose exponentially in the leaf. This process was catalyzed by the vacuolar sucrose transporter SUT2, which mediates the export of sucrose from the vacuole into the cytoplasm in both rice (OsSUT2) and barley (HvSUT2) (Endler et al. 2006; Eom et al. 2011). This regulatory mechanism substitutes the circadian clock for temporal control of carbohydrate supply during the night and regulates sucrose depletion in response to photoperiod independently from the oscillator. This is different to the regulation of the starch degradation rate. The regulation of the starch degradation rate at night requires real-time information about the starch content and the time left until next dawn (Graf et al. 2010; Stitt & Zeeman 2012; Scialdone et al. 2013; Seaton et al. 2014). While the information about starch content is presumably provided by phosphorylation marks at the starch granule surface (Scialdone et al. 2013), the temporal information is provided by the circadian clock (Graf et al. 2010). Both sources of information are proposed to be integrated by arithmetic division carried out by so far unknown molecules to calculate the appropriate starch degradation rate at night (Scialdone et al. 2013). This sophisticated level of system regulation is not required for the carbohydrate supply from storage sucrose. The reason is that the accumulation of sucrose in the vacuole during the day increases the sucrose concentration in the vacuole, which, at night, drives the depletion kinetics of sucrose from the vacuole into the cytoplasm. As this depletion is exponential and follows first-order kinetics, higher concentrations of sucrose deplete faster during the first parts of the night compared to lower sucrose concentrations, but in both cases the kinetics converges to near depletion of vacuolar sucrose at around 24h after dawn. This

mechanism adjusts sucrose supply to the length of the night in 24h cycles independently from the circadian clock, even under unexpected variation of day length for 4h. Consistent with the absence of clock control on the regulation, transcription of SUT2 in barley was not under control by the circadian clock but inducible by sucrose signaling. In addition, HvSUT2 protein abundance did not cycle during the night and followed day length-dependent regulation.

As Arabidopsis does not store high amounts of sucrose in the leaf at night, this regulation is of limited importance in Arabidopsis. Thus, the Arabidopsis homologue to OsSUT2/HvSUT2, which is termed AtSUT4/SUC4 (Schneider et al. 2012), does not have a physiological role comparable to barley or rice. This conclusion is consistent with the finding that Arabidopsis *sut4/suc4*-mutants do not reveal a growth phenotype (Schneider et al. 2012), but *sut2*-mutants in rice (Eom et al. 2011). In summary, several pieces of evidence demonstrate that the carbohydrate physiology is different between Arabidopsis and sucrose storing species like barley or rice.

6.3 The concentration-driven, clock-independent carbohydrate supply from sucrose explains differential growth phenotypes between barley and Arabidopsis

The differences in nocturnal regulation of carbohydrate supply from storage sucrose or transitory starch contribute to the differential effects of the clock component ELF3 on growth in Arabidopsis and barley. Only Arabidopsis but not barley depends on carbohydrate supply from transitory starch at night. Thus, the defective temporal control of starch degradation in the *elf3*-mutants only interfered with carbohydrate supply in Arabidopsis but not barley during the night and caused reduced growth. As a consequence, biomass in Arabidopsis depends on the clock component ELF3, but ELF3 does not control biomass in barley. This suggests that the role of the circadian clock on metabolism and growth as demonstrated in Arabidopsis (Stitt & Zeeman 2012; Greenham & McClung 2015) is not ubiquitous in plants and depends on the carbohydrate stored for the night.

Similarly, the differences in nocturnal regulation of carbohydrate supply from sucrose or starch explain why barley but not Arabidopsis reveals reduced biomass after one week of growth in cool nights. Starch degradation is tightly controlled by an internal program set by the circadian clock so that carbohydrate supply at night is compensated against cool temperatures. As a consequence, carbohydrate supply from starch is comparable between cool and entrained night temperatures. This is in contrast for carbohydrate supply from sucrose which is not controlled by the circadian clock but a depletion kinetics. This depletion kinetics is, like all biochemical reactions of first order, temperature dependent and reduces turnover at lower temperatures. Therefore, cool night temperatures reduced sucrose export from the vacuole into the cytoplasm throughout the night and resulted in reduced carbohydrate availability for growth. As barley metabolism at night depends primarily on vacuolar sucrose but not starch, growth in cool nights is only reduced in sucrose storing barley but not starch storing Arabidopsis. Thus, temperature but not the circadian clock is one of the major regulators of carbohydrate supply in barley at night due to the predominant storage of sucrose as carbohydrate source.

6.4 Role of the circadian clock on metabolism and growth is not universal in plants

The differences in the circadian control of metabolism and growth between barley and Arabidopsis are remarkable, given the assumption that the circadian clock generally contributes to improved physiological performance and growth in plants (Greenham & McClung 2015; Harmer 2009). This assumption is based on the high degree of conservation of circadian clock genes across different species (Song et al. 2010) and similarities in the diel regulation of the transcriptome between monocot and dicot species (Filichkin et al. 2011). However, this work demonstrates that the role of the circadian clock for metabolism and growth is not universal in plants and can differ between species. The reason is that the storage of sucrose or starch as carbohydrate source for the night is species specific. As the carbohydrate supply from sucrose and starch is regulated by two different pathways of which only one

depends on the circadian clock, the circadian control on metabolism is not determining growth in all species in the same way.

It remains to be understood how and why species like barley and rice partition photosynthetic assimilates primarily into sucrose but still build up little amounts of transitory starch. Apparently, these questions cannot be answered in the Arabidopsis model. It is well possible that the simultaneous use of the sucrose and the starch storage metabolism allows increased carbohydrate turnover at night which would facilitate higher metabolic activity and faster growth. Based on this study it can be speculated that barley uses the clock controlled degradation of starch to mitigate growth reduction under cool night temperatures when carbohydrate supply from sucrose is inefficient. On the other hand barley might benefit from the easy regulation of carbohydrate supply from sucrose that could facilitate faster growth under favorable conditions.

For these reasons, the knowledge transfer from the Arabidopsis model to the cereal crops and further grasses is very restricted in the field of metabolic physiology and growth. Therefore, attempts to improve growth, biomass and yield in the cereal crops wheat, barley and rice will have to acknowledge the physiological and regulatory characteristics of the sucrose depletion metabolism that cannot be deduced from the Arabidopsis model.

7 Material and methods

7.1 Plant material and growth conditions

The spring barley cultivar Bowman (BW) was used as barley wild type plants. In addition I analyzed the introgression line BW290 which carried an introgression of the eam8.k allele in the background of Bowman. The eam8.k allele is characterized by a base pair mutation leading to a premature stop codon in HvELF3, which is orthologous to ELF3 in Arabidopsis (Faure et al. 2012). This genotype is denoted as hvelf3 mutant in this work. For Arabidopsis the elf3-4 mutant in the WS background was used (Anwer et al. 2014). Both barley and Arabidopsis were grown in growth chambers at 20°C at 350µmol m^{-2} s^{-1} photon flux density (PPFD) during the day and 18°C during the night. Barley and Arabidopsis seeds were directly sown into the soil (Einheitserde Classic) and stratified for 4 days at 4°C.

The rice mutant ossut2 was in the Hwayoung background and was, together with the wild type seed, provided by Jong-Seong Jeon (Eom et al. 2011). The rice seeds were surface sterilized with 70% EtOH for 5min, then shaked in a 3% sodium hypochlorite (NaOCl) solution for 40 min followed by washing five times with sterile water. Seeds were not stratified but pre-germinated in constant light at 28°C for one week on autoclaved half strength Murashige Skoog medium with 3% sucrose (for 1l: 2,2g Murashige-Skoog medium, 30g sucrose, 8g phyto agar, 0.25g MES, pH5.7 adjusted by KOH). Seedlings were then transplanted to normal potting soil (Einheitserde Classic) and cultured in growth chambers at 12h light at 28°C and 350µmol m^{-2} s^{-1} PPFD (photosynthetic photon flux density) and 12h dark at 25°C. Air humidity was around 60%.

7.2 Diel/circadian sampling for metabolites and RNA

Barley and/or rice plants were grown to the three leaf stage. The whole second leaf of one barley or rice plant was cut at the base (around 150-200mg) and sampled into 2ml Eppendorf tubes supplied with 2 milling beads. At least 3 different plants were sampled per time point and snap frozen in liquid nitrogen for further analysis. In the case of Arabidopsis, plants were grown for 4 weeks before complete rosettes from at least 4 plants were pooled to one biological sample (100-200mg fresh weight) and processed as barley and rice. The time points of sampling applied as given in the respective diagrams of the individual experiments.

7.3 Biomass measurements

The biomass of 3-4 week old barley and Arabidopsis plants was measured as fresh weight of the shoot directly after cutting from the rootstock on an accuracy balance. Individual shoots were collected, oven-dried and measured for dry weight. At least 15 biological replicates were measured per specie for biomass analysis.

7.4 Measurement of sugars and starch

Frozen samples were ground to powder in 2ml tubes in a Retsch-mill (Retsch/Quiagen) and weighed to determine the individual fresh weight per sample. The soluble sugar fraction of sucrose, glucose, fructose, maltose and fructan was separated from insoluble starch by boiling the sample 2 times in 1ml 80%EtOH at 85°C for 30min. The soluble fraction of 2ml was transferred to a new tube and the chlorophyll was precipitated by adding 0,5ml chloroform and 1 ml water. 100µl of the clear solution was concentrated in a vacuum concentrator (speedvac) (Eppendorf) until all solvent had evaporated before resuspended with 100µl sterile water. The content of sucrose, glucose, fructose and maltose was measured in a photometer (BioTek) at 340nm wavelength against the glucose background and

quantified against a glucose standard curve using the enzyme kits from r-biopharm (Cat. No. 11 113 950 035 and Cat. No. 10 139 106 35) adapted to 96-well format.

For measurement of fructan, the speedvac precipitate was resuspended with 80µl of sterile water. Fructan was hydrolyzed to fructose by adding 10µl of 37% HCl and incubating for 20min at 80°C before neutralized with 10µl 7,67M NaOH. Then, the fructan was measured as fructose against the fructose background and quantified against a fructose standard curve using the enzyme kit for fructose (r-biopharm, Cat. No. 10 139 106 35).

For measurement of starch, the insoluble fraction was boiled in 500µl KOH at 95°C for 45min before neutralized with 90µl 1N HAc. To digest the starch to glucose, 17µl (3.5U) α-amylase (Roche, Art. Nr. 10102814001) and 83µl (2.5U) amyloglucosidase (Roche, Art. Nr. 10102857001) were added and incubated at room temperature over night before glucose was measured using the enzyme kit for glucose (r-biopharm, Cat. No. 11 113 950 035).

The absolute content of each metabolite was calculated for the 3.5ml extraction solution and referred to the initial fresh weight.

7.5 Assay to test sucrose-inducible SUT2 expression

Barley plants (Bowman) in the three leaf stage were depleted from storage sucrose by transfer to 4h of extended darkness at the end of the night. Leaf blades of 15cm from the second youngest leaf were cut at the base and transferred to 15ml tubes filled with either 3ml of 30mM sucrose as treatment or 3ml water as control so that the solutions could be taken up by the transpiration stream. Each leaf was placed in an individual tube and 6 tubes per treatment and time point were prepared. These tubes were kept in the dark at room temperature until sampling. An additional leaf supplied with blue ink confirmed that the incubation solution was taken up by the transpiration stream after 15min and distributed across the whole leaf. The upper 5cm of the leaf tips were cut with a scissor at the time points given in the diagram and processed for qPCR analysis.

7.6 Quantitative real time PCR

The diurnal/circadian and sucrose induced transcription of HvSUT2 was analyzed by qRT-PCR. Total RNA was extracted from 100-150mg leaf material using Trizol (Invitrogen) following the manufacturers protocol. The samples were DNAse treated with the Ambion kit (Invitrogen) following the manufacturers protocol before cDNA was synthesized from 1µg of RNA with Superscript II (Invitrogen) following the manufacturers protocol. Quantitative real time PCR reactions were performed in 384 well format on a Light Cycler 480 System (Roche) and contained 1µl of half-diluted cDNA, 1U of GoTaq Polymerase (Promega), 0.2 mM dNTPs, 2.5mM MgCl2, 0.2 µM of each primer and 1µl EvaGreen (Biotinum) using the following amplification conditions: 95°C for 5min, 40 cycles of 95°C for 10sec, 60°C for 10 sec, 72°C for 10 sec.

Expression of HvSUT2 (UniGene Hv.20993, accession number AJ272308) using the forward primer 5'-TGTTGCAGACAAGGAAGGAAGGC-3' and reverse primer 5'-CGGAACCGGTGTGCAAAGAATG-3' was tested against actin expression (forward primer 5'-CGTGTTGGATTCTGGTGATG-3', reverse primer 5'-AGCCACATATGCGAGCTTCT-3'). Expression was processed as absolute quantification based on a actin standard curve.

7.7 Protein quantification

Protein extraction

200 mg of leaf material sampled as described before were ground to fine powder with mortar and pestle. The powder was incubated with pre-heated SDT lysis buffer (4% w/v SDS, 100mM TRIS/HCl pH 7.6, 0.1M DTT) at 95°C for 5min. Thereafter, the sample was sonicated in a sonication bath for 1min to shear the DNA and reduce viscosity of the sample. The sonicated sample was centrifuged at 5,000rpm for 10min at room temperature on a table centrifuge and the supernatant transferred to new tubes. The supernatant was again centrifuged at 14,000rpm for 30min and transferred to new

tubes. This centrifugation step was repeated twice. After that, the protein concentration was determined by Bradford assay.

Proteolytic digestion and desalting

50µg of protein in the extracts were processed using the FASP method (Wisniewski 2009) as described in detail in Hartl et al. (2015). Cysteines were alkylated with chloroacetamide and successively hydrolyzed with LysC and trypsin. Trifluoroacetic acid (TFA) was added to a final concentration of 0.5%. Peptides were desalted and pre-fractionated prior LC-MS/MS into three fractions using the Empore Styrenedivenylbenzene Reverse Phase Sulfonate material (SDB-RPS, 3M) as described in detail in Kulak et al. (2014).

LC-MS/MS data acquisition

Dried peptides were redissolved in 2%ACN, 0.1% TFA for analysis and adjusted to a final concentration of 0.2µg/µl. Samples were analyzed using an EASY-nLC 1000 (Thermo Fisher) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher). Peptides were separated on 16cm frit-less silica emitters (New Objective, 0.75µm inner diameter), packed in-house with reverse-phase ReproDil-Pur C18 AQ 3µm resins (Dr. Maisch). Peptides (1µg) were loaded on the column and eluted for 130 min using a segmented linear gradient of 0% to 95% solvent B (solvent A: 5% ACN, 0.5% FA; solvent B 100% ACN, 0.5% FA) at a flow rate of 300nL/min. Mass spectra were acquired in the Orbitrap analyzer with a mass range of 300-1750 *m/z* at a resolution of 70.000 FWHM and a target value of $1x10^6$ ions. Precursors were selected with an isolation window of 1.3 *m/z*. HCD fragmentation was performed at a normalized collision energy of 25. MS/MS spectra were acquired with a target value of 10^5 ions at a resolution of 17,500 FWHM and a fixed first mass of *m/z* 100. Peptides with a charge of +1 or with unassigned charge state were excluded from fragmentation for MS2, dynamic exclusion for 30s prevented repeated selection of precursors.

Data analysis

Raw data were processed using MaxQuant software (version 1.5.1.2, http://maxquant.org) (Cox and Mann, 2008) with label-free quantification (LFQ) and iBAQ enabled (Cox et al., 2014). MS/MS spectra were searched by the Andromeda search engine against the MIPS database (http://mips.helmholtz-muenchen.de). Sequences of 248 common contaminant proteins and decoy sequences were automatically added during the search. Trypsin specificity was required and a maximum of two missed cleavages allowed. Minimal peptide length was set to seven amino acids. Carbymidomethylation of cysteine residues was set as fixed, oxidation of methionine and protein N-terminal acetylation as variable modifications. Peptide-spectrum-matches and proteins were retained if they were below a false discovery rate of 1%. Subsequent quantitative statistical analysis was performed in Perseus (version 1.5.2.6, http://maxquant.org; Cox and Mann 2012). Hits were only retained if they were quantified in at least to of three replicates in any of the four conditions.

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