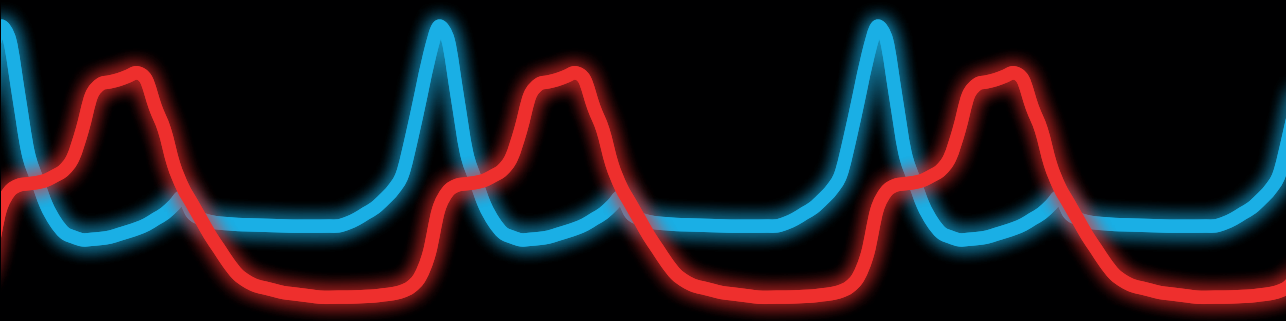
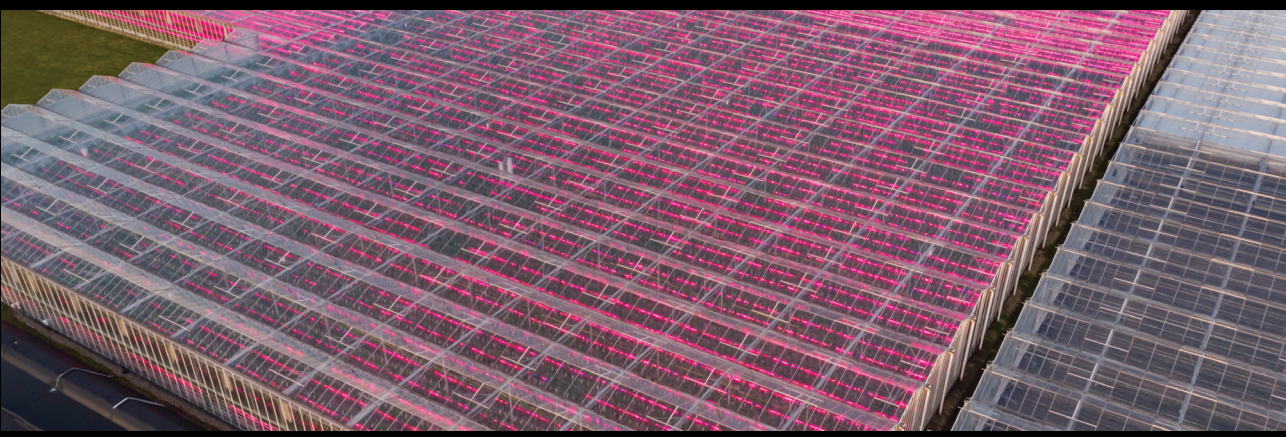
From lab to greenhouse

Molecular mechanisms of physiological control of plant growth





Mark van Hoogdalem

From lab to greenhouse:

Molecular mechanisms of physiological control of plant growth

Mark van Hoogdalem

Thesis committee

Promotor

Prof. Dr C. Testerink

Professor of Plant Physiology

Wageningen University & Research

Co-promotor

Dr A.R. van der Krol

Associate professor, Laboratory of Plant Physiology

Wageningen University & Research

Other members

Prof. Dr L.M. Trindade, Wageningen University & Research

Dr K. Kajala, Utrecht University

Dr C. de Groot, Bejo Zaden, Warmenhuizen

Dr M. van Zanten, Utrecht University

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

From lab to greenhouse:

Molecular mechanisms of physiological control of plant growth

Mark van Hoogdalem

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Tuesday 18 February 2020

at 1:30 p.m. in the Aula.

Mark van Hoogdalem

From lab to greenhouse: Molecular mechanisms of physiological control of plant growth,

236 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2020)

With references, with summary in English and Dutch

ISBN: 978-94-6395-276-7

DOI: https://doi.org/10.18174/511416

Table of contents

Chapter 1

General introduction

Plant growth regulation in horticulture: -DIF as alternative for chemicals

In horticulture it is important to control elongation of stems and grow compact shaped plants. Especially in the production of ornamental plants a compact shape is a desired (visual) quality trade, but also allows maximal production per unit area and reduces transportation costs. Growing high-quality, compact-shaped plants year-round can be challenging due to high crop density in greenhouses, especially on cloudy and/or warm days, since plant cell elongation is generally stimulated under these conditions (Smith, 1982; Martinez-Garcia et al., 2014; Fankhauser and Batschauer, 2016; Quint et al., 2016; Casal and Balasubramanian, 2019). Chemical plant growth retardants (PGRs) are commonly used in horticulture to control plant growth. Most of the registered PGRs control plant growth through targeting Gibberellin (GA) status, a group of plant hormones that stimulates stem elongation (Carvalho et al., 2008; Rademacher, 2017). However, due to environmental and health safety concerns the use of chemical PGRs is increasingly restricted by government regulations (Bergstrand, 2017). Therefore, alternative methods of plant growth control are needed that do not rely on the use of chemicals. One method commonly used in horticulture to control plant growth without the use of chemicals is realizing a negative day-night temperature difference (cold days/warm nights). Such a regime is referred to as a negative DIF (-DIF) regime, as opposed to a naturally occurring positive DIF (+DIF) regime in which temperatures are higher during the day than during the night (Erwin et al., 1989). A -DIF regime suppresses elongation in many plant species, including the model species Arabidopsis (Figure 1; (Myster and Moe, 1995; Moe and Heins, 2000; Bours et al., 2013). It was shown that suppression of plant growth under -DIF is mainly during the cold photoperiod (Stavang et al., 2005; Bours et al., 2013; Bours et al., 2015). In pea, the suppression of stem elongation under -DIF is associated with a reduction in bioactive GA levels due to increased GA catabolic activity (Grindal et al., 1998; Stavang et al., 2005). However, so far this could not be shown in Arabidopsis (Thingnaes et al., 2003). The phytochrome B (PHYB) photoreceptor is thought to play an important role in the growth inhibition effect of -DIF (Thingnaes et al., 2008; Patil and Moe, 2009; Bours et al., 2013) and *phyB* mutant lines of Arabidopsis show reduced suppression of leaf elongation in response to -DIF (Thingnaes et al., 2008; Bours et al., 2013).

In 2009 a research project started at the Laboratory of Plant Physiology of Wageningen University & Research (Wageningen, The Netherlands) that focused on increasing our understanding of the molecular processes that regulate elongation responses to -DIF: the TTI-Compact Plants project (Bours, 2014). This project lead to the discovery that -DIF suppresses cell elongation in Arabidopsis through limiting auxin biosynthesis and signaling, leading to reduced auxin-induced ethylene biosynthesis and signaling (Bours et al., 2013; Bours et al., 2015). It was found that two members of the growth-promoting PHYTOCHROME INTERACTING FACTOR family of transcription factors (PIF4 and PIF5) act upstream to regulate auxin biosynthesis, and that another member (PIF3) acts downstream of ethylene signaling to regulate cell elongation responses to diurnal light/temperature conditions (Bours et al., 2013; Bours et al., 2015). The -DIF treatment alters clock-controlled rhythmic leaf movement in young sink leaves of Arabidopsis, suggesting that the circadian clock might also be affected under -DIF (Bours et al., 2013).

Indeed, preliminary results indicate differences in activity of firefly-luciferase (ffLUC) reporter plants for clock gene promoter activity under +DIF and -DIF, indicating that expression of core clock genes may be affected by -DIF (Bours, 2014). However, major questions relating clock-gene regulation under -DIF remained, which are addressed in this thesis. Finally, results also suggested that -DIF leads to altered starch levels and it was shown that -DIF leads to induction of an ffLUC reporter for sugar starvation status (Bours, 2014). It was not determined how -DIF affects carbohydrate status, and this also became part of this thesis research.

The following paragraphs summarize the interaction of the main components involved in the -DIF response as known at the onset of this project. It involves the regulation of three major processes involved in the growth response to -DIF: transcriptional regulation of growth by PIFs and the PIF-antagonist ELONGATED HYPOCOTYL 5 (HY5), entrainment of the circadian clock by light and temperature and regulation of carbohydrate metabolism. Aim of the thesis research was to get a better understanding of how these different processes involved in the growth response to -DIF interact and may be manipulated by additional light treatments to enhance the effect of -DIF in greenhouses. In addition, the insights into the molecular regulation of the growth response to -DIF may also lead to alternative treatments to be used in greenhouses to control plant growth.

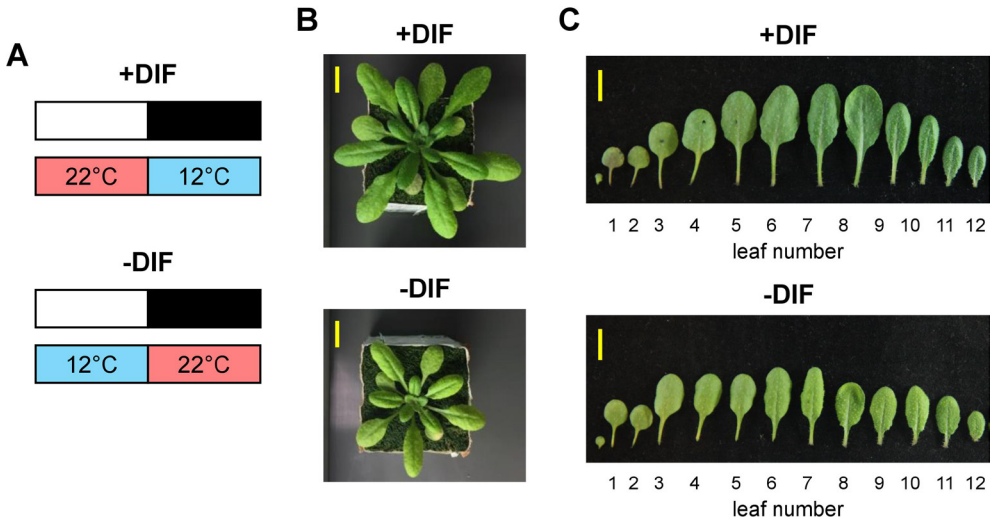


Figure 1: The effect of +DIF and -DIF on growth of adult Arabidopsis. A: Schematic representation of +DIF (12hL 22°C/12hD 12°C) and -DIF (12hL 12°C/12hD 22°C) regimes used in this thesis. White bars represent photoperiod; black bars represent dark period; blue bars represent 12°C; red bars represent 22°C. **B**+**C**: Representative top-view images (**B**) and leaf profiles (**C**) of Arabidopsis Col-0 WT plants grown for 31 days under +DIF conditions (+DIF) or under +DIF for 21 days and then transferred to -DIF for 10 days (-DIF). Numbering of leaves in **C** is based on order of emergence. Yellow scale bar=1cm.

PIFs mediate light/temperature regulation of plant growth

Central components in the regulation of elongation in Arabidopsis are the PIF transcription factors. The PIFs form a group of basic helix-loop-helix (bHLH) transcription factors that promote cell elongation by transcriptional activation of genes involved in processes related to cell elongation, such as cell wall modification, auxin biosynthesis, signaling and transport (Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018). PIFs are also involved in repressing transcription of genes involved in light-regulated suppression of elongation (photomorphogenesis). The activity of PIFs is regulated by light and temperature cues, both at the transcriptional and post-transcriptional level. The underlying mechanisms through which PIFs are regulated by light and temperature cues as known at the onset of this thesis work are summarized below and presented in Figure 2. Although Figure 2 focuses on the regulation of PIF4, in many cases the same regulatory mechanisms apply to other PIF members.

Regulation of PIF activity by phytochromes in response to changes in R:FR light ratio

As their name suggests, PIFs are able to interact with the phytochrome (PHY) photoreceptors. Arabidopsis has five different *PHY* genes: *PHYA*-*PHYE* (Clack et al., 1994), however only interactions of PHYB and PHYA with PIF proteins have been studied in more detail. All PIF proteins can interact with light-activated PHYB, but PIF3 can also interact with light-activated PHYA (Leivar and Quail, 2011). The PHY photoreceptors are synthesized in an inactive red (R) light-absorbing Pr state in the cytosol, but illumination with R light leads to the conversion of the Pr form into the active far red (FR) light-absorbing Pfr form (Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018). Absorbance of FR light converts the active Pfr form back into the inactive Pr form again. Conversion of active Pfr back into inactive Pr can also happen spontaneously in the absence of light, a process referred to as dark reversion (Furuya and Schäfer, 1996). Only active PHY Pfr is able to enter the nucleus, where it can interact with PIFs and trigger their degradation (Rockwell et al., 2006; Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018). The R:FR light ratio determines how much of the PHY pool is in its active state and indirectly determines the stability of the present PIF protein pool. For example, a low R:FR light ratio leads to a reduction in the amount of active PHYs and increased PIF accumulation in the nucleus, which triggers cell elongation (Lorrain et al., 2008). This response is referred to as the shade avoidance response (SAR) (Smith, 1982; Martinez-Garcia et al., 2014; Fankhauser and Batschauer, 2016). At the onset of this thesis research it was not known how the R:FR light ratio affects the transcription of the different *PIF* genes or the transcriptional activity of the genes encoding the PHY proteins that can affect the stability of PIF proteins. Therefore, transcriptional regulation of *PIF* genes and how this can be influenced by light also became one of the research targets in this project. In addition, light regulation of the *PHY* genes became the topic of the matching thesis research of Umidjon Shapulatov (Shapulatov, 2019).

Regulation of PIF activity in response to B light

Low levels of blue (B) light can also induce SAR, which suggests that B light also regulates PIF protein activity (Pierik et al., 2009; Keuskamp et al., 2011; de Wit et al., 2016; Pedmale et al., 2016). Indeed, the elongation response to low B light is dependent on PIF4, PIF5 and the cryptochrome photoreceptors cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) (Pierik et al., 2009; de Wit et al., 2016). CRYs are activated by B light and are involved in inhibition of elongation and stomatal opening (Pierik et al., 2009; Yu et al., 2010; de Wit et al., 2016). The elongation response to low B light is through CRY1/CRY2-mediated regulation of PIF protein activity, as B light-activated CRY1 was shown to bind to PIF4 and blocks transcriptional activity of PIF4 protein at PIF4 target genes (Ma et al., 2016). Interestingly, under low levels of B light CRY1 and CRY2 appear to enhance transcriptional activity of PIF4 and PIF5 (Pedmale et al., 2016; Xu et al., 2016).

Downregulation of PIF activity by B light may also be indirectly through the bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5), as B light leads to stabilization of HY5 protein. HY5 activity suppresses elongation and thus acts antagonistically to PIFs (Kami et al., 2010; Lau and Deng, 2010; Leivar and Quail, 2011; Gangappa and Botto, 2016). While PIFs act as positive regulators of skotomorphogenesis, HY5 promotes photomorphogenesis by stimulating expression of genes regulating processes such as photosynthetic machinery assembly, pigment production and chloroplast development (Lee et al., 2007; Zhang et al., 2011). Stability of the HY5 protein is regulated in the nucleus by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), which forms a protein complex with SUPPRESSOR OF PHYA1-4 (SPA1-4) that targets HY5 for degradation in the absence of light (Osterlund et al., 2000; Saijo et al., 2003; Zhu et al., 2008). In the light, the COP1-SPA-HY5 interaction is destabilized by both light-activated PHYs and CRYs. As a result, HY5 protein is stabilized in the light. Since HY5 stimulates its own transcription, the HY5 protein levels are increased in the light compared to those in the dark. B light regulates HY5 stability, since HY5 dissociates from COP1/SPA1 as activated CRY1 interacts with SPA1 (Lian et al., 2011; Liu et al., 2011). Later, during this thesis work it was also shown that, besides attenuation of COP1/SPA activity by CRY1, CRY1 is also able to prevent binding of the G-protein β subunit AGB1 to HY5, which blocks HY5 transcriptional activity (Lian et al., 2018). Thus, B light regulates HY5 protein stability and HY5 transcriptional activity, which includes promoting transcription of the *HY5* gene itself (Abbas et al., 2014; Binkert et al., 2014; de Wit et al., 2016).

PIF protein and HY5 protein can bind to specific promoter sequences (E-box and/or G-box). Several studies have shown that HY5 and PIF4 may compete for the same binding site in promoters of genes involved in Reactive Oxygen Species (ROS) responses (Chen et al., 2013), photosynthesis (Toledo-Ortiz et al., 2014) and cell elongation (Gangappa and Kumar, 2017). Whether PIF or HY5 proteins act as positive or negative regulators of transcription depends on the promoter context, but for promoters at which both PIF and HY5 are able to bind these transcription factors often have opposite function. The *PIF4* gene itself contains both an E-box and G-box (Ibañez et al., 2018). Research suggests that HY5 negatively regulates *PIF4* transcription, as *PIF4* transcript levels are increased

in *hy5* mutants and HY5 is able to directly bind the *PIF4* promoter (Lee et al., 2007; Delker et al., 2014). During this research many other factors were identified in the regulation of *PIF* gene transcription and regulation of PIF protein transcriptional activity (e.g. BZR1, TCPs, MED25). These will be discussed in more detail in the general discussion chapter.

Regulation of PIF activity in response to low (non-freezing) temperatures

It is known that low (non-freezing) temperatures result in reduced growth of plants. For instance, low temperatures result in shorter hypocotyls and smaller, more compact shaped rosettes (Scott et al., 2004; Fiorani et al., 2005; Atkin et al., 2006; Patel and Franklin, 2009; Legris et al., 2017). Because of the role of PIFs in plant elongation, this suggests lowered PIF activity under low temperature. Indeed, it was previously shown that at low temperatures GA levels are reduced. GA signaling targets DELLA protein for destruction, and under -DIF DELLA proteins may therefore be more stable. This has consequences for PIF protein activity, as DELLA proteins can bind to PIFs, sequestering PIF proteins for transcriptional activity (Achard et al., 2008; de Lucas et al., 2008; Feng et al., 2008). The reduced GA levels and growth suppression in response to low temperatures is mediated by a small group of C-repeat Binding Factors (CBFs), which activate genes encoding enzymes for GA degradation and genes encoding DELLA proteins (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004; Achard et al., 2008; Miura and Furumoto, 2013). Besides blocking PIF4 transcriptional activity by DELLAs, the transcription of the *PIF4* gene itself is also reduced at low, non-freezing temperatures (Mizuno et al., 2014). This may be through increased suppression of *PIF4* transcription by HY5 (Lee et al., 2007; Delker et al., 2014), as *HY5* transcription as well as HY5 protein levels are increased in response to cold temperatures in a CBF-independent manner (Catala et al., 2011). During the period of this thesis work it became known how PHYB functions as a temperature sensor, which links to activity of PIF proteins (Jung et al., 2016; Legris et al., 2016; Qiu et al., 2019). This will be discussed in more detail in the final discussion chapter.

In this project, transcription of both *PIF*s and *HY5* was measured as function of different light quality treatments. However, the post-transcriptional regulation of PIF and HY5 proteins means that high transcription of the encoding genes does not always translate to high protein activity. However, low transcription of these genes can still reliably be interpreted as resulting in reduced protein activity. One way in which post-transcriptional regulation of protein stability can be included in ffLUC reporter studies is by making use of reporter fusion constructs in which the transcription factor is fused to the ffLUC protein. However, the intrinsic low stability of transcription factors has severely limited the use of such fusion reporters in this research. Another way in which the remaining transcription factor protein activity (e.g. PIF4) can be monitored is through monitoring the activity of specific (PIF4-)target genes. In this project we used the target-of-PIF4/5 *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) as reporter for integral PIF activity in response to different light conditions, by constructing p*IAA29::LUC* reporter plants.

The role of the circadian clock in the regulation of plant growth

For optimal plant growth correct sequential timing of the different cellular processes required for growth is essential (Braidwood et al., 2014). Moreover, these sequential steps should be coordinated with diurnal fluctuations in environmental conditions resulting from earth's rotation (e.g. light/dark cycles and associated temperature cycles). Indeed, it has been shown that expression of genes that encode the proteins involved in different steps in plant growth (cell wall loosening, water uptake, cell wall crosslinking) is orchestrated by the endogenous circadian clock, which is daily entrained by light and temperature (Harmer et al., 2000; Nozue et al., 2007; de Montaigu et al., 2010). The circadian clock maintains proper timing and coordination of gene expression with anticipated changes in the environment, to allow for optimal plant growth (McClung et al., 2002; de Montaigu et al., 2010; Greenham and McClung, 2015; Henriques et al., 2018). The genes that are under control of the circadian clock in plants are often studied by transferring plants from an environment with diurnal entrainment by light and/or temperature cycles to constant (free-running) conditions (Johansson and Koster, 2019). In Arabidopsis these studies lead to the identification of a set of interacting transcription factors that form the core of the circadian clock, often referred to as the central oscillator. The interconnected positive and negative feedback interactions between these transcription factors are required for generating and sustaining endogenous rhythms of approximately 24 hours (circadian rhythm). These core clock genes show wave-like expression patterns with a period of 24h and peak activities (named phase) at different times of the day (de Montaigu et al., 2010; Greenham and McClung, 2015; Webb et al., 2019).

The central oscillator in Arabidopsis: The MYB-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are core clock components with peak expression in the morning. CCA1/LHY suppress the expression of several members of the *PSEUDORESPONSE REGULATOR* (*PRR*) gene family, including *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *PRR5*, *7* and *9* (Hsu and Harmer, 2014). As the day progresses, *TOC1* and *PRR* expression is stimulated during the afternoon by the MYB-like protein REVEILLE 8, and TOC1 and PRR proteins suppress expression of *CCA1* and *LHY* (Nakamichi et al., 2010; Rawat et al., 2011). At night, TOC1 is targeted for degradation by the F-box protein ZEITLUPE (ZTL) (Más et al., 2003). GIGANTEA (GI), of which expression peaks in the afternoon, is involved in the stabilization of ZTL during the day and prevents its interaction with TOC1 until night (Cha et al., 2017). In the evening, the Evening Complex (EC), which consists out of EARLY FLOWERING 3 (ELF3), 4 (ELF4) and LUX ARRHYTHMO (LUX), act as transcriptional repressors of several core clock components, including PRR5, 9 and GI (Nusinow et al., 2011; Herrero et al., 2012; Mizuno et al., 2014).

The phase of core clock component expression is fine-tuned by environmental signals such as photoperiod and temperature cycle, but also by internally generated signals such as hormones and sugars (McClung et al., 2002; Oakenfull and Davis, 2017; Gil and Park, 2019; Webb et al., 2019). In turn, the circadian clock regulates expression of the genes that play key roles in the regulation of growth-related processes, including regulation of

the cell cycle (Masri et al., 2013; Fung-Uceda et al., 2018), hormone signaling (Thain et al., 2004; Covington and Harmer, 2007), starch metabolism (Graf and Smith, 2011; Izumi, 2019) and PIF-mediated cell elongation (Figure 2; (Nusinow et al., 2011; Sun et al., 2019)).

Besides regulating the phase of clock-target gene expression, the clock components may also regulate growth-related output processes by regulating protein activity. For example, during the course of this thesis work, several studies have shown that some of the clock components are able to regulate PIF protein activity by direct interaction with PIFs and/or PIF-target gene promoter regions (Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). The findings of these studies are discussed in the general discussion chapter of this thesis (Chapter 6). The fact that the clock is entrained by light and temperature and that growth is under control of the clock provides a direct mechanistic link between the effect of -DIF treatment on growth. Indeed, in this thesis research we provide solid evidence of how -DIF differentially affects the phase of individual clock genes, and how in turn this affects regulation of starch metabolism and growth.

Link between the clock, carbohydrate metabolism and growth

The previous research on the effects of -DIF in plants indicated that a carbohydrate (CH) starvation status may be reached at the end of the night under -DIF (Bours, 2014). Growth depends strongly on sugar availability as sugars ultimately provide the carbon building blocks and the energy needed for growth and maintenance of plant structures. Therefore, plant growth has to be balanced with sugar availability and a CH starvation status may trigger signaling towards inhibition of growth. In Arabidopsis, the protein kinases target of rapamycin (TOR) and sucrose non-fermenting 1(SNF1)-related kinase 1 (SnRK1) play a central role in the regulation of growth and development in response to sugar availability (Lastdrager et al., 2014). TOR acts in a protein complex (TORC1) and sugars activate TORC1, which promotes plant growth and development through positive regulation of processes such as cell proliferation, protein translation and metabolic pathways (Shi et al., 2018). In contrast, SnRK1 activity is suppressed by sugars. Therefore, SnRK1 is mainly activated under stress conditions that cause limited sugar availability, resulting in induction of CH starvation status (Baena-González et al., 2007). In response to CH starvation, SnRK1 negatively regulates energy consuming processes that are stimulated by TORC1 such as protein translation, and positively regulates pathways involved in energy production (Hardie, 2007; Tome et al., 2014). This is at least partially through transcriptional reprogramming (Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008; McGee and Hargreaves, 2008). Mair et al. (2015) showed that the KIN10 subunit of SnRK1 can accomplish transcriptional reprogramming by phosphorylation and activation of the basic leucine zipper 63 (bZIP63) transcription factor (Mair et al., 2015). A recent study suggests that SnRK1 also suppresses TORC1-stimulated processes through suppression of TORC1 activity, as KIN10 was shown to physically interact with and phosphorylate RAPTOR (regulatory-associated protein of TOR), a subunit of TORC1 (Nukarinen et al., 2016). Phosphorylation of RAPTOR in plants probably leads to TORC1 inactivation, similar to RAPTOR regulation of the TORC1

mammalian ortholog (Gwinn et al., 2008). It is possible that TORC1 and SnRK1 complexes regulate plant growth through modulation of PIF activity (Figure 2). Indeed, studies that were published during this project revealed a link between PIF activity and protein stability and increased activity of TORC1 and SnRK1, respectively. This will be discussed in more detail in the general discussion of this thesis (Chapter 6).

It is thought that not cellular levels of sugars such as sucrose or glucose determine TORC1 and/or SnRK1 activity, but that conversion of (excess) sugars into the signaling molecule trehalose-6-phosphate (T6P) acts as an indicator for excess carbohydrate availability. Thus, it is the T6P level that reciprocally regulates TORC1/SnRK1 activity (Figure 2). This is supported by the observation that levels of T6P show direct correlation with sucrose levels in Arabidopsis (Paul et al., 2008) and that production of T6P by trehalose-6-phosphate synthase 1 (TPS1) is essential for proper control of vegetative growth and plant development (van Dijken et al., 2004; Gómez et al., 2006). It has been shown that T6P stimulates growth and development when sugars are abundant through suppression of SnRK1 activity (Zhang et al., 2009; Figueroa and Lunn, 2016).

Photoperiod control of starch utilization

Sugars are produced during the day through photosynthesis. Part of the sugars produced by photosynthesis during the day are stored in the form of linked glucose polymers (starch) that form starch granules (Streb and Zeeman, 2012). During the night, starch is broken down in order to provide sugars to support maintenance and growth in the absence of light. Since sugar availability for growth during the night depends on starch biosynthesis during the day, starch utilization during the night is strictly coordinated with the growth demand during the night to prevent CH depletion. Under constant temperature of ~20°C and light/dark cycles of 24h (typical lab conditions), starch is synthesized during the day in an almost linear way, reaching maximal levels at the end of the photoperiod. Starch breakdown at night also follows a near-linear pattern and is regulated by the circadian clock in such a way that starch reserves are almost, but not completely, mobilized at the end of the night (Graf and Smith, 2011; Streb and Zeeman, 2012). Previously it was shown that depletion of starch reserves before the end of the night induces a so-called CH starvation status in the plant, which results in a period of growth suppression (Gibon et al., 2004). The essential requirement of proper regulation of starch metabolism for vegetative growth is also illustrated by the growth-phenotype of several Arabidopsis starch mutants. For example, rosettes of the Arabidopsis *gwd1* (*sex1*) mutant, which contains very high starch levels because it lacks activity of the starch breakdown enzyme glucan water dikinase, as well as the *pgm1* mutant, which contains strongly reduced starch levels due to lack of activity of the starch synthesis enzyme phosphoglucomutase, show a reduction in biomass and leaf length compared to WT (Caspar et al., 1985; Caspar et al., 1991; Streb and Zeeman, 2012). Induction of the CH starvation marker gene *DARK INDUCIBLE 6 (DIN6*/*ASN1*) at night indeed suggests that the growth phenotype of the *pgm1* and *gwd1* mutants results from low sugar availability for growth due to the inability to mobilize starch (*gwd1*) or lack of starch reserves for the production of sugars (*pgm1*) (Paparelli et al., 2013). In this thesis research on the effects

of -DIF on plant growth, starch metabolism was one of the focal points. Eventually, the insights into the link between starch metabolism and growth contributed to a new protocol option for controlling plant growth in greenhouses.

STW project compact plants: From fundamental insights to new growth protocols

Although -DIF allows for growth control without chemical PGRs, there are some limitations to this method. For example, realizing lower day-time temperatures in order to create an effective -DIF regime in greenhouses is only possible when outside temperatures are already relatively low. On the other hand, increasing night-time temperatures in greenhouses will increase energy consumption, which has a negative environmental impact (Tantau, 1998; Bergstrand, 2017). As a result, using -DIF in greenhouses to control plant growth is only feasible during periods of moderate temperatures (e.g. fall and early spring for The Netherlands). Therefore, alternative treatments, or treatments that extend the use of -DIF are needed that can be used to control plant growth in greenhouses without the use of chemicals. Better understanding of plant growth regulation by light and temperature may help identify such treatments. Therefore, in 2014 the STW project compact plants started. This project was a collaboration between the Laboratory of Plant Physiology and the Horticulture and Production Physiology department (both of Wageningen University & Research) and intellectually and financially supported by 22 companies that are active in the horticultural sector. While I focused on increasing our knowledge on plant growth regulation by light and temperature on a molecular level, potential applications were tested in a more realistic setting (e.g. in SOLINATOR climate cabinets or greenhouses) in collaboration with our project partners.

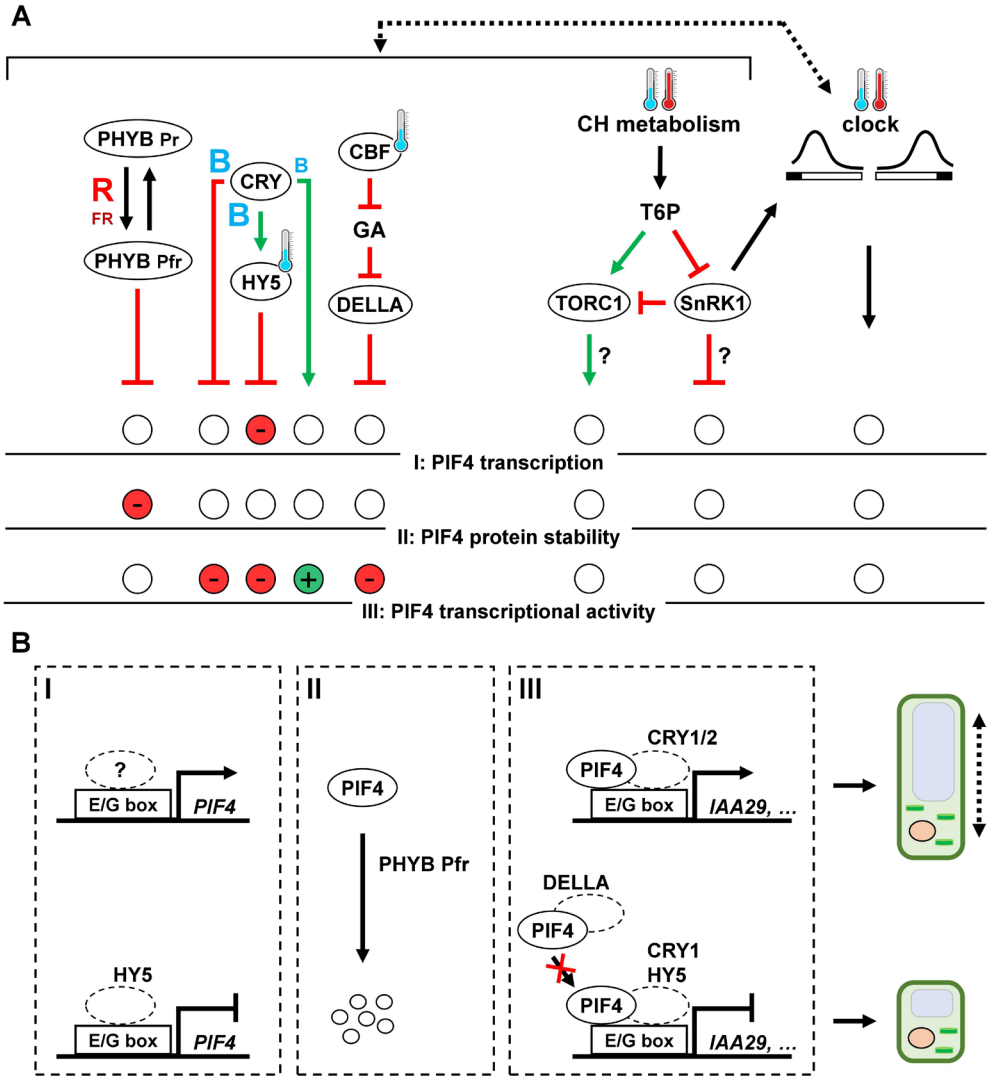


Figure 2: Model for the regulation of PIF4 in plant elongation responses at the onset of this thesis. **A**: Model for regulation of molecular components that mediate light/temperature control of PIF4 on three different levels: *PIF4* transcription (I), PIF4 protein stability (II) and transcriptional activity of PIF4 at PIF4 target genes (III). **B**: Model showing control of plant cell elongation through regulation of *PIF4* transcription (I), PIF protein stability (II) and PIF4 transcriptional activity (III) by regulatory components shown in A. Details on regulatory mechanisms are mentioned in the main text. Possible feedback interactions of PIF4 on upstream components in model are not shown. Ovals represent proteins or protein complexes. Black solid arrows indicate known regulation; black dashed arrow indicates regulation is not fully elucidated; green arrows indicate positive regulation; red bars indicate negative regulation. Question marks indicate possible regulation. Color and sign in circles in A indicate whether regulatory component enhances (green; +), suppresses (red; -) or does not influence (white) activity of PIF4 on different levels. Thermometers in A indicate whether regulation/component is stimulated at high (red thermometer) or low (blue thermometer) temperature. Colored letters in A indicate regulation is stimulated by high blue light (large B), low blue light (small B) or by a high red/far-red light ratio (large R, small FR).

Thesis outline

In order to increase our understanding of the molecular mechanisms behind regulation of plant growth responses to light/temperature signals, we aimed to use ffLUC reporters for promoter activity of genes involved in plant growth (e.g. clock genes and PIFs). In **Chapter 2** we describe the development of LUMINATOR: a system that allows for monitoring diurnal ffLUC reporter activity in up to 36 Arabidopsis rosette plants under controlled light and temperature conditions. It is shown that LUMINATOR can be used to study transcriptional regulation of *PIF4*, *HY5* and *GI* under 12h light/12h dark cycles by using ffLUC reporters for the promoter activity of these genes. The system was also used to study transcriptional responses of *PIF4*, *HY5* and *GI* to one hour added light at the start of the photoperiod. The observed transcriptional responses were predictive for long-term plant growth responses of tomato to the added light, which suggests that LUMINATOR may be used to help predict plant growth responses to additional light treatments. LUMINATOR also allows for studying ffLUC reporter activity at different temperatures. However, the effect of temperature on activity of the ffLUC enzyme itself make results difficult to interpret.

One of the main goals of this thesis project was to increase our understanding of the mechanisms underlying the plant growth response to -DIF. For this purpose, we performed RNA-sequencing (RNAseq) analysis of mRNA and miRNA of Arabidopsis Col-0 WT and *phyB-9* mutant plants growing under +DIF or -DIF conditions. Plant material was harvested at two timepoints: end of day (ED) and end of night (EN). The results of this study are described in **Chapter 3**. Analysis of differentially expressed mRNAs indicates that -DIF leads to upregulation of cold acclimation processes at ED and a low energy status at EN. Processes that link to promotion of cell growth, such as cell wall modification and water transport, are downregulated at ED or EN under -DIF. Differential regulation of PIF target genes suggests that cell growth under -DIF is limited through reduced PIF protein activity. Most of the genes involved processes linked to cell growth were not differentially expressed at EN in the *phyB-9* mutant, suggesting that they may be causal for growth suppression under -DIF and that PHYB plays a role in conveying temperature information at night. Analysis of differentially expressed miRNAs and their putative target mRNAs suggests that part of the transcriptional response to -DIF is through regulation of transcript stability by miRNAs.

The plant circadian clock plays an important role in the regulation of growth. Previous studies showed that -DIF results in altered clock-controlled leaf movement in Arabidopsis, suggesting that the circadian clock itself may be affected by -DIF. Since our RNAseq experiment only included two timepoints, it makes it difficult to use our RNAseq data to determine whether -DIF affects rhythmic expression of clock-regulated genes. Therefore, in **Chapter 4** the role of the clock under -DIF is studied in more detail. It is shown that expression of core clock genes is altered under -DIF. The altered clock gene expression under -DIF is associated with changes in starch metabolism, which is known to be regulated by the clock. As a result, starch levels in source leaves and sucrose levels in sink leaves are strongly reduced at the end of the night, leading to induction of marker genes for CH starvation. Since it is known that plant growth is suppressed in response

to CH starvation, this suggests that the induction of CH starvation at EN may be causal for growth suppression under -DIF.

The RNAseq data analysis in Chapter 3 suggests a link between reduced growth and reduced PIF expression/protein activity under -DIF, which is consistent with previous studies. In our search for additive light treatments that could be used to control plant growth we therefore aimed to use LUMINATOR to identify additive light treatments that lead to reduced PIF expression and/or protein activity. **Chapter 5** describes how LUMINATOR was used to study transcriptional responses of *PIF3*/*4*/*5*, the PIF4/5-induced target gene *IAA29* (indicator for PIF protein activity) and *HY5* (PIF antagonist) to additional blue (B) or red (R) LED light at the start and end of the photoperiod. Both additive light treatments triggered different transcriptional responses for the selected reporter genes. However, in both cases the transcriptional responses suggested reduced PIF protein activity and/or expression. In several cases the additive light treatments resulted in reduced plant length when provided for two weeks in SOLINATOR climate cabinets or greenhouses. However, results suggest that the effectiveness of additive B/R light treatments to reduce plant growth may depend on the overall light conditions and/or the intensity of the added light.

In **Chapter 6** we provide an updated version of the model for PIF regulation shown in Figure 2, based on studies that were published during the course of this project. In addition, we discuss how our findings related to the regulation of the clock, CH metabolism and cold acclimation may explain for reduced PIF activity and growth under -DIF. At the end of the chapter we provide a number of suggestions for new protocols that may be used to control plant growth, and several recommendations for future use of LUMINATOR in the search for more sustainable methods for regulating plant growth and development.

Chapter 2

LUMINATOR: monitoring transcriptional responses to light pulses to predict long-term plant elongation responses

Mark van Hoogdalem
Maarten Wassenaar
Habtamu Giday
Umidjon Shapulatov
Jacqueline Busscher-Lange
Marielle Schreuder
Casper van den Abeele
Wim van Ieperen
Alexander R. van der Krol

Under review

Abstract

Transcriptional control of hypocotyl elongation by transcription factors (TFs) PIF4 and HY5 has been well studied. However, activity of these TFs has been less well studied in mature Arabidopsis plants. Moreover, these TFs are affected by temperature and/or light conditions at both transcription and post-transcriptional level. The effect of short-term light treatments on PIF4 or HY5 activity is therefore still difficult to predict. For this reason, the selection of light treatments (for both color and timing) in greenhouses for control of plant growth is still very much an empirical art. Here we tested whether transcriptional responses of *PIF4*, *HY5* and other relevant genes to specific short-term light treatments can be used to predict long term plant growth responses. For this, LUMINATOR was used. A self-built system for monitoring firefly luciferase (ffLUC) reporter gene activity in response to different light treatments in mature Arabidopsis rosette plants. Transcriptional responses were measured in reporter plants expressing a key reporter for elongation (*PIF4::LUC*), for suppression of elongation (*HY5::LUC*) and for the circadian clock (*GIGANTEA::LUC*).

Results show that transcriptional effects of one-hour additional light in morning and evening can be detected, and that short-term light treatments have long lasting effects throughout the remainder of the day. Responses of ffLUC activity measured by LUMINATOR were confirmed by qRT-PCR analysis of the corresponding endogenous genes. The transcriptional response to the light treatment in Arabidopsis was predictive for long-term growth responses in tomato. LUMINATOR was also used to test the effect of different diurnal temperature regimes on transcriptional activity of ffLUC reporters. However, the effect of temperature on the ffLUC reaction itself make results more difficult to interpret. Still, comparison of ffLUC activity profiles in plants grown under different temperature regimes can provide valuable information on temperature effects on gene transcription.

We conclude that LUMINATOR can be used to study short-term transcriptional responses to different light treatments. Furthermore, these short-term transcriptional responses were shown to be predictive for long-term growth responses in Tomato. Results obtained with LUMINATOR may therefore be used to select effective light treatments for regulation of plant growth.

Introduction

A major advantage of growing plants in greenhouses or other indoor horticulture facilities is that environmental conditions can be regulated to a certain extent to affect plant performance. This allows growers to use light and/or temperature to control plant developmental processes, such as the timing of flowering (Erwin et al., 1989; Runkle and Heins, 2001; Craig and Runkle, 2016). Light and temperature treatments may also be used to control plant growth, for instance to generate a compact plant stature, which for ornamentals is considered a quality trait. Increasing the fraction of blue light using LEDs (light-emitting diodes), or dropping the temperature for a couple of hours after sunrise are examples of light and temperature treatments that can be used to grow compact-shaped plants in greenhouses (Cuijpers and Vogelezang, 1992; Buwalda et al., 2007; Ouzounis et al., 2014). However, reliable scoring of the effect of such treatments can take up to several weeks when evaluated by growth phenotype. Since there are endless variations in light colors, intensities and durations of potential added light treatments, identifying a most effective light regime for plant growth control in greenhouses is still very much based on practical experience rather than on scientific insight into the molecular control processes of plant growth. The molecular principles of control of hypocotyl elongation have been well studied in Arabidopsis, which has resulted in the identification of key transcriptional regulators and response genes that may be used as markers for elongation. Here we tested whether the direct effect of specific light/temperature treatments on the expression of such key growth-related genes can be monitored directly using firefly-luciferase (ffLUC) reporter plants. This can then be used to determine whether these short-term effects on gene expression are predictive for long-term effects on growth. In such way, many different light conditions and photoperiods can be screened much more efficient to help identify and develop new light/temperature treatments to control plant growth in greenhouses.

The ffLUC reporter system was initially developed to study gene expression dynamics related to the biological clock in plants (De Wet et al., 1985; Ow et al., 1986; Millar et al., 1992). When plants are sprayed with the substrate luciferin, the ffLUC activity in ffLUC reporter plants can be detected over several days in (semi) real-time with a sensitive camera. Indeed, the different clock gene ffLUC reporter plants have been instrumental in visualizing the dynamic interactions between a specific set of transcription factors that together form the core of the circadian clock in Arabidopsis (reviewed in (Johansson and Koster, 2019)). Most ffLUC reporter expression studies are performed in seedlings or young plants grown in microtiter plates, which are intermittently placed in a dark imaging chamber in which a CCD camera or other device captures the bioluminescent signal from the plants (Millar et al., 1992; Okamoto et al., 2005; Kamioka et al., 2016; Shalit-Kaneh et al., 2018). Commercially available set ups for imaging in planta ffLUC activity are available. In these set ups seedlings or small plants can be grown and imaged for several days under artificial LED lights and climate control (e.g. Nightshade LB 985, Berthold Technologies, Bad Wildbad, Germany). However, such set ups are not suited to monitor ffLUC activity in a population of mature Arabidopsis plants under various light/temperature conditions. Since the relative contribution of different transcription factors targeting key growth-related genes may change over development, analysis of

transcriptional responses in mature rosette plants provides new information on control of key genes related to growth.

To allow for monitoring ffLUC reporter activity in mature Arabidopsis rosette plants, the custom-made LUMINATOR system was built. LUMINATOR allows for (semi) continuous monitoring of ffLUC activity in seeds, seedlings, or up to 36 mature Arabidopsis ffLUC reporter plants, grown under climate-controlled temperature (T) and relative humidity (RH) and top-illuminated by a combination of Blue (B), Red (R) and Far Red (FR) LED lights. In LUMINATOR the intensities of R (5-600 µmole m^{-2} s^{-1}), B (8-400 µmole m^{-2} s^{-1}) and FR (3-500 µmole m^{-2} s^{-1}) light can be regulated independently, allowing to monitor gene expression as a function of light quality and intensity. The ffLUC activity images are captured by a cooled high-performance camera. A typical experiment with mature rosette plants is performed with an array of 5x5 plants grown on rockwool blocks, using 7 min. of exposure time, and images are taken every 30 min. Luciferin was provided by spraying the plants twice a day. Since delayed chlorophyll fluorescence can interfere substantially with the weak light produced by ffLUC activity a ZBPB074 Bandpass Filter is used (Asahi Spectra, Sumida, Tokyo, Japan), which blocks red chlorophyll fluorescence and passes the yellow/green light of ffLUC activity. Here we demonstrate that LUMINATOR can be used to study the effect of different light treatments on short-term gene expression. As basic illumination during the photoperiod we used a light-step profile (light ramping) using mixed LED lights, crudely mimicking the changing light conditions during the day. The transcriptional responses to this light regime are compared with transcriptional responses to a simple lights-on-lights-off block light regime (light on/off). The effect of these two different light regimes on expression of three genes that are known to play a major role in plant growth regulation was determined: *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), *ELONGATED HYPOCOTYL 5* (*HY5*) and *GIGANTEA* (*GI*). For this purpose we used 4-week-old Arabidopsis *PIF4::LUC*, *HY5::LUC* and *GI::LUC* reporter plants.

The basic helix-loop-helix (bHLH) transcription factor PIF4 promotes skotomorphogenesis and cell elongation in response to low R:FR and high temperatures (Huq and Quail, 2002; Leivar et al., 2008; Lorrain et al., 2008; Koini et al., 2009; Franklin et al., 2011). In contrast, HY5, a bZIP transcription factor, promotes photomorphogenesis and inhibits cell elongation (Koornneef et al., 1980; Chattopadhyay et al., 1998; Osterlund et al., 2000; Gangappa and Botto, 2016; Gangappa and Kumar, 2017). *GI* is a circadian clock gene with peak expression in the afternoon (Fowler et al., 1999). Changes in *GI::LUC* reporter activity as function of the two light treatments may be indicative of changes in clock regulation of genes. Although the molecular function of the GI protein is still not fully understood it has been shown to be required for temperature compensation of the circadian clock and is required for normal flowering time and plant growth (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Gould et al., 2006; Mishra and Panigrahi, 2015). Results show that the light on/off treatment resulted in higher *GI::LUC* and *HY5::LUC*, but in lower *PIF4::LUC* activity compared to the default light regime using light ramping. Such short-term transcriptional responses suggest that under a long-term light on/off regime plants will be more compact. This was tested in a long-term growth experiment using tomato plants (*Solanum lycopersicum* cv. ‘Money maker’). Indeed, tomato plants grown under a light on/off regime were smaller than those grown

under a more natural light regime with increasing and decreasing light intensity and R:FR at beginning- and end-of-day, respectively. These results thus demonstrate that short-term transcriptional responses of ffLUC reporters for key components of plant growth can be predictive for long-term plant growth responses to a given light treatment.

LUMINATOR was also used to test the effect of different diurnal temperature regimes on reporter gene activity. However, since the ffLUC enzymatic activity is sensitive to temperature (Koksharov and Ugarova, 2011; Feeney et al., 2016), changes in ffLUC activity are difficult to relate to changes in transcriptional activity or the reporter. Indeed, validation by endogenous gene activity measurements indicated that ffLUC reporter results were not always consistent with changes in endogenous gene expression level. Despite such complications, the ffLUC activity profiles may still provide valuable information on qualitative changes in gene expression under different temperature regimes. With LUMINATOR we now have the tool to assess direct effects of light treatments on key gene expression to help predict long term effects of such treatments on plants growth in greenhouses and other indoor horticulture facilities.

Material and Methods

Plant material

Arabidopsis Col-0 (N1092), *35S::LUC* (N9966) and *GI::LUC* (N9961) seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). *PIF4::LUC* seeds were provided by Salomé Prat (CNB-CSIC, Madrid, Spain). *HY5::LUC* seeds were generated as described below. All reporter lines are in Col-0 background. Tomato seeds were of the line *Solanum lycopersicum* cv. 'Money maker'.

Growth conditions

Arabidopsis seeds were stratified in the dark for three days at 5°C, after which they were sown on 4x4x4cm rockwool blocks (Grodan, Roermond, The Netherlands) soaked in Hyponex nutrient solution (Unifarm, Wageningen, The Netherlands). Plants were pre-grown in a climate chamber (12hL/12hD; 150 µmole m^{-2} s^{-1} light provided by fluorescent tubes; 22°C; relative humidity (RH) at 65%). Directly before transfer to LUMINATOR reporter plants were watered by soaking the rockwool blocks in Hyponex solution, which allows growth for 4 days without additional watering. Growth conditions in LUMINATOR cabinet are described below.

Tomato seeds were sown on 4x4x4cm rockwool blocks, which were soaked in 0.5x Tomato nutrient solution (Yara Benelux BV, Vlaardingen, The Netherlands) before sowing, and stratified in the dark for 24h at 5°C. Seeds were then transferred to a climate chamber (12hL/12hD; 150 µmole m^{-2} s^{-1} light provided by fluorescent tubes; 25°C; RH75%). After 8 days plants were transferred to either of two custom-made climate cabinets (SOLINATOR), in which light was provided by LED artificial sunlight research modules generation 1 (Specialty Lighting Holland B. V., Breda, The Netherlands). In both

cabinets the plants were grown under cycles of 12hL 25°C / 12hD 15°C, at constant RH65%. In one cabinet light intensity and quality were variable during each 12h photoperiod, mimicking naturally occurring variations in light intensity (depending on wavelength) and resulting phytochrome photostationary state (PSS) over the day (Supplemental Figure S1). Maximum total light intensity reached at mid-day was 146 µmole m^{-2} s^{-1}, PSS varied between 0.65 and 0.72. In the other climate cabinet light intensity was kept constant at 100 µmole m^{-2} s^{-1} and PSS at 0.72 during each photoperiod. Total light sum of both treatments was the same. Plants were watered three times a week with 0.5x Tomato nutrient solution. After two weeks plants were harvested for morphological measurements.

Generation of transgenic *HY5::LUC* line

The 756 bp intergenic region upstream of the *HY5* (AT5G11260) start codon was PCR-amplified (primers listed in Supplemental Table S1) using Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and cloned by TOPO® Cloning reaction (Invitrogen, Carlsbad, CA, USA) into the pENTR™ TOPO® entry vector. To generate *HY5::LUC* expression construct the entry vector containing the *HY5* promoter sequence was recombined into the pGREEN-GW-Luc68 destination vector by LR recombination using Gateway® LR Clonase® II enzyme mix (Invitrogen, Carlsbad, CA, USA). The *HY5::LUC* expression construct was transformed to electrocompetent *Agrobacterium tumefaciens* (AGL0) and Arabidopsis Col-0 plants were transformed by floral dip transformation (Clough and Bent, 1998). Positive transformants were selected on 1xMS agar plates supplemented with 10mg/L BASTA and transferred to soil for propagation until homozygous lines were obtained.

RNA extraction and qRT-PCR for gene expression analysis

For gene expression analysis shoots of 4-week-old Col-0 plants were harvested separately and immediately frozen in liquid nitrogen. The frozen material was homogenized using a Mixer Mill MM 400 (Retsch, Haan, Germany) and total RNA was isolated using the InviTrap® Spin Plant RNA Mini kit (Stratec, Birkenfeld, Germany) according to manufacturer's instructions. Subsequently genomic DNA was removed with the TURBO DNA-*free*™ kit (Invitrogen, Carlsbad, CA, USA). Quality of the RNA was checked by running 2µl on agarose gel and concentration and purity was determined by NanonDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA). For each sample 1µg RNA was used to synthesize cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR reactions were performed on the CFX Connect™ Real-Time PCR Detection System using SYBR® Green qPCR mix and CFX Maestro™ software was used to analyse the data (all Bio-Rad, Hercules, CA, USA). Expression levels were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), relative to expression levels of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780). Primers used are listed in Supplemental Table S2.

The LUMINATOR cabinet

The LUMINATOR is a custom-made climate cabinet containing a high performance PIXIS: 1024 CCD camera (Princeton Instruments, Roper technologies, Sarasota, FL, USA) fitted with a 35mm f/1.4 Nikkor SLR lens (Nikon, Shinjuku, Tokyo, Japan) for imaging of bioluminescence in reporter plants (Figure 1). The camera+lens combination provides a field of view that may vary between 7 cm x 7 cm (distance to camera lens ~15cm) and 45 cm x 45 cm (distance to camera lens ~120 cm). The frame of LUMINATOR consists out of a BLOCAN aluminium frame (RK Rose+Krieger, Minden, Germany) fitted with polyvinyl chloride (PVC), extruded polystyrene (XPS) foam for insulation and transparent polycarbonate panels. The inside of the cabinet is lined with aluminium foil to maximize even light distribution and to block low phosphorescent light emission from the insulation material. The ceiling is fitted with LED lights, emitting either blue (B; 420-500nm), red (R; 590-660nm) or far red (FR; 680-760nm) light (light spectra in Supplemental Figure S2), mounted on an aluminium plate with space in the middle for the camera and lens. The intensity of the different LEDs is controlled by an Arduino Uno microcontroller (www.arduino.cc). The temperature in LUMINATOR is controlled by two 380W thermoelectric cooling units (uwe electronic, Unterhaching, Germany) mounted on either side of the climate cabinet. Relative humidity in LUMINATOR is controlled by a custom-made humidifier which blows humidified air through an opening in the back of the climate cabinet. The thermoelectric units and humidifier are both regulated by Arduino Uno microcontrollers that are connected to a sensor in the centre of the climate cabinet that measures temperature and RH every 5 seconds and adjusts the settings of the thermoelectric units and humidifier accordingly. Supplemental Figure S3 shows climate conditions in LUMINATOR cabinet during the experiments presented here. The total costs of this self-build LUMINATOR is less than 50k€.

Light regime in LUMINATOR

For typical imaging of ffLUC activity in plants under diurnal light regime we used a one-hour step illumination gradient (light ramping) at start-day and end-day, to mimic altered light intensity and quality in morning and evening under natural light conditions. During this ramp light intensity is 30 µmole m^{-2} s^{-1} and during the remaining hours of the photoperiod 100 µmole m^{-2} s^{-1} (Supplemental Figure S1A, right panel). The ratio B:R:FR light during ramping is 1:2:1 and during the remaining hours of the photoperiod 3:6:1 (Supplemental Figure S4A). As a result of the change in B:R:FR ratio, PSS during light ramping (0.75) was lower than during the remainder of the photoperiod (0.82) (Supplemental Figure S1C, right panel). Light spectra of the two light settings are shown in Supplemental Figure S2. Using ramping of light intensity and quality allows for more effective studying of additional light treatments during morning or end afternoon for effects on ffLUC reporter activity in plants.

Imaging ffLUC activity in LUMINATOR

Reporter plants are placed in LUMINATOR in a custom-made aluminium grid with 5x5 compartments that each hold one reporter plant on a rockwool block (Figure 1C). The compartments prevent light contamination of plants surrounding reporter plants with strong ffLUC activity. Different ways of applying the substrate luciferin were tested (see below). Best results were obtained by spraying plants with 1mM D-luciferin (Promega, Fitchburg, WI, USA) twice daily, immediately after imaging at 2 and 8.5 hours after onset of lights (ZT(h)=2 and 8.5). Plants were allowed to acclimate to conditions in LUMINATOR for 24 hours before ffLUC activity imaging was initiated. Camera control and lights-on/lights-off control is by MetaMorph software (Molecular Devices, San Jose, CA, USA). ffLUC activity images are taken every 30 min. with an exposure time of 7 min. LEDs are switched off 30 seconds prior to imaging to allow for chlorophyll fluorescence decay. Light from chlorophyll fluorescence of plants is blocked by using a ZBPB074 Bandpass Filter (Asahi Spectra, Sumida, Tokyo, Japan). Figure 1D shows an example of image taken with LUMINATOR.

Application of luciferin substrate to ffLUC reporter plants during multiple day imaging

In many publications ffLUC reporter activity is monitored over several days by growing seedlings in liquid medium supplemented with luciferin (Okamoto et al., 2005; Kamioka et al., 2016). For bigger plants on soil or rockwool the uptake of luciferin may be through the shoot (when luciferin is supplied by foliar spray) or roots (when luciferin is added to nutrient solution), respectively. However, in bigger plants luciferin becomes limiting more rapid than for seedlings in liquid medium, especially when reporter gene activity is very high. We tested different ways of luciferin application on reporter activity in 4-week-old rosette plants. Luciferin supplied to the roots was by soaking the rockwool blocks on which the plants were grown in 20ml 1mM D-luciferin in Hyponex nutrient solution 24 hours before the start of imaging in LUMINATOR. Alternatively, luciferin was supplied to the shoot by spraying twice per day with 1 mM luciferin. ffLUC activity was imaged every 30 min (7 min. acquisition time) during a 12-hour photoperiod. The ffLUC activity of plants that receive luciferin through the roots is generally lower than ffLUC activity of plants receiving luciferin by foliar spray, indicating that uptake through the roots may limit luciferin availability in the shoot (Supplemental Figure S5). For the *GI::LUC* plants the spraying of luciferin had only small immediate effect on bioluminescence (Supplemental Figure S5A), indicating that this application of substrate keeps levels close to saturating levels. Indeed, the profile obtained from *GI::LUC* plants sprayed twice a day with luciferin matches the diurnal profile of endogenous *GI* expression in seedlings and three week old rosette plants (Mockler et al., 2007; Bordage et al., 2016). In the *35S::LUC* reporter plants the effect of daily spraying with luciferin was more pronounced (Supplemental Figure S5A), indicating that in this reporter plant available luciferin becomes limiting for the bioluminescence reaction faster. However, because activity profiles in plants sprayed with luciferin are more representative of endogenous gene activity, in all of our experiments the substrate was supplied by twice daily spraying with 1mM luciferin.

Quantifying ffLUC activity in plants

Luminescence was analyzed using ImageJ software (imagej.nih.gov/ij). Images were imported as stack into ImageJ, equal square areas covering each individual plant were defined in the ROI manager of ImageJ and mean grey value was measured for each plant throughout the image stack. Luminescence was calculated by subtracting background values from the mean grey values. Background values were determined by determining mean grey values of Col-0 WT plants grown under the same experimental conditions as the reporter plants.

Relative luminescence (normalized to luminescence on ZT(h)=0 or ZT(h)=11) was calculated for each individual plant to deal with differences in general luminescence level between individual reporter plants from the same line that may be caused by lens properties, reporter activity, and/or differences in shape of individual plants (e.g. petiole length and leaf width). When indicated, relative ffLUC activity on day 3 was normalized to relative ffLUC activity on day 1 to correct for changes in ffLUC activity during three days growth under control conditions. Integral luminescence was calculated by adding up all luminescence values during day or night. To obtain relative integral luminescence, integral luminescence on day 3 was normalized to integral ffLUC activity on day 1.

Plant growth measurements

Rosette area and projected leaf length of Arabidopsis were measured using ImageJ software, using the same images that were used for quantification of ffLUC activity (see above). The projected leaf lengths were obtained by measuring the distance between rosette center and leaf tip on pictures. Tomato hypocotyl and epicotyl lengths were measured by ruler. Total leaf area was determined using a leaf area meter (Li-3000-100, Li-Cor Biosciences, NE, USA).

Statistical analysis

Statistical significance of differences in ffLUC activity, endogenous mRNA transcript levels and plant-growth parameters was determined by Student's *t*-test ($p<0.05$).

Results

LUMINATOR for monitoring diurnal ffLUC reporter activity in plants

The aim of the experiments described here is to determine whether in principle the effect of short-term light treatments, which may be used in greenhouse or closed cultivation chambers to steer plant growth, can be detected in transcriptional responses in luciferase reporter plants that are representative of key genes related to growth or the clock. LUMINATOR was used to monitor luciferase activity in mature ffLUC reporter plants under controlled light, temperature and humidity (Figure 1; for full description see Material and Methods). For ffLUC activity, the reporter plants require the substrate luciferin. We

tested the best way for application of luciferin to mature rosette plants grown on rockwool blocks. Results show that when luciferin is directly sprayed on the shoot, ffLUC activity is about twice as high compared to when luciferin is taken up solely by roots (Supplemental Figure S5). This indicates that uptake of luciferin through roots is limiting the potential ffLUC activity in the shoot of mature plants. Therefore, for mature rosette plants luciferin was applied twice a day by spraying of shoot with 1 mM luciferin-D in water. Before ffLUC activity imaging, the reporter plants are pre-grown for 4 weeks in growth cabinets under diurnal 12h light/12h dark regime. One day before transfer to LUMINATOR the plants are sprayed with luciferin to inactivate previously accumulated luciferase protein. In LUMINATOR, individual rosette plants are separated by a metal grid to prevent light contamination of neighboring plants (Figure 1C). The ffLUC activity is imaged every 30 min. during 12h light/12h dark cycles at T=22°C and RH=70% for three days (see log of conditions in Supplemental Figure S3A). The default light regime provided during the photoperiod is a combination of blue (B), red (R) and far red (FR) light provided by LEDs. To mimic changes in light intensity and R:FR ratio under natural light conditions during the day a one-hour light ramp during the first and last hour of the photoperiod is used. The total light intensity during ramping is 30 µmole m^{-2} s^{-1} and 100 µmole m^{-2} s^{-1} during the remaining hours of the photoperiod (Supplemental Figure S1A). The intensity ratio B:R:FR light during ramping is 1:2:1 and during the remaining hours of the photoperiod 3:6:1 (Supplemental Figure S4A). As a result, the PSS is 0.75 during light ramping and 0.82 during the remaining hours of the photoperiod (Supplemental Figure S1C, right panel). These variations in light conditions in LUMINATOR are selected based on the observation of changes in natural light conditions during dawn/dusk and mid-day (Supplemental Figure S1, left panels and (Smith, 1982; Mortensen and Moe, 1992; Urban et al., 2012; Li et al., 2014)).

Diurnal ffLUC reporter activity in mature rosette plants

To monitor key transcriptional processes in plants under different light and temperature conditions we selected a reporter plant expressing *PIF4::LUC* (López Salmerón, 2013), representative of activity stimulating elongation, a reporter plant expressing *HY5::LUC*, representative for suppression-of-elongation activity, and a reporter plant expressing *GI::LUC* (Edwards et al., 2010), representative for the biological clock activity in plants. In addition, we monitored activity in a control reporter plant expressing a *35S::LUC* construct (Edwards et al., 2010). Here we show diurnal activity of these ffLUC reporters in mature Arabidopsis rosette plants. The *GI::LUC* activity shows regular oscillations with a peak around ZT(h)=10 (Figure 2), which is later than the peak for *GI* mRNA levels in Arabidopsis seedlings (ZT(h)=8) (Mockler et al., 2007; Edwards et al., 2010). The *HY5::LUC* activity shows a more irregular activity pattern during the day and peaks at ZT(h)=3 (Figure 2). In contrast, *HY5* mRNA levels peak at dawn in seedlings (Mockler et al., 2007). Also *PIF4::LUC* activity shows irregular activity during the day. The peak in *PIF4::LUC* is around ZT(h)=8, compared to a peak in *PIF4* mRNA levels at ZT(h)=6 in seedlings (Mockler et al., 2007; Nusinow et al., 2011) (Figure 2). The activities of *HY5::LUC* and *PIF4::LUC* anticipate dawn, indicating regulation by the circadian clock.

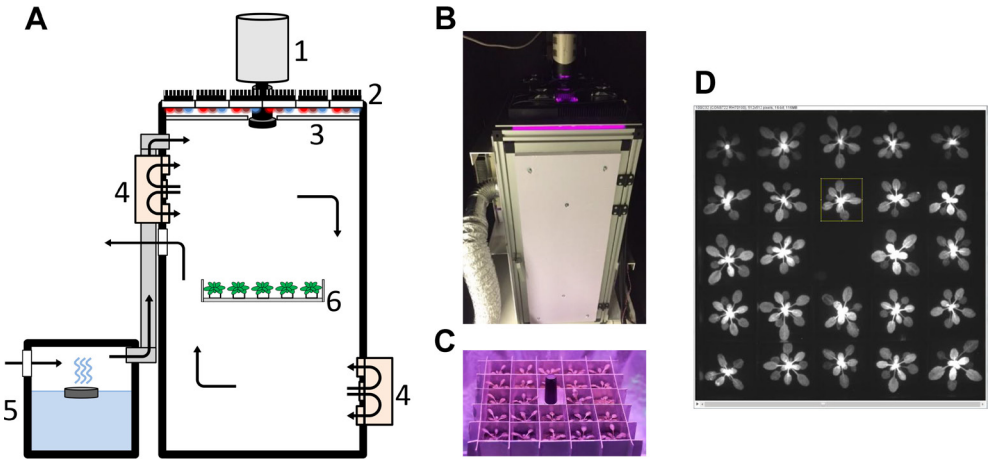


Figure 1: LUMINATOR set-up. A: Schematic drawing of LUMINATOR with high performance CCD camera on top (1) of which the lens sticks through a hole in the ceiling fitted with red, blue and far red LEDs (2) and a transparent plate covering the LEDs (3). Temperature is regulated by two thermoelectric cooling units (4) and humidity by a custom made humidifier (5) blowing humidified fresh air in the cabinet. Grid holding reporter plants to be imaged is placed in the centre of the cabinet (6). Arrows represent airflow through the system. **B**: LUMINATOR system. **C**: Custom made aluminium grid that holds Arabidopsis reporter plants. Data logger placed in the centre of the grid. **D**: Screenshot of image taken with LUMINATOR and adjusted with ImageJ software.

The direct response to the day light condition is different for the four reporter genes. While *GI::LUC* does not respond to the ramping light conditions, both *PIF4::LUC* and *35S::LUC* activity shows a direct upregulation of activity in response to light (Figure 2). In contrast, the *HY5::LUC* reporter activity decreases during ramping light conditions, but increases during the full light condition (Figure 2). The sharp peaks in *35S::LUC* activity at ZT(h)=2.5 and ZT(h)=8 coincide with the times of re-spraying with luciferin. A small transient increase in ffLUC activity at these time points are observed for all reporters. This indicates that luciferin may become limiting in some cells of the leaf, especially when ffLUC activity is very high as in the *35S::LUC* reporter plants. When luciferin is continuously supplied through the roots the peaks *GI::LUC* and *35S::LUC* activity are not observed (Supplemental Figure S5). However, in this situation the overall ffLUC activity remains much lower, indicating a continuous limiting supply of luciferin for the potential ffLUC activity in the shoot.

The expression profiles of *GI::LUC*, *HY5::LUC* and *PIF4::LUC* were validated at selected time points by qRT-PCR on endogenous gene mRNA transcript levels. For this, mRNA was isolated from the shoot of Col-0 plants grown in LUMINATOR under the same light and temperature conditions as used for the ffLUC activity measurements. Results show a good match between profile in *PIF4::LUC* and *GI::LUC* activity and profile of corresponding endogenous mRNA expression levels (Figure 3). For *HY5::LUC* the activity profile shows a smaller amplitude then for the endogenous *HY5* mRNA profile (Figure 3). This indicates that the *HY5* promoter used in the reporter construct may by

lacking elements required for downregulation at some parts of the day. In general, the peak ffLUC activities are about two hours delayed compared to the peaks in mRNA level.

We note that the ffLUC activity is quantified from top-view images of rosettes and that the movement of leaves can affect the efficiency at which ffLUC activity is captured. Supplemental Figure S6A shows the projected leaf area of rosette plants over a period of 24 hours and Figure S6B shows the projected leaf length of selected leaves in rosette plants over 24 hours. Comparison of the projected rosette area or the pattern of projected leaf length (both indicative for leaf movement) with the pattern of ffLUC activity of the different reporters indicates that leaf movement is not dominant over the ffLUC activity profiles determined by promoter activity of the reporter constructs. This is also due to the fact that the highest ffLUC activity is usually associated with the rosette center containing the sink leaves (Figure 1D). Indeed, excluding moving (source) leaves by only measuring luminescence in the center of the rosette did not lead to large qualitative changes in the ffLUC activity profiles (Supplemental Figure S7B+C).

The ffLUC activity images were also used to determine whether the phase in reporter activity is different in sink and source tissues. For this, ffLUC activity was quantified separately in source and sink leaves (Supplemental: Figure S7A+B). Results show that for all reporters the qualitative expression pattern (timing of peaks) was similar in source and sink. However, these results do show that the relative ffLUC activity in source and sink tissue differs for each reporter and may change over the day, as demonstrated by the plotted ratio $ffLUC_{source}$:$ffLUC_{sink}$ changes over the day (Supplemental Figure S7D).

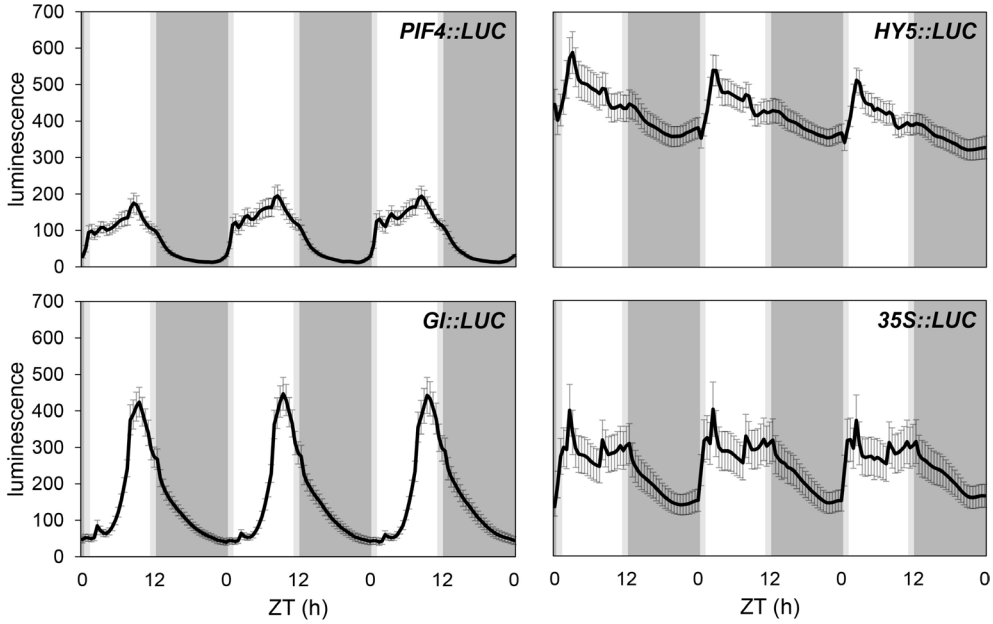


Figure 2: Diurnal ffLUC profile at constant temperature. Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* Arabidopsis reporter plants grown three subsequent days (12hL/12hD) under light ramping regime at constant temperature (22°C) and RH (70%). Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6). Data are mean luminescence ± SE (n=6), measured every 30 min.

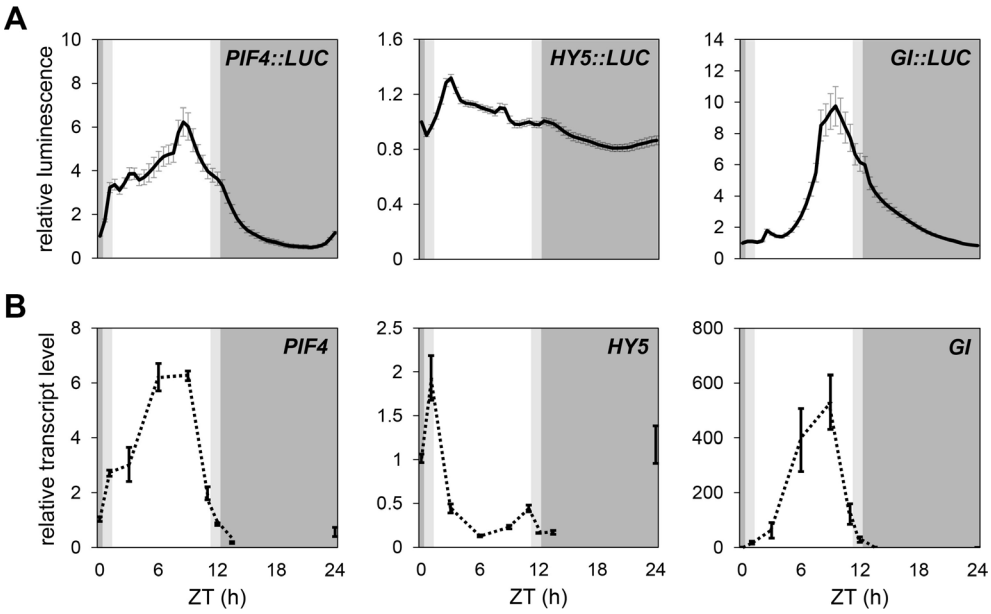


Figure 3: Normalized ffLUC profile and endogenous gene expression. A: Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC* and *GI::LUC* Arabidopsis reporter plants. Data are mean relative luminescence ± SE (n=6) calculated using data day 1 of Figure 2. **B**: *GI*, *HY5* and *PIF4* expression at ZT(h)=0, 1, 3, 6, 9, 11, 12 and 14 in 4-week-old Arabidopsis Col-0 WT plants grown under same conditions as reporter plants in A. Data are mean relative transcript levels ± SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780). Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6).

Light on/off regime results in lower *PIF4::LUC* but higher *HY5::LUC* activity

The activity of the two reporters representing key genes in control of elongation (*PIF4::LUC* and *HY5::LUC*) both show strong activation in the light, but opposite responses to the light ramping conditions (Figure 2 and 3A). This indicates that expression of these genes (and possibly elongation) may be controlled by short, well-timed, treatments in light intensity and/or quality. Indeed, selection of such light treatments may be based on the direct transcriptional responses of *PIF4* and *HY5*. For this purpose we tested whether LUMINATOR can be used to detect the effect of added light treatments on *HY5::LUC* and *PIF4::LUC* reporter activity. The set of reporter plants was imaged for one day growing under the default light profile with light ramping at dawn and dusk, followed by two days light without light ramping (lights on/off; Supplemental Figure S4B). This is effectively a treatment with added R+B light during dawn and dusk.

The ffLUC activity in the different reporter plants was again determined every half hour (Supplemental Figure S8). The direct short term effects (Figure 4A+B) and the effect on *PIF4::LUC* and *HY5::LUC* activity integrated over-day or over-night period (Figure 4C) were quantified. For the short-term responses of the reporters the relative ffLUC activity at day one (default light ramping condition) is compared to the relative ffLUC activity at

day three (light on/off) (Figure 4A+B). Results show that the short term response to the added light treatment is a downregulation of *PIF4::LUC* activity at dawn and dusk, while *HY5::LUC* activity is higher at dawn and dusk. Supplemental Figure S9 shows the same results, but here the data from Figure 4A and B were normalized to the ffLUC activity at dawn/dusk on day one and day three of ffLUC reporter plants grown under light ramping conditions for three subsequent days (Supplemental Figure S10) to correct for changes in ffLUC activity over three days of growth under light ramping (control) conditions.

For the dawn period, when effects were most pronounced, the changes in ffLUC reporter activity were validated for endogenous *PIF4* and *HY5* transcription by quantifying mRNA levels at selected time points (Supplemental Figure S11). This confirms that the light treatment results in significant (opposite) changes in *PIF4* and *HY5* mRNA levels after 1 hour of light treatment at dawn. Compared to the short-term effect of the additional light treatment on reporter activity in the morning, the effect on *PIF4::LUC* and *HY5::LUC* reporter activity at end-of-day is very small (Figure 4B). Indeed, *PIF4* transcript levels were not significantly different at end-of day in response to the additional light treatment. However, qRT-PCR analysis shows that *HY5* transcription is significantly upregulated at the end of day by the additional light treatment (Supplemental Figure S11B). The discrepancy between the response of the *HY5::LUC* reporter and endogenous *HY5* transcription at dusk seems to be caused by the limited downregulation of *HY5::LUC* activity during control (ramping) conditions. This is consistent with the *HY5::LUC* reporter missing regulatory elements that are required for downregulation of the *HY5* promoter in the light. The same analyses of the *GI::LUC* and *35S::LUC* reporters show that the short-term activity of these reporters is higher at dawn and dusk under a lights on/off regime, with a most pronounced effect at dawn (Figure 4A+B).

Elongation responses of the plant not only depends on short term responses of *PIF4* but also on the prolonged activity of *PIF4*, especially at night, when PIF4 protein is stable. During the day PIF4 protein is targeted for destruction by interaction with the active Pfr form of light receptor Phytochrome B (Lorrain et al., 2008). In contrast, suppression of elongation in the plant is dependent on prolonged activity of *HY5,* especially during the day when the HY5 protein is stable. At night HY5 protein is targeted for destruction by interaction with COP1 (Osterlund et al., 2000). We therefore also quantified the integrated activity of *PIF4* and *HY5* during the full day and full night period. For comparison, the *PIF4::LUC* activity and *HY5::LUC* activity was integrated over the day and night period of the first day under default light conditions (light ramping) and normalized to integrated reporter activity during the day and night period of the third day under the added R+B light treatment (lights on/off). The same was done for the *PIF4::LUC* activity and *HY5::LUC* activity of plants grown for three subsequent days under light ramping conditions. Results indicate a significant reduction in integrated *PIF4::LUC* activity during the night under the lights on/off regime, while the integrated activity of *HY5::LUC* during the day was the same under both light regimes (Figure 4C). When the same analysis is done for the *GI::LUC* and *35S::LUC* reporters no difference in integral day and night activity is detected for the two light treatments (Figure 4C). The lower integrated *PIF4::LUC* activity during the night in response to a short term light

treatment, indicates that a short light treatment has long lasting effects and may be potentially be used to control plant elongation responses.

Light on/off regime results in shorter Tomato plants

To determine whether the short-term light treatment tested under LUMINATOR can be correlated to plant growth responses under more realistic growth conditions, the effect of the added light at dawn and dusk was tested for Tomato plants (*Solanum lycopersicum* cv. 'Money maker'), as tomato is an important greenhouse crop species. Tomato plants were pre-grown for eight days under a default light regime mimicking a more natural day light spectral composition with natural changes in light intensity and PSS than can be obtained under LUMINATOR (Supplemental Figure S1, middle panels). Subsequently, plants were grown for an additional two weeks under the same light conditions or under a light on/off regime, effectively resulting in added R+B light at dawn and dusk. To make growth conditions even more realistic, temperature was not constant but kept at a diurnal cycle of 25°C during the day and 15°C at night. Figure 5 shows the effect of the two light regimes on growth of Tomato plants after two weeks. Plants grown under the simple lights on/off regime had shorter hypocotyls, shorter epicotyls and reduced total leaf area compared to those grown under a more natural light regime. This suggests a lower activity for the *PIF4* ortholog in tomato in response to the added light treatment, similar as was found for the integral *PIF4* reporter activity in Arabidopsis under added R+B light.

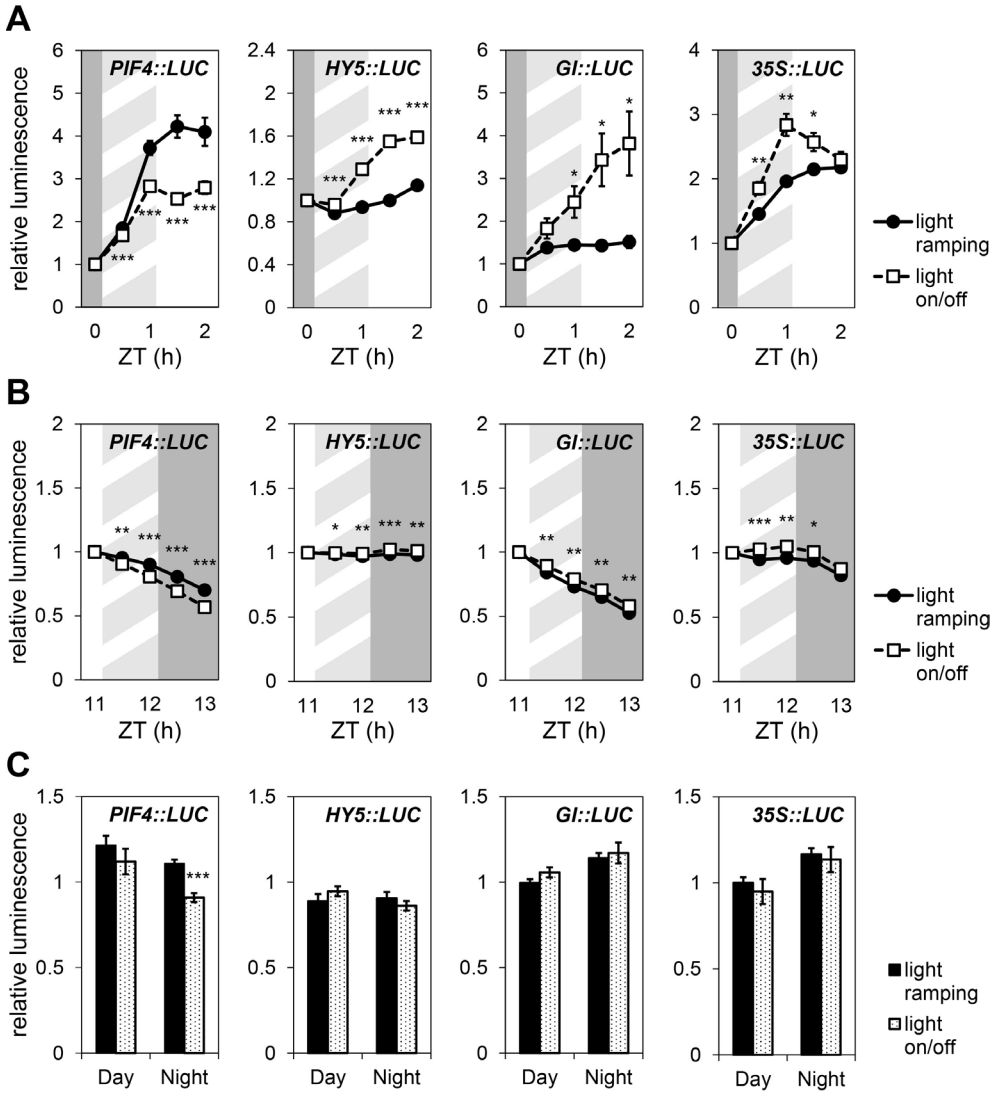


Figure 4: Effect of lights on/off regime on immediate and integral ffLUC activity. A+B: Reporter plants were grown three subsequent days (12h light / 12h dark) under light ramping regime on day 1 and light on/off regime on day 2 and 3, at constant temperature (22°C) and RH (70%). Graphs show relative changes in luminescence of *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* during start (**A**) and end (**B**) of day 1 (light ramping; solid line, black dots) or day 3 (light on/off; dashed line, white squares). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 3 of Supplemental Figure S9. Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6). **C**: Relative integral ffLUC activity of *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* reporters during day or night period on day 3 compared to day 1. Reporters were grown under light ramping regime on day 1-3 (black bars), or under light ramping regime on day 1 and light on/off regime on day 2 and 3 (white bars). Data are mean relative integral luminescence ± SE (n=6) calculated using data day 1 and 3 of Figure 2 and Supplemental Figure S9. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative luminescence during light ramp.

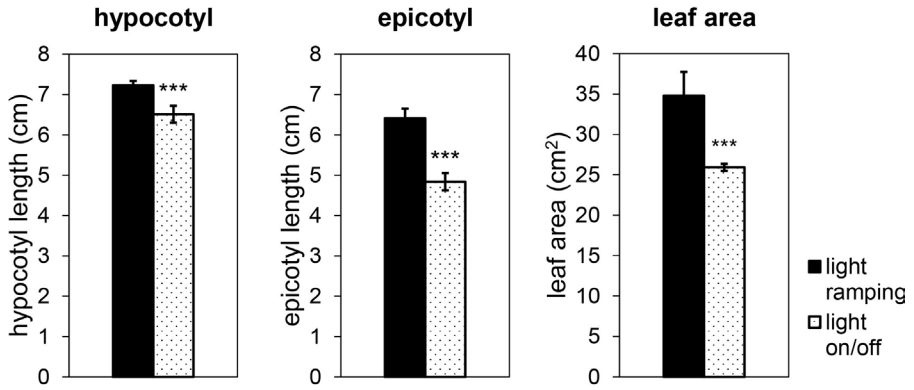


Figure 5: Effect of lights on/off regime on growth of tomato plants. Hypocotyl length, epicotyl length and leaf area of 22-day-old tomato (*Solanum lycopersicum* cv. 'Money maker') plants that were grown under 12h light/ 12h dark cycles. Light conditions included variable PSS and light intensity (mimicked natural light; black bars) or constant PSS and light intensity (lights on/off; white bars). For detailed description of light conditions see materials and methods. Temperatures were always 25°C during light period and 15°C during dark period and RH constant 65%. Data are means ±SE (n=15) ***p<0.001 (Student's t-test) significantly different from plants grown under variable PSS and light intensity (ramping).

Responses of different ffLUC reporter lines to temperature changes

Characterization of ffLUC *in-vitro* indicates that the kinetics of ffLUC enzyme activity has a maximum between 20°C and 30°C (Koksharov and Ugarova, 2011) and like all enzymes, also the enzyme activity of ffLUC is sensitive to temperature. Therefore, in-planta ffLUC activity profiles under conditions of changing temperature are difficult to interpret as changes in activity may not be caused by changes in reporter promoter activity. However, LUMINATOR can be used to test the effect of different constant temperatures on ffLUC activity in the different reporter plants. For this, the ffLUC activity in reporter plants was imaged for one day at 22°C, followed by either two days at 27°C or two days at 12°C (see logs in Supplemental Figure S3C+D). For each experiment the temperature switch was at ZT(h)=0. Due to technical limitations RH was kept at 40% for the switch from 22 to 27°C, while RH was kept at 70% for the temperature switch from 22°C to 12°C. To discriminate between effect of temperature on ffLUC enzyme activity and effects of temperature on the reporter promoter activity, the ffLUC activities were again compared to changes in endogenous *GI*, *HY5* and *PIF4* transcript levels (Supplemental Figures S12 and S13).

As expected, all reporters show a short-term increase in activity when temperature is raised from 22°C to 27°C, while a decrease in temperature results in an immediate decrease in ffLUC activity (Figure 6). The relative short-term response to lower temperature is very similar for all reporters (Figure 7) and therefore may be due to a general effect of lower temperature on enzymatic ffLUC activity rather than an effect on promoter activity. In contrast, the reporters showed differences in their short-term relative responses to higher temperatures (Figure 7). This suggests that at least part of these

warm temperature responses is mediated by different temperature control of reporter activity.

The long-term effect of the temperature switch was analyzed by integrating the ffLUC activity of each reporter over the day or night period (Figure 8). These analyses indicate that temperature has a more pronounced effect on overall *PIF4::LUC* than on overall *HY5::LUC* activity: while integral *PIF4::LUC* activity is higher during the day, overall *HY5::LUC* activity during the day remains the same at 27°C and 22°C (Supplemental Figure S12A; Figure 8A). For the night period, both integral *PIF4::LUC* and *HY5::LUC* are lower at 27°C then at 22°C (Figure 8A).

At 12°C, *PIF4::LUC* activity is low compared to at 22°C, while *HY5::LUC* activity is actually higher (after the initial drop in activity, leading to higher integrated *HY5::LUC* activity during night (Supplemental Figure S13A+B). For the *GI::LUC* reporter, the increase in temperature from 22°C to 27°C resulted in small shift in ffLUC peak activity from ZT(h)=10 to ZT(h)=9 (Supplemental Figure S12A). During both day and night integrated *GI::LUC* levels were lower at 27°C compared to 22°C (Figure 8A). In contrast, *GI::LUC* activity at 12°C is lower during day but higher during the night (Figure 8B). The main phase in *GI::LUC* activity is advanced to ZT(h)=6 at 12°C (compared to ZT(h)=10 at 22°C) (Supplemental Figure S13A).

The *PIF4::LUC* reporter activity and *PIF4* mRNA levels qualitatively show similar changes at the different temperatures (Supplemental Figure S12 and Supplemental Figure S13). However, the higher *PIF4::LUC* reporter activity at 27°C (compared to at 22°C) suggest a higher *PIF4* transcription rate at 27°C, which is not confirmed by the *PIF4* mRNA measurements, except for the end-of-night timepoint. At 12°C, the *PIF4::LUC* activity is strongly reduced, and is representative of a strong reduction in endogenous *PIF4* gene transcription at 12°C (Supplemental Figure S13). Also the correlation between *HY5::LUC* and *GI::LUC* reporter activity at 27°C and the corresponding endogenous mRNA levels is more or less consistent (Supplemental Figure S12) when an added effect of higher temperature on ffLUC activity is taken into account (no higher endogenous gene transcription).

Similar to the discrepancy between *HY5::LUC* and *HY5* mRNA level for light responses, there is also a discrepancy between the *HY5::LUC* and *HY5* mRNA level in response to 12°C. While the *HY5::LUC* reporter shows a direct and strong down regulation at the onset of the day at 12°C, the endogenous *HY5* gene shows an upregulation at 12°C (Supplemental Figure S13). This suggests that the factor(s) acting on the regulatory elements that seem to be missing in the *HY5::LUC* reporter are not only light sensitive, but also strongly influenced by temperature.

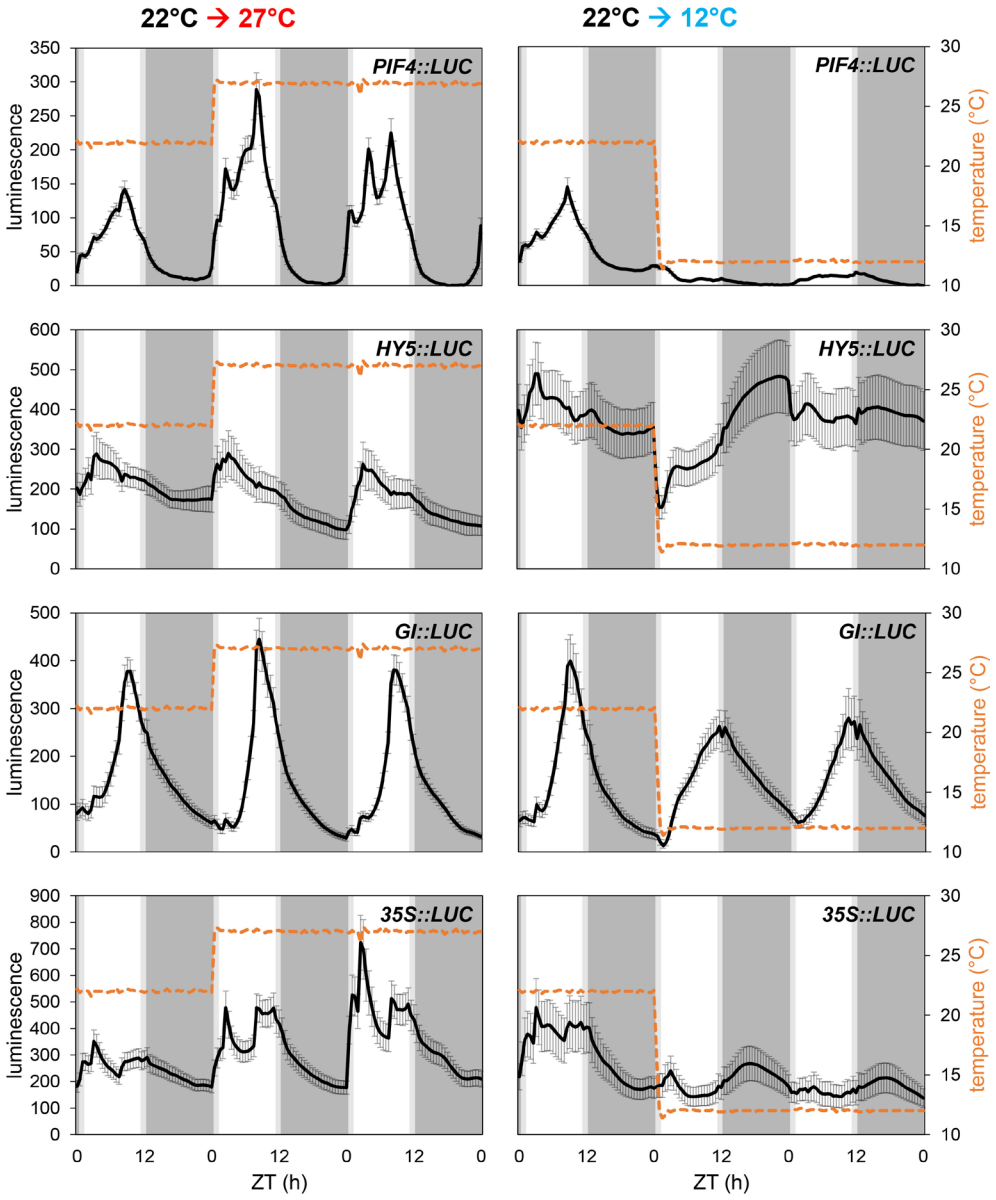


Figure 6. Effect of temperature changes on ffLUC activity. Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* Arabidopsis reporter plants grown three subsequent days (12h light / 12h dark) under light ramping regime. Temperature on day 1 was constant 22°C and changed to either constant 27°C (left) or 12°C (right) on day 2 and 3. Black line shows luminescence, orange line temperature (°C). Dark grey graph areas represent dark period, light grey areas represent light ramp conditions (30 µmole m^{-2} s^{-1}) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}). Data are mean luminescence ± SE (n=6), measured every 30 min.

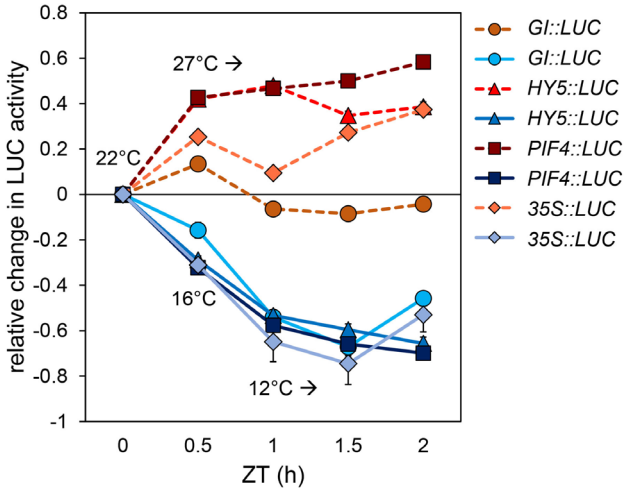


Figure 7: short-term effect of temperature changes on ffLUC activity. Relative change in ffLUC activity of *GI::LUC* (circles), *HY5::LUC* (triangles), *PIF4::LUC* (squares) and *35S::LUC* (diamonds) in response to temperature increase to 27°C (dashed red/brown lines) or temperature decrease to 12°C (solid blue lines). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 2 of Figure 2 and Figure 6.

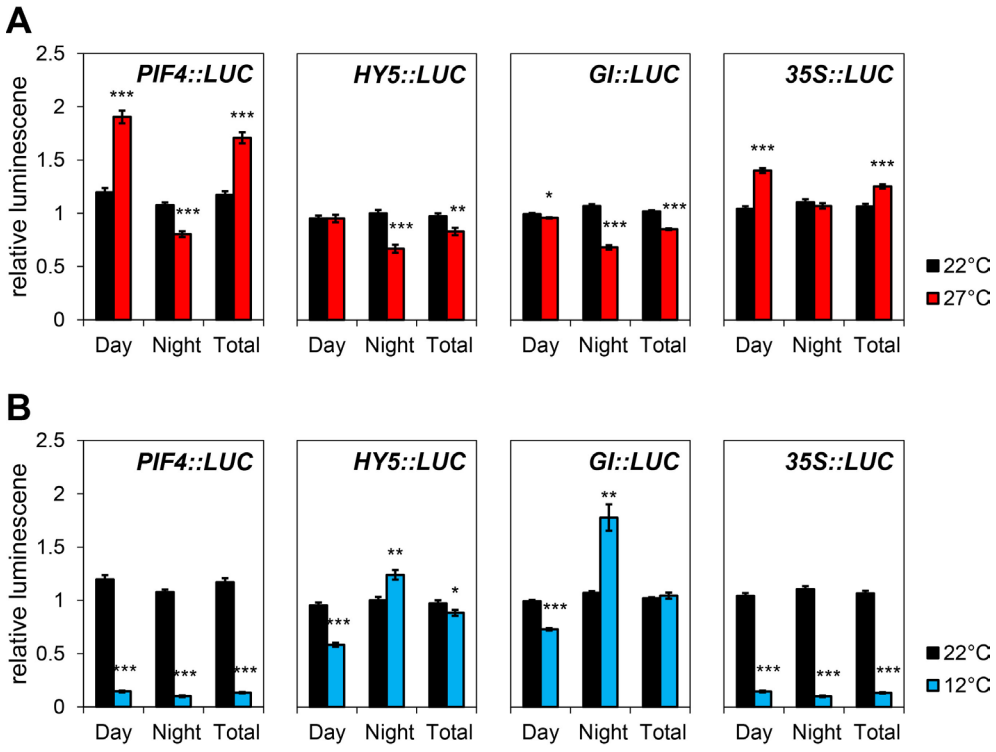


Figure 8: Effect of temperature changes on integral ffLUC activity. Relative integral ffLUC activity of *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* reporters during day, night or total 24h cycle on a day at T=27°C (**A**; red bars) or at T=12°C (**B**; blue bars) compared to a day at T=22°C (black bars). Reporters were grown at constant 22°C on day 1-3, or temperature was switched to 27 °C or 12°C at start day 2. Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 2 of Figure 2 and Figure 6. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative luminescence during light ramp.

Discussion

Analyzing ffLUC reporter activity in mature rosette plants

Here we tested whether in principle the transcriptional activity of selected ffLUC reporter genes can be used to screen the effect of a light treatment to determine the long-term effect on plant growth. For this we developed LUMINATOR, a self-made system to image ffLUC activity in reporter plants under controlled light and temperature conditions. We determined that when the supply of luciferin is maintained by daily spraying of the shoot with luciferin, LUMINATOR can be used to study short- and long-term transcriptional responses to light or temperature treatments. In contrast, supply of luciferin through roots is not sufficient to reveal the full potential of ffLUC activity in the shoot (Supplemental Figure S5). Translation of ffLUC reporter activity to the activity of the corresponding endogenous gene needs to be done with caution: Comparison of ffLUC activity profiles with corresponding endogenous gene transcript profiles shows a delay in ffLUC activity peak compared to the peak in mRNA levels of around 2h when plants were grown at 22°C. At low temperature the delay in peak ffLUC activity compared to related endogenous gene expression is even more pronounced, which is likely the consequence of reduced *ffLUC* mRNA translation, reduced ffLUC enzymatic activity and increased *ffLUC* mRNA stability at 12°C. The delays between peak ffLUC activity and phase of related endogenous gene should be taken into account when *in planta* ffLUC profiles are used to guide sampling for mRNA measurements. Integrating ffLUC activity over the day and night may therefore provide more reliable information on how overall (reporter) gene activity is affected by different treatments (Figure 4C and Figure 8).

The measurement of ffLUC reporter activity in mature rosette plants may also be influenced by leaf movement. The activity of clock reporter ffLUC genes has often been measured in seedlings, while also seedling cotyledons show circadian oscillations (Somers et al., 1998; Dowson-Day and Millar, 1999), while the potential effect of cotyledon movement on the ffLUC activity profile has never been determined. For mature Arabidopsis rosette leaves the effect of leaf movement may be stronger than for seedlings, and both light and temperature affect hyponastic leaf movement (Koini et al., 2009; Bours et al., 2012; Bours et al., 2013; Pierik and de Wit, 2014; Pantazopouloua et al., 2017). Therefore, the fine structure in the overall ffLUC activity profile may not solely be due to changes in promoter activity, but also due to changes in the efficiency at which leaf ffLUC activity can be imaged. However, our analysis indicates that changes in projected leaf length or projected rosette area over time do not dominate the changes in ffLUC activity of the reporters measured from LUMINATOR images. This is mainly due to the fact that the bulk of ffLUC activity is from the smaller sink leaves with no or very low amplitude in leaf movement. Although the use of ffLUC reporters in mature rosette plants is not without drawbacks, monitoring gene activity in mature rosette plants provides the added benefit of gene expression analysis as function of local sink or source activity in leaves. Indeed, dissecting the relative ffLUC reporter activity in source and sink provide insights into the signaling between source and sink leaves that may be revealed by some reporters (Supplemental Figure S7).

The *HY5::LUC* reporter activity profile does not fully correlate to *HY5* mRNA profile

Some promoter fragments used in the ffLUC reporter lines may not contain all relevant regulatory elements. This seems to be the case with the *HY5::LUC* reporter, for which only a relatively short promoter fragment of 756 bp was used. However, it is also possible that the partial discrepancy between the activity of *HY5::LUC* and endogenous *HY5* is caused by a position effect on the *HY5::LUC* transgene (Van Leeuwen et al., 2001). The discrepancy between *HY5::LUC* reporter and endogenous *HY5* mRNA transcript levels is most pronounced during the ramping period in the morning, where *HY5::LUC* activity is downregulated, while the endogenous *HY5* expression is upregulated. Also, expression of the *HY5::LUC* reporter is not downregulated, like the endogenous *HY5* gene, during the night. This suggests that the *HY5::LUC* reporter lacks an element for repressor activity at night and an activator activity at the onset of the light period. These could be two separate factors acting on the endogenous *HY5* promoter, perhaps targeting the same regulatory element for *HY5* that seems to be lacking in the *HY5::LUC* reporter.

Relative reporter activities in sink and source tissues reveals sink/source relations

Most luciferase imaging systems are only used for monitoring ffLUC activity in Arabidopsis seedlings or very young plants (Millar et al., 1992; Okamoto et al., 2005; Kamioka et al., 2016; Shalit-Kaneh et al., 2018). Being able to also monitor gene activity dynamics in mature rosette plants is important as regulation of gene expression may change over development. Moreover, regulation of key growth genes is also a function of local hormone and local sugar status (Blasing et al., 2005; Usadel et al., 2008; Sairanen et al., 2012; Zhao et al., 2018), resulting in potential altered transcriptional regulation in source and sink leaf tissue. Indeed, ffLUC activity in source and sink tissue is not the same for each reporter (Supplemental Figure S7). Previously is was shown that sugars can increase auxin through PIF4 (Sairanen et al., 2012). Indeed, relative *PIF4::LUC* activity in source and sink leaves shows indeed the strongest diurnal changes and increasing sugar availability during the day correlates with relative higher *PIF4::LUC* activity in sink leaves during the day, while decreasing sugar availability during the night correlates with decreasing *PIF4::LUC* activity in sink leaves (Supplemental Figure S7D). The entrainment of the circadian clock in plants may be through light, temperature, and sugars (Somers et al., 1998; McClung et al., 2002; Salome and McClung, 2005; Thines and Harmon, 2010; Haydon et al., 2013; Oakenfull and Davis, 2017; Frank et al., 2018). Of these, sugars are endogenous signals that may differ between source and sink leaves. The relative *GI::LUC* activity in sink and source leaves suggest that, in contrast to the effect on *PIF4*, sugars are a negative regulator of *GI* activity with increasing sugar levels during the day correlating with increasing suppression of relative *GI::LUC* activity in sink leaves compared to source leaves during the day, and decreasing sugar levels at night with decreasing suppression of relative *GI::LUC* activity in sink leaves (Supplemental Figure S7D). Interestingly, the ratio of relative activity in source and sink leaves changes about two hours before the actual end of the twelve hour photoperiod, both for *PIF4::LUC* and *GI::LUC*, consistent of a common regulatory factor with opposite effects on *PIF4* and

GI promoter activity. Currently we are pursuing a more comprehensive analysis of different reporter activity in source and sink tissues of mature Arabidopsis rosette plants in relation to local carbohydrate metabolism.

Added R+B light suppresses overall *PIF4* transcription at night

The effect of added R+B light on *PIF4::LUC* or *HY5::LUC* activity was quantified for mature rosette plants. Both growth related genes show a strong but opposite direct response to the light treatment in the morning (Figure 4A), while the direct effect for the evening light treatment was absent or very small (Figure 4B). However, when integrated over the full photoperiod, the effect of the morning light treatment on day-integral of either *PIF4::LUC* or *HY5::LUC* activity was not significant. However, the light treatment did produce a significant lower night-integral *PIF4::LUC* activity (Figure 4C). The transcriptional responses in the morning as reported by ffLUC reporter activity were in agreement with changes in endogenous *PIF4*, *HY5* and *GI* mRNA transcript levels. For the interpretation of the effect of the added light treatment on transcription of *PIF4* and *HY5*, and translation to potential effects on plant elongation responses, additional factors acting on protein stability or protein activity need to be taken into account. The result of decreased *PIF4* transcription during the night is therefore especially relevant since PIF4 protein is more stable at night.

The higher *HY5* and *GI* promoter activity in direct response to added R+B light suggests that transcription of these genes is sensitive to light intensity and/or increased R:FR. Indeed, several publications demonstrate stimulation of these promoters by light (Paltiel et al., 2006; Abbas et al., 2014; Binkert et al., 2014; de Wit et al., 2016). The *HY5* promoter activity is under positive feedback regulation by HY5 protein (Abbas et al., 2014; Binkert et al., 2014), while HY5 protein level is determined by nuclear COP1, a negative regulator of HY5 protein stability (Osterlund et al., 2000). The direct stimulation of *HY5* promoter activity in the morning upon added R+B light may therefore be due to increased HY5 protein stability resulting from a more rapid inactivation of nuclear COP1 activity. The results are consistent with B light affecting *HY5* transcription in seedlings (de Wit et al., 2016) and positive regulation of HY5 promoter binding activity under blue light (Lian et al., 2018). Moreover, as the HY5 protein can bind to the *PIF4* promoter and is a negative regulator of *PIF4* transcription in seedlings, the increased *HY5* and decreased *PIF4* promoter activity in the first hour of added R+B light may causally be related (Lee et al., 2007; Delker et al., 2014; Toledo-Ortiz et al., 2014; Zhang et al., 2017). Thus, determining integral *HY5* transcription may help interpret the actual relevance of *PIF4* transcription for plant elongation responses.

The overall response to R+B is a decreased *PIF4* activity during the night with little or no effect on overall *HY5* activity, and therefore the light treatment is predicted to result in more compact plants. This was tested in tomato because Tomato is an important crop species for greenhouse horticulture and has functional homologues of many light-signaling components that control cell growth in Arabidopsis, including HY5 and PIFs (Liu et al., 2004; Rosado et al., 2016). More realistic light and growth conditions were used in

the tomato experiment than is possible in LUMINATOR to determine the validity of results obtained under 'laboratory' conditions in practice in greenhouses. As predicted from the two-day transcriptional responses in Arabidopsis, the treatment of young Tomato plants by a simple lights on/off regime instead of a more natural light regime with variable light intensity and quality over the day resulted in more compact plants (Figure 5). Thus, short-term responses of reporter activity to light treatments were indeed predictive for long-term growth responses. LUMINATOR therefore provides a tool to help identify novel light treatments that potentially can be used to control plant growth.

No higher *PIF4* transcription at 27°C

LUMINATOR was also used to test ffLUC activity at different temperatures. In response to a temperature changes from 22°C to 12°C and 22°C to 27°C all reporters showed an immediate drop or increase in ffLUC activity, respectively (Figure 7). This is expected since temperature affects enzymatic activity of ffLUC, which has an optimal activity between 20°C and 30°C (Koksharov and Ugarova, 2011). However, reporters showed different diurnal ffLUC activity profiles, which indicates additional differential regulation of the promoters by temperature (Figure 6). The difference in *PIF4::LUC* activity between 22°C and 27°C is not reflected in changes in *PIF4* mRNA. The light conditions in LUMINATOR apparently are sufficient to prevent the high ambient temperature induction of *PIF4* transcription as has been shown for thermomorphogenic responses at 27°C under different light intensities (Qiu et al., 2019). Indeed, it was shown that the transcriptional upregulation of *PIF4* during thermomorphogenesis is affected by light quality and intensity through COP1 and CRY1 (Ma et al., 2016; Park et al., 2017; Qiu et al., 2019). Moreover, the integral activity during the day of *PIF4::LUC*, but not of *HY5::LUC*, is increased at 27°C, and may be indicative of higher elongation activity at 27°C. The equal transcriptional activity of *PIF4* at 22°C and 27°C does not exclude a higher PIF4 protein activity and may still induced enhanced elongation responses: Although the consensus is that PIF4 protein is not stable in the light due to interaction with light-activated phytochrome B, recently it was shown that increased PIF4 activity during the day at high temperature may still contribute to elongation responses (Zhu et al., 2016; Qiu et al., 2019). In contrast, the low temperature of 12ºC decreased integral *PIF4::LUC* activity both during day and night, while integral *HY5::LUC* activity during the night is increased at 12ºC. HY5 protein is destabilised at night by COP1, while COP1 is stabilized at low temperature (Jang et al., 2015). The reduced elongation of Arabidopsis at low temperature may therefore be mostly due to the reduced *PIF4* transcription and PIF4 protein activity.

Conclusions

LUMINATOR, together with appropriate ffLUC reporter plants, may provide a tool to help find new light treatments that can be used to control plant growth in greenhouses in a much more efficient way. Monitoring ffLUC reporter activity provides a high temporal resolution of transcriptional activity of key genes for growth in the same plant and

provides an overview of local activity within the plant. Obtaining similar type of data using mRNA measurements would require an impractical number of independent plant samples, individual RNA isolations and sequencing, and would be far more time consuming and costly. Although the use of ffLUC reporters in mature plants does come with drawbacks as discussed above, they still provide a useful screening method for which promising treatments can be validated with more direct endogenous gene measurements. Transcriptional responses to light treatments can be directly monitored in LUMATOR with ffLUC reporters, but effects of temperature on ffLUC protein activity and post-transcriptional regulation of protein activity should be taken into account in interpretation of results. With the appropriate reporter plants effective timing, intensity and color of short-term light treatments may be determined for most cost-efficient application to control plant growth in greenhouses.

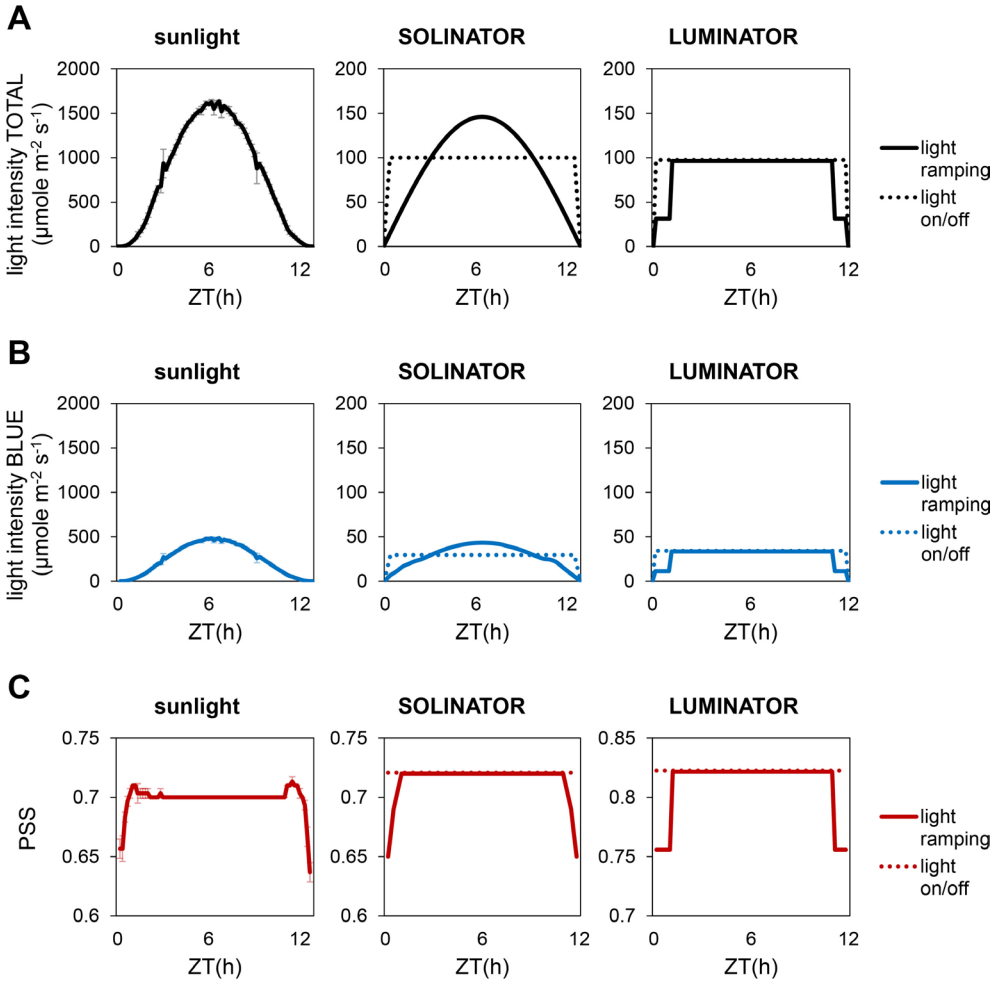
Supplemental Data

Supplemental Table S1: Primers used for cloning. First column shows primer name, second primer sequence.

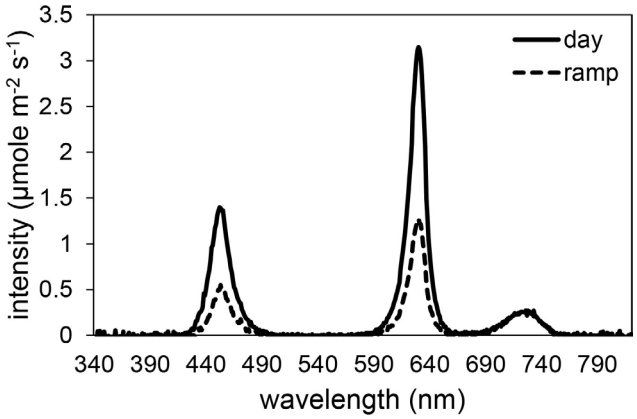
primer name	*sequence*
HY5 promoter fw	CACCTCTAATGTTAACGTTGAGATGGC
HY5 promoter rev	TTTTCTTACTCTTTGAAGATCGATCA

Supplemental Table S2: Primers used for qRT-PCR. First column shows primer name, second column shows TAIR id of gene and third column shows primer sequence.

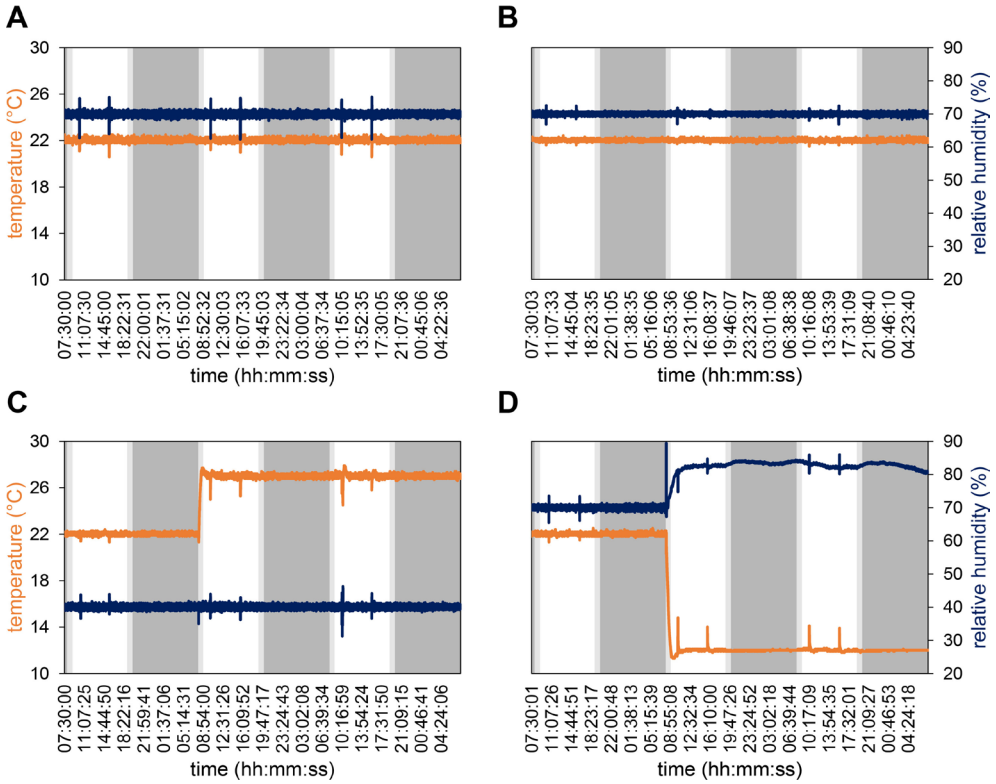
primer name	*TAIR id*	*sequence*
GI fw	AT1G22770	TTTCTCCGATATTCGTCGATCTC
GI rev	AT1G22770	CTGAATCAAACAGCTAAACCC
HY5 fw	AT5G11260	GTCGGAGAAAGTCAAAGGAAGC
HY5 rev	AT5G11260	TTCCTCTCTCTTGCTTGCTGAG
PIF4 fw	AT2G43010	ACCTCAGCAGTTCATACGTCAG
PIF4 rev	AT2G43010	TGTACCGGGTTTTGGCAAAC
YLS8 fw	AT5G08290	TTACTGTTTCGGTTGTTCTCCATTT
YLS8 rev	AT5G08290	CACTGAATCATGTTCGAAGCAAGT
IPP2 fw	AT3G02780	CATGCGACACACCAACACCA
IPP2 rev	AT3G02780	TGAGGCGAATCAATGGGAGA



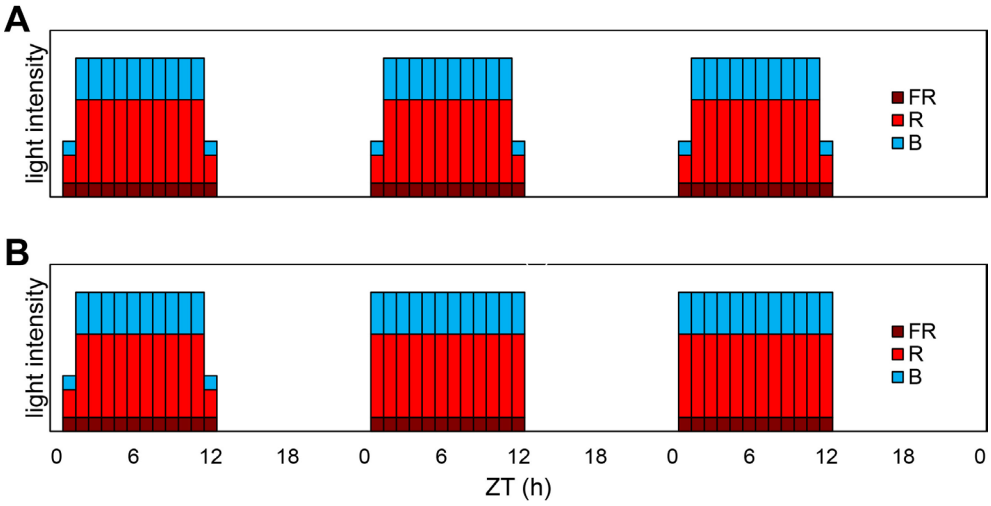
Supplemental Figure S1: Light conditions LUMINATOR and SOLINATOR compared to natural light. Daily variations in total light intensity (**A**), blue light intensity (**B**) and photostationary state (PSS; **C**) under natural sunlight (left panels), in SOLINATOR (middle panels) and in LUMINATOR (right panels). Natural sunlight was measured outside at Haarweg, Wageningen, The Netherlands (N 51° 58.2', E 5° 40.0') from the 28th until the 30th of September, 2011 (cloudless days). Graphs show average light intensities and PSS ±SE. Variations in light intensities and PSS under light ramping conditions (solid lines) in SOLINATOR and LUMINATOR were based on natural sunlight measurements, but not identical due to technical limitations of the systems. During light on/off conditions (dashed lines), light intensities and PSS were kept constant in SOLINATOR and LUMINATOR. In SOLINATOR, daily light integral under both light conditions was the same.



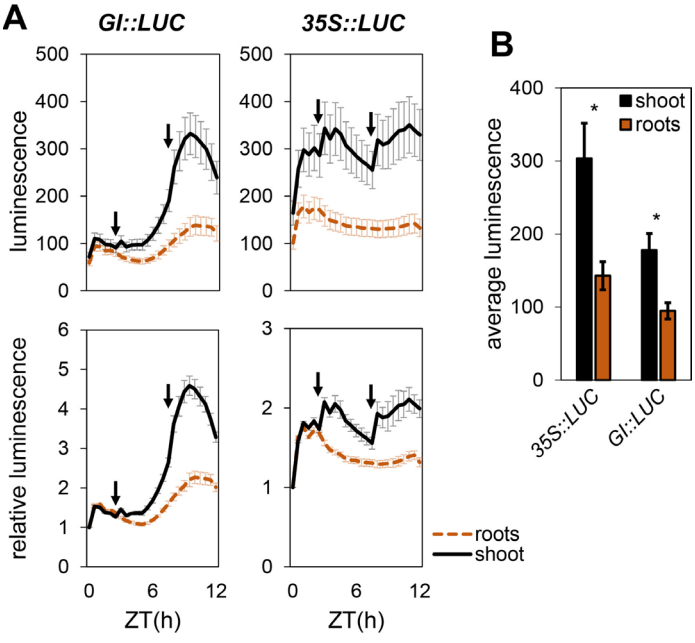
Supplemental Figure S2: Light spectrum LUMINATOR. Spectrum of day (solid line) and ramp (dashed line) light conditions. Spectra were measured using a Flame-T spectroradiometer (Ocean Optics, Duiven, The Netherlands). For measurements sensor was placed at plant height in the center of the grid holding reporter plants.



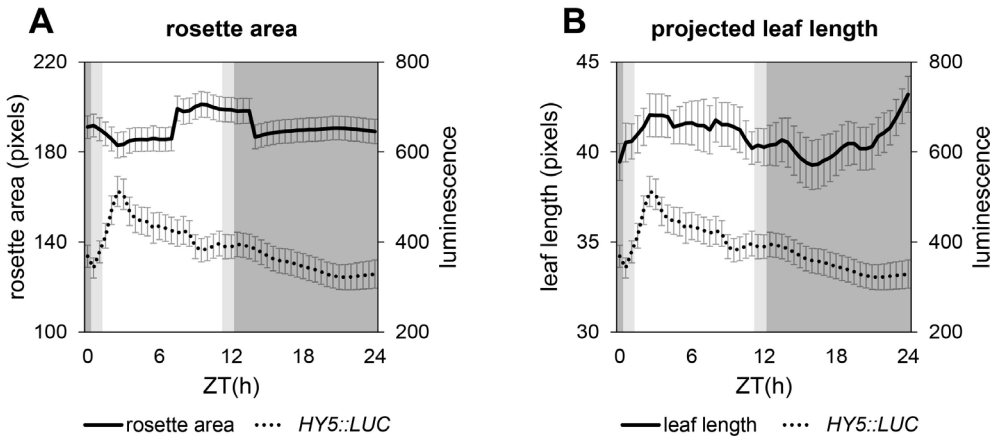
Supplemental Figure S3: Temperature and relative humidity logs LUMINATOR climate cabinet. Temperature (°C; orange line) and relative humidity (%; blue line) in center of LUMINATOR cabinet measured every 5 seconds during light ramping regime (control) experiment (**A**), light on/off regime experiment (**B**), increased temperature experiment (**C**) and temperature drop experiment (**D**). Dark grey graph areas represent dark period, light grey areas represent light ramp conditions (30 µmole m^{-2} s^{-1}) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}).



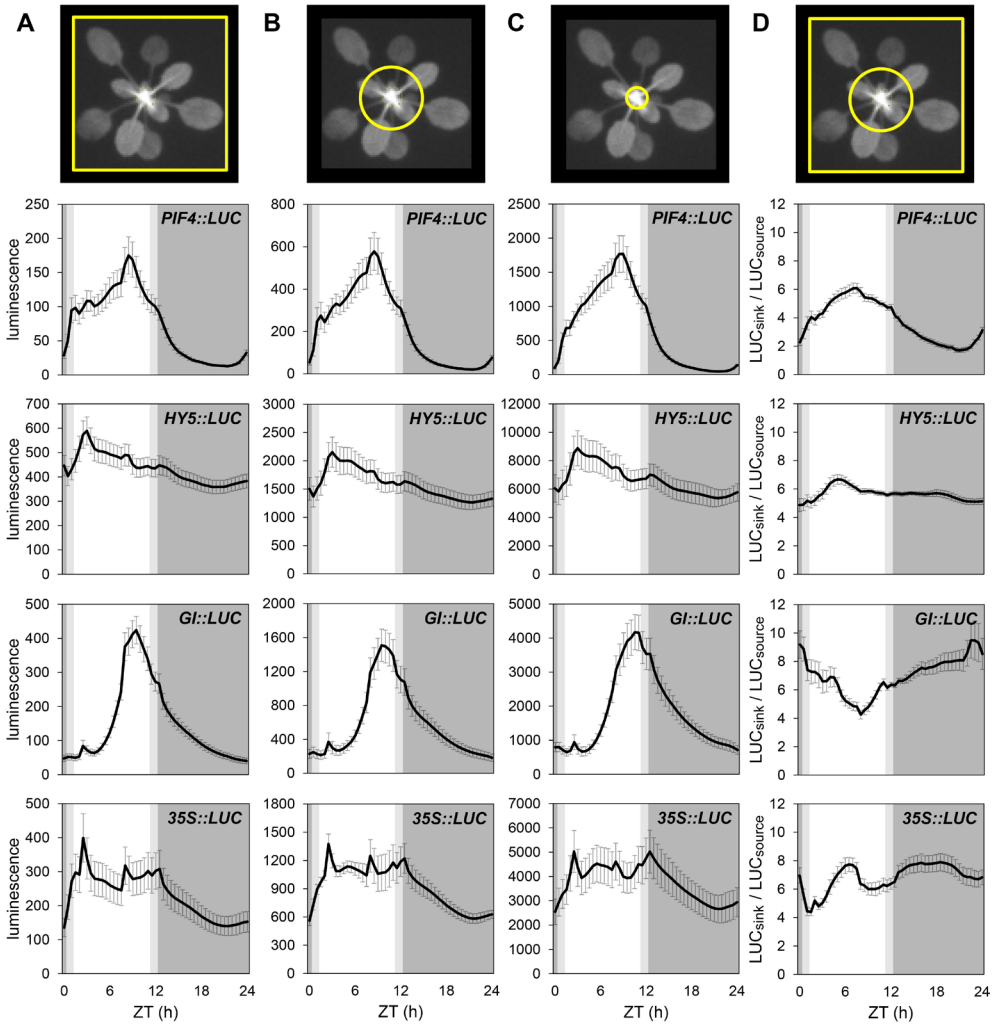
Supplemental Figure S4: Schematic representation of different light treatments in LUMINATOR. A: Light conditions light ramping regime (every day 1h light ramp during first and last hour of photoperiod). **B**: Light conditions lights on/off regime (no light ramp on day 2 and 3). Dark red bars represent light intensity FR LEDs, red bars light intensity R LEDs and blue bars light intensity B LEDs. Max. light intensity is 100 µmole m^{-2} s^{-1}.



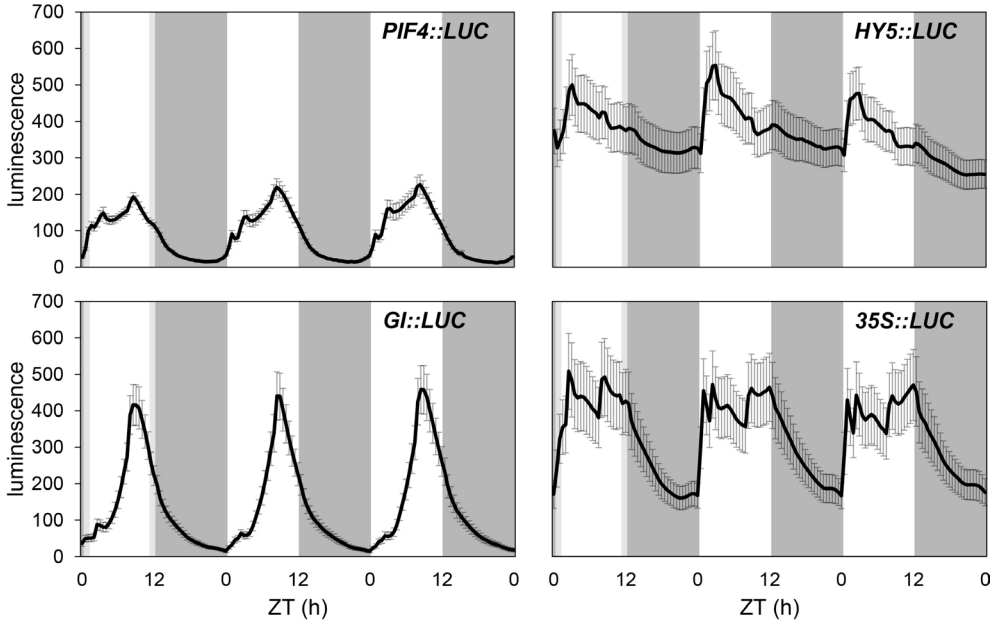
Supplemental Figure S5: Effect of different ways of luciferin application on ffLUC activity. A: ffLUC profiles of *GI::LUC* and *35S::LUC* Arabidopsis reporter plants during 12 hour photoperiod. Upper graphs show actual luminescence, lower graphs show relative luminescence (to ZT(h)=0). Luciferin was supplied to the rockwool on which plants were grown (brown dashed line) 24h prior to start imaging, or by spraying it on the leaves (black solid line) twice during imaging. Arrows indicate moment of luciferin application by spray. Data are mean luminescence ± SE (n=6). **B**: Average ffLUC activity of *35S::LUC* and *GI::LUC* reporter plants from **A** during the 12h photoperiod. Luciferin was supplied to the rockwool on which plants were grown (brown bar) 24h prior to start imaging, or by spraying it on the leaves (black bar) twice during imaging. Data are mean luminescence ± SE (n=6). *p<0.05 (Student's t-test) significantly different from relative luminescence in plants that got luciferin through roots.



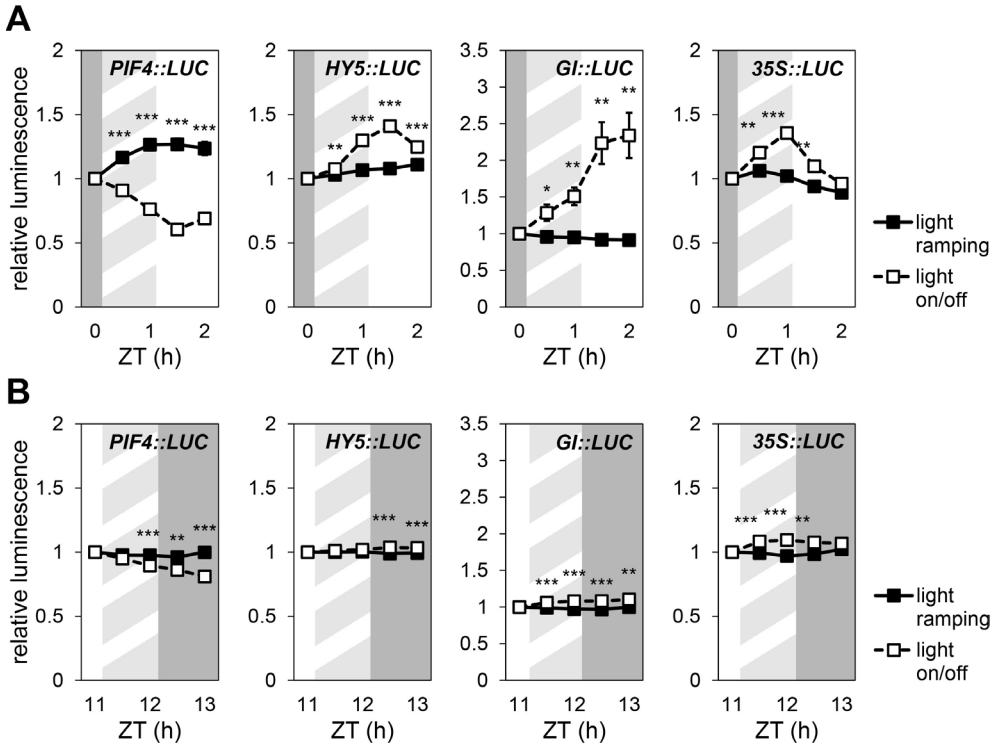
Supplemental Figure S6: Variations in rosette area and leaf length compared to *HY5::LUC* profiles. **A**: Projected rosette area (solid line) and *HY5::LUC* activity (dashed line) of 4-week-old *HY5::LUC* reporter plants during 24 hours at 22°C, measured from LUMINATOR images used to obtain ffLUC activity profiles in Figure 2. Data are mean projected rosette area ± SE (n=6). **B**: Projected leaf length (solid line) and *HY5::LUC* activity (dashed line) of 4-week-old *HY5::LUC* reporter plants during 24 hours at 22°C, measured from same images used for graphs in **A**. Data are mean projected rosette area ± SE (n=8). Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6).



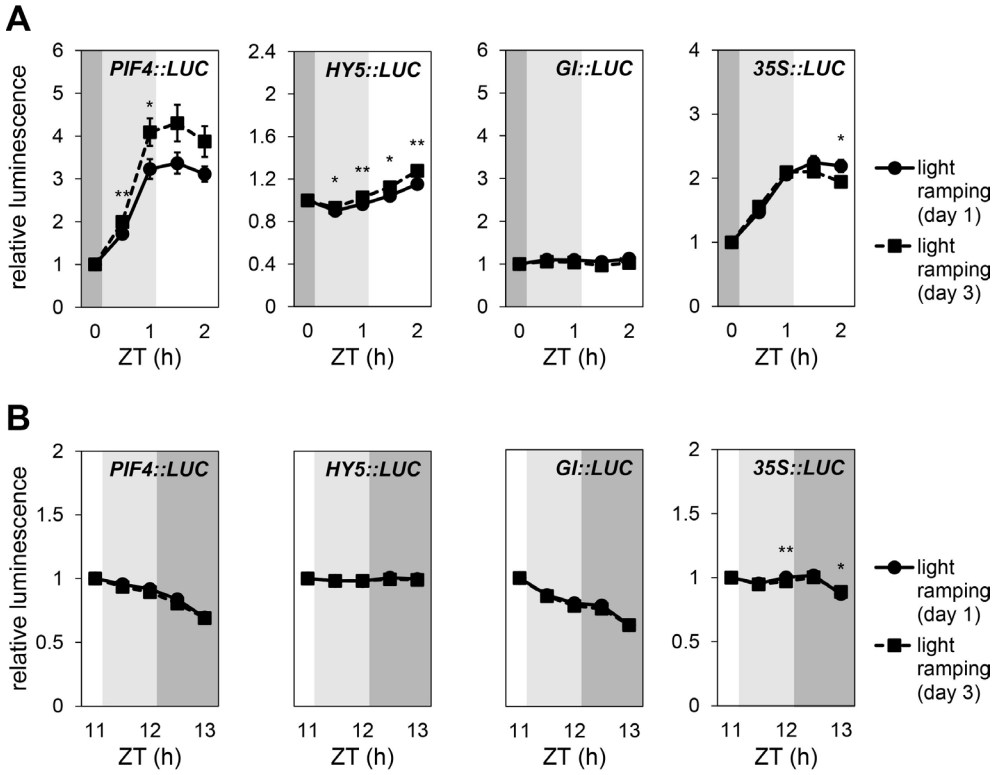
Supplemental Figure S7: ffLUC profile of different rosette areas. A-C: Diurnal ffLUC profiles of 4-week-old *GI::LUC*, *HY5::LUC*, *PIF4::LUC* and *35S::LUC* Arabidopsis reporter plants. Luminescence in whole plant (**A**), sink area (**B**) or center-part of rosette (**C**) was measured from LUMINATOR images used to obtain ffLUC activity profiles Figure 2 (day 1). Top images show area that was used for measuring luminescence. Data are mean luminescence ± SE (n=6). **D**: ratio between luminescence in source and sink for different reporters. Top picture shows area used as sink (circle) and source (area between square and circle). Data are mean luminescence source : sink ± SE (n=6), calculated using data **A** and **B**. Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6).



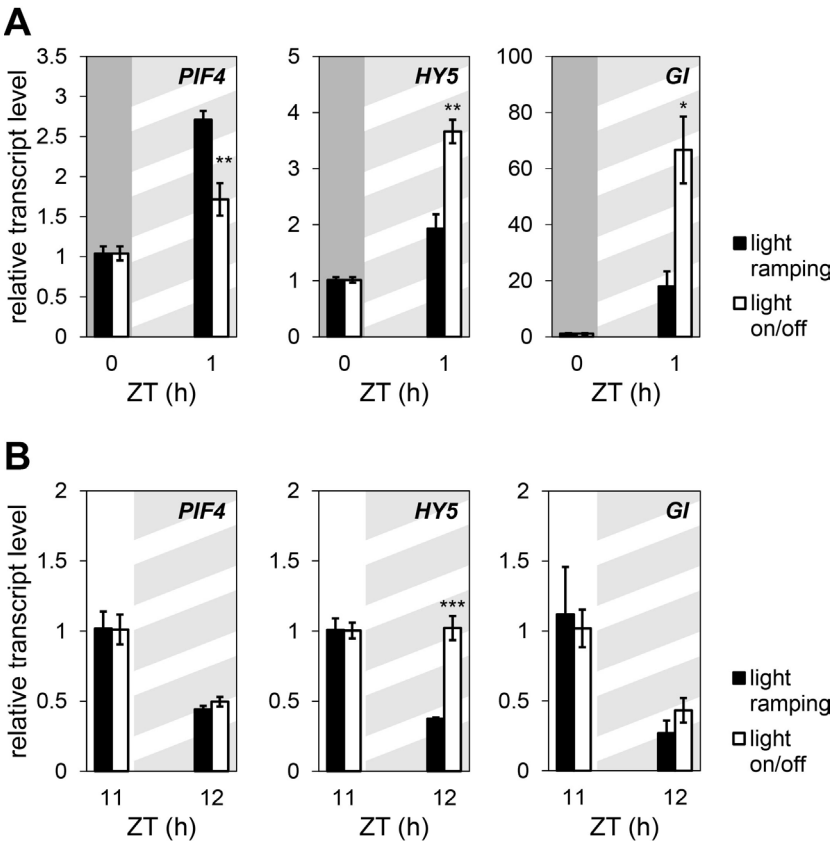
Supplemental Figure S8: Effect of changes in diurnal light conditions on diurnal ffLUC activity. Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* Arabidopsis reporter plants grown three subsequent days (12h light / 12h dark) under light ramping regime on day 1 and light on/off regime on day 2 and 3, at constant temperature (22°C) and RH (70%). Dark grey graph areas represent dark period, light grey areas represent light ramp conditions (30 µmole m^{-2} s^{-1}) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}). Data are mean relative luminescence ± SE (n=6).



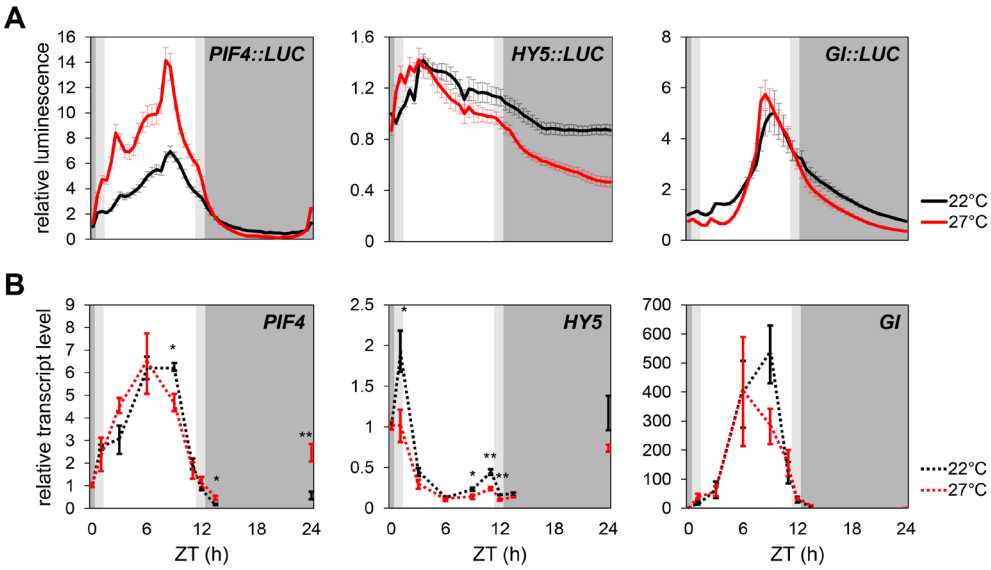
Supplemental Figure S9: Relative ffLUC activity under lights on/off and light ramping regime. Reporter plants were grown three subsequent days (12h light / 12h dark) under light ramping regime on day 1-3 (light ramping treatment, black squares), or under light ramping regime on day 1 and light on/off regime on day 2 and 3 (light on/off treatment, white squares). Temperature in both experiments was constant temperature (22°C) and RH (70%). Graphs show relative ffLUC activity of *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* during start (**A**) and end (**B**) on day 3 normalized to relative ffLUC activity on day 1 to corrected for changes in ffLUC activity during 3 days under light ramping regime. Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m-2 s-1; R:FR=2) and white areas represent day light conditions (100 µmole m-2 s-1; R:FR=6). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and day 3 of Figure 2 and Supplemental Figure S9. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative luminescence during light ramp.



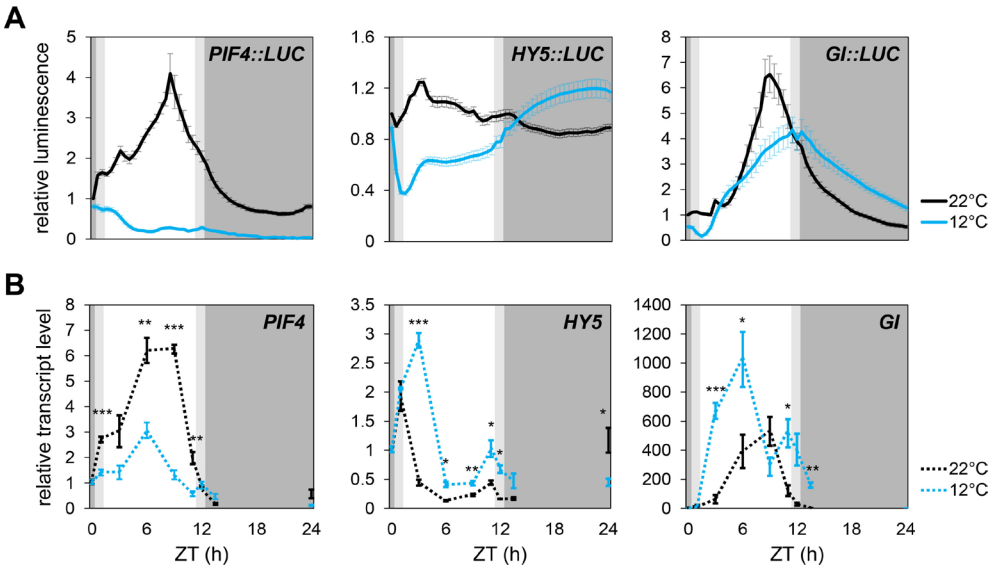
Supplemental Figure S10: Reproducibility of ffLUC activity over three days. Reporter plants were grown three subsequent days (12h light / 12h dark) under constant temperature (22°C) and RH (70%). Light conditions included a 1h ramp at the start and end of the photoperiod. Graphs show relative changes in luminescence of *PIF4::LUC*, *HY5::LUC*, *GI::LUC* and *35S::LUC* during start (**A**) and end (**B**) of photoperiod on day 1 (solid line, circles) and day 3 (dashed line, squares). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 3 of Figure 2. *p<0.05, **p<0.01 (Student's t-test) significantly different from relative luminescence on day 1.Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6).



Supplemental Figure S11: Effect of lights on/off regime on endogenous gene expression. Expression of *PIF4*, *HY5* and *GI* at dawn (ZT(h)=0 and ZT(h)=1; **A**) and dusk (ZT(h)=11 and ZT(h)=12; **B**) in Arabidopsis Col-0 WT plants grown in LUMINATOR under same conditions as reporter plants in Supplemental Figure S9. Graphs show gene expression in plants grown under light ramping regime (black bars) or light on/off regime (white bars). Data are mean relative transcript levels ± SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from relative transcript level during light ramp. Dark grey graph areas represent dark period, light grey areas represent light ramping conditions (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6).



Supplemental Figure S12: ffLUC activity profile and related endogenous gene expression at 22°C and 27°C. A: Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC* and *GI::LUC* Arabidopsis reporter plants during 24h (12h light / 12h dark) under light ramping conditions, at constant 22°C, RH 40% (black line) or constant 27°C, RH 40% (red line). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 2 Figure 6. **B**: Expression of *PIF4*, *HY5* and *GI* at ZT(h)=0, 1, 3, 6, 9, 11, 12 and 14 in Arabidopsis Col-0 WT plants grown in LUMINATOR under same conditions as reporter plants in **A**. Black lines show gene expression under constant 22°C, red lines show gene expression under 27°C. Data are mean relative transcript levels ± SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780). *p<0.05, **p<0.01 (Student's t-test) significantly different from relative transcript level at 22°C. Dark grey graph areas represent dark period, light grey areas represent ramp conditions (30 µmole m^{-2} s^{-1}) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}).



Supplemental Figure S13: ffLUC activity profile and related endogenous gene expression at 22°C and 12°C. A: Diurnal ffLUC profile of 4-week-old *PIF4::LUC*, *HY5::LUC* and *GI::LUC* Arabidopsis reporter plants during 24h (12h light / 12h dark) under light ramping conditions, at constant 22°C, RH 70% (black line) or constant 12°C, RH 70% (blue line). Data are mean relative luminescence ± SE (n=6) calculated using data day 1 and 2 Figure 6. B: Expression of *PIF4*, *HY5* and *GI* at ZT(h)=0, 1, 3, 6, 9, 11, 12 and 14 in Arabidopsis Col-0 WT plants grown in LUMINATOR under same conditions as reporter plants in **A**. Black lines show gene expression under constant 22°C, red lines show gene expression under 12°C. Data are mean relative transcript levels ± SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780). *p<0.05, **p<0.01 (Student's t-test) significantly different from relative transcript level at 22°C. Dark grey graph areas represent dark period, light grey areas represent ramp conditions (30 µmole m^{-2} s^{-1}) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}).

Chapter 3

Global transcriptional response of Arabidopsis to inversed light and temperature cycles

Mark van Hoogdalem*

Umidjon Shapulatov*

Jan van Haarst

Alexander R. van der Krol

*These authors contributed equally to this work

Abstract

Normal diurnal light/temperatures cycles (warm day and cold night: +DIF) stimulate plant growth. In contrast, plant growth is suppressed under a regime of cold days and warm nights (-DIF). In horticulture -DIF is commonly used to control plant architecture in greenhouses, but knowledge on how -DIF affects plant growth is limited. To obtain an overview of processes affected under -DIF, we performed RNA sequencing (RNAseq) analysis of total mRNA and miRNA of adult Arabidopsis rosettes grown under +DIF (12h L 22°C/12h D 12°C) and -DIF (12h L 12°C/12h D 22°C) at two timepoints: end of day (ED) and end of night (EN). As the photoreceptor phytochrome B (PHYB) was shown to play a key role in temperature sensing and the *phyB-9* mutant shows a reduced response to -DIF, our study includes the Arabidopsis *phyB-9* mutant.

Results show that -DIF leads to differential expression of in total 2706 genes in WT. Of these genes, 550 are temperature-regulated in a timepoint-independent manner and often involved in cold acclimation. Genes only upregulated at ED are also associated with cold acclimation processes, indicating that the induction of cold acclimation is partially restricted to the light-period. Genes that are only upregulated at EN are associated with processes linked to low energy status. Many genes that are only downregulated at ED or EN are associated with processes that link to the promotion of cell growth, such as cell wall modification, water transport, energy status and expression of PHYTOCHROME INTERACTING FACTORs (PIFs). Most of the genes involved in these processes were not differentially expressed at EN in the *phyB-9* mutant, suggesting that they may be causal for growth suppression under -DIF. Furthermore, it indicates that mainly during the night PHYB plays an important role in regulation of gene transcription under -DIF conditions. On the other hand, more genes show differential expression in the *phyB-9* mutant than in WT under -DIF at ED, suggesting that in WT PHYB plays a role in buffering transcriptional changes under -DIF during the day. In total, 96 miRNAs also showed differential expression in response to -DIF. For several miRNAs, differential expression of their predicted target genes was observed. These are involved in many different processes, including some of the processes linked to growth regulation that were found to be affected under -DIF. This suggests that part of the transcriptional response to -DIF is through regulation of transcript stability by miRNAs.

In conclusion, our results on the global transcriptional response of Arabidopsis to -DIF reveal the impact of -DIF on a wide range of processes linked to growth and cold acclimation and show that the transcriptional response to -DIF is strongly dependent on PHYB activity.

Introduction

Temperature is a major regulator of plant growth, as has been shown in numerous experiments performed at different constant temperatures. For instance, in Arabidopsis, growth of hypocotyls is positively correlated with temperature between 10°C and 30°C degrees (Legris et al., 2017). In more mature rosette plants, higher temperatures (24-30°C) generally result in increased petiole elongation, hyponastic growth and thinner leaves compared to plants grown under 20-22°C (Koini et al., 2009; Kumar and Wigge, 2010; Crawford et al., 2012; Delker et al., 2014; Ibañez et al., 2015; Raschke et al., 2015), while lower (non-freezing) temperatures result in smaller, more compact shaped rosettes of Arabidopsis plants with increased leaf thickness (Scott et al., 2004; Fiorani et al., 2005; Atkin et al., 2006; Patel and Franklin, 2009). Growth responses of Arabidopsis to temperature are gated by the circadian clock, which means that the response to temperature is restricted to a specific time of the day (Fowler et al., 2005; Zhu et al., 2016). For example, the increased expression of a small group of C-repeat Binding Factors (CBFs) in response to low temperatures is restricted to the morning (Fowler et al., 2005). The cold-induced CBF expression negatively regulates plant growth by reducing GA levels (Achard et al., 2008; de Lucas et al., 2008; Feng et al., 2008). Gating of plant growth responses to temperature by the circadian clock is consistent with the observation that growth of Arabidopsis and other species is reduced when plants are subjected to cold days, and warm nights instead of naturally occurring warm days and cold nights, even when the temperature sum and light sum of the two treatments are the same (Went, 1944; Myster and Moe, 1995; Moe and Heins, 2000; Bours et al., 2013; Bours et al., 2015). Indeed, inversed day/night temperature treatments are applied in commercial greenhouses to keep plants compact and are referred to as -DIF conditions, in contrast to the normal high temperature during day and low temperature at night (referred to as +DIF) (Erwin et al., 1989; Carvalho et al., 2008). To get insight into the altered gene activity that may correlate with the reduced elongation response under -DIF, we investigated the transcriptional response to -DIF (12hL 12°C/12hD 22°C) compared to a +DIF (12hL 22°C/12hD 12°C) treatment for two timepoints: end-of-night and end-of-day.

Previously it has been shown that the plant growth response to -DIF is affected by the ratio between red (R) and far red (FR) light, suggesting that the R/FR-absorbing phytochrome B (PHYB) photoreceptor is involved in the responses to -DIF (Myster and Moe, 1995). Indeed, the Arabidopsis PHYB knock-out mutants *phyB-1* and *phyB-9* do not show reduced leaf elongation in response to -DIF, suggesting that suppression of plant growth under -DIF depends on PHYB activity (Thingnaes et al., 2008; Bours et al., 2013). Moreover, PHYB has been identified as major temperature sensor in the regulation of plant growth in response to elevated temperatures (Jung et al., 2016; Legris et al., 2016). Light-activated PHYB interacts in the nucleus with a set of basic helix-loop-helix (bHLH) transcription factors called PHYTOCHROME INTERACTING FACTORs (PIFs), which target genes involved in elongation responses (Leivar and Quail, 2011; Leivar and Monte, 2014; Xu et al., 2015; Pham et al., 2018). PHYB suppresses PIF protein activity by targeting PIFs for degradation and/or inhibition of PIF binding to target promoters (Leivar and Quail, 2011; Leivar and Monte, 2014; Ma et al., 2016; Pham et al.,

2018). The spontaneous reversion of active PHYB Pr to its inactive PHYB Pfr form, which is referred to as dark-reversion but also occurs in light, is temperature-dependent, rendering the effect of PHYB on PIF proteins temperature sensitive (Jung et al., 2016; Legris et al., 2016). In addition, the action of PHYB in the light on PIF proteins in cooperation with the co-factor Hemera (HMR) is temperature sensitive (Qiu et al., 2019). Combined, this identifies PHYB as an important thermosensor for growth control through action on PIFs.

Previously we have shown that growth under -DIF results in lower PIF4 activity, with PIF4 acting upstream in a signal transduction cascade involving the hormones auxin and ethylene. The reduced ethylene signaling under -DIF eventually also results in lower *PIF3* promoter activity, which links directly to elongation responses (Bours et al., 2015). The lower PIF activity, combined with slower dark reversion of PHYB at low temperature during the day, relates to the reduced elongation of Arabidopsis plants under -DIF. To gain further insight into the molecular processes that are affected by -DIF we performed RNA-sequencing on material of 4-week-old Arabidopsis Col-0 wild-type plants that were grown for 3 days under +DIF or -DIF. Because the growth of young sink leaves and petioles of older leaves are most responsive to -DIF, only the center part of the rosette plants were sampled. Sampling was done at two timepoints: at end-of-day (ED) and end-of-night (EN). To determine which of the molecular processes that are affected by -DIF are dependent on PHYB function we also sampled material of the *phyB-9* mutant, which does not show reduced elongation of young leaves in response to -DIF (Bours et al., 2013). In addition to sequencing mRNA transcripts, the same samples were also used for isolation and sequencing of microRNAs (miRNA). miRNAs can target specific mRNA transcripts for degradation, and several studies have shown that small RNAs play a role in the regulation of plant growth and adaptation in response to temperature (Alonso-Peral et al., 2010; May et al., 2013; Shriram et al., 2016). By determining changes in expression of miRNAs in response to -DIF we may identify alternative mechanisms besides transcriptional regulation to alter gene expression in response to -DIF. Our results identified key processes affected by -DIF that relate to the observed reduced elongation response under -DIF. Only few miRNAs with differential response to -DIF were identified, and some of these correlated with differential expression of their putative target gene(s).

Material and Methods

Plant material, growth conditions and sampling

Seeds of Arabidopsis Col-0 WT and the *phyB-9* mutant line (Reed et al., 1993) were stratified in the dark at 5°C for three days and then sown on 4x4x4cm rockwool blocks (Grodan, Roermond, The Netherlands) that were pre-soaked in Hyponex nutrient solution (Unifarm, Wageningen, The Netherlands). Germination and plant growth was in a Weiss Technik climate cabinet under +DIF conditions (12hL 22°C/12hD 12°C; RH65%) as described before (Bours et al., 2012), except that light intensity was 180 µmole m^{-2} s^{-1} and plants were watered three times a week with 0.5x Hyponex nutrient solution. After 4 weeks, half of the plants were transferred to a second Weiss cabinet and grown under -

DIF conditions (12hL 22°C/12hD 12°C; RH65 %) for 3 more days. On the 32nd day after sowing, plants growing under +DIF or -DIF conditions were harvested at the end of the day (ED; ZT(h)=12) and at the end of the night (EN; ZT(h)=24). Shoots were cut from the rockwool blocks, placed on a flat surface and a punch (2 cm diameter) was used to cut out the center part of the rosette. Because -DIF suppresses growth in leaves and petioles, harvesting the center of rosettes grown under +DIF and -DIF using the same size puncher could potentially result in samples having a different tissue composition, which may complicate interpretation of RNAseq data. However, it was shown that during the first four days after transfer from +DIF to -DIF conditions, the effect of -DIF on leaf elongation was still limited (Bours et al., 2013). Thus, by harvesting the material before the end of the fourth day after plants were transferred to -DIF we strongly reduce the chance that any differences in gene expression between samples is due to differences in sample tissue composition. The center and outer shoot parts were put in separate containers and immediately frozen in liquid nitrogen. For each sample that was used for RNA isolation, center parts of 10 individual plants were pooled. The experimental procedure was replicated twice in time in order to obtain 3 biological replicates for each sample.

mRNA and small RNA extraction, library preparation and sequencing

Samples were ground manually in liquid nitrogen by using a mortar and pestle and were used for both mRNA and small RNA extraction. Total RNA in samples was isolated using the InviTrap® Spin Plant RNA Mini kit (Stratec, Birkenfeld, Germany) according to manufacturer's instructions. Genomic DNA was subsequently removed by using the TURBO DNA-free™ kit (Invitrogen, Carlsbad, CA, USA). The resulting RNA was eluted in Ambion™ diethylpyrocarbonate (DEPC)-treated water (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until use. Quality of the RNA was checked by running 2µl on agarose gel and concentration and purity was determined by NanonDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA). In addition, mRNA quality of samples was tested with Agilent 2100 Bioanalyzer RNA Nano Kit (Agilent, Santa Clara, CA, USA). The mRNA library preparation was done by using the Illumina® TruSeq™ RNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA). Sequencing was done with Illumina MiSeq™ (Illumina Inc., San Diego, CA, USA).

Isolation of small RNA in samples was performed by using mirPrimier® microRNA Isolation Kit (Lot#SLBL6958V, Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. All small RNA samples were confirmed to contain 20-24 nucleotide size products by electrophoresis using 15% polyacrylamide (19:1) gel with 7 M urea and buffered with 1×TBE, using an SE600 standard vertical gel electrophoresis system. Purity and concentration of small RNA samples was determined by NanonDrop™ spectrophotometer and quality was tested with Agilent 2100 Bioanalyzer RNA Nano Kit. Library preparation for small RNA samples was done by using the Illumina® TruSeq™ Small RNA Library Prep Kit (Illumina Inc., San Diego, CA, USA). Sequencing of small RNA was done using Illumina HiSeq™ 2000 high throughput sequencing technology (Illumina Inc., San Diego, CA, USA).

RNAseq analysis

The Illumina sequence reads were mapped to the TAIR10 Arabidopsis reference genome (https://www.arabidopsis.org/) using Tophat (Trapnell et al., 2013) and Bowtie (Langmead et al., 2009) software. Sequence reads of small RNA samples were mapped to Arabidopsis microRNA sequences of miRbase (Kozomara and Griffiths-Jones, 2014). Assembly and quantification of mRNA transcripts and identification of differentially expressed genes (DEGs; false discovery rate Q-value<0.05) was done with Cufflinks (Trapnell et al., 2013). Due to high variability in small RNA read counts between biological replicates for each sample we decided to add up read counts of biological replicates for further analysis. Small RNAs were considered differentially expressed if total small RNA read count was ≥100 in at least one of the samples and fold change was ≥2. Prediction of Arabidopsis miRNA targets was done with psRNATarget (Dai and Zhao, 2011).

Identification of common elements in different lists of DEGs and construction of Venn diagrams was done with the Venny 2.1 webtool (Oliveros, 2007-2015). Gene ontology (GO) enrichment analyses were done using the online analysis tool of the PANTHER Classification System (Mi et al., 2013). Analysis type: PANTHER Overrepresentation Test (Released 2019-06-06). Test Type: Fisher's exact test with FDR correction. Reference list included 23784 genes that were expressed in at least one of the samples. Semantic similarity analysis and construction of TreeMaps was done using the Revigo webtool (Supek et al., 2011) (allowed similarity: small (0.5); semantic similarity measure: SimRel) and R software (v3.6). Mapping of DEGs to metabolic pathways was done with the KEGG mapper webtool (Kanehisa and Goto, 2000).

Analysis effect -DIF on leaf length and shoot weight

Arabidopsis were grown under +DIF conditions as described above. After three weeks, half of the plants were transferred to -DIF conditions and 10 days later (31 days after sowing) plants growing under +DIF or -DIF conditions were harvested in the morning. For leaf/petiole length measurements, shoots were cut from the rockwool blocks and rosette leaves were separated and ordered based on order of emergence. The resulting leaf profiles were photographed and used to measure leaf/petiole lengths using ImageJ software (imagej.nih.gov/ij). For weight measurements, shoots were cut from the rockwool blocks and fresh weight (FW) of 5 pooled shoots was immediately determined. Subsequently, the shoot material was transferred to a 12ml polypropylene tube, oven-dried at 70°C for 48 hours and weighted to obtain shoot dry weight (DW).

Results

RNA sampling in WT and *phyB-9* mutant plants grown under +DIF and -DIF

In order to determine the transcriptional response to a -DIF treatment, Arabidopsis plants were grown for 29 days in growth cabinets under +DIF, after which half of the plants were transferred to growth cabinets with -DIF conditions. Tissues were harvested when plants

were 32 days old. The effect of -DIF on leaf length of adult Arabidopsis plants is strongest in small developing (sink) leaves (Supplemental Figure S1A+C) and leaf petioles. The latter is illustrated by the reduced ratio between petiole length and leaf blade length (P:L ratio) under -DIF (Supplemental Figure S1B). Therefore, the center of the rosettes, containing sink leaves and petioles of source leaves, were harvested for RNA isolation (Figure 1A). Material of Arabidopsis Col-0 WT and *phyB-9* mutants (Reed et al., 1993) grown for three days under +DIF or -DIF was sampled at end-of-day (ED; ZT(h)=12) or end of night (EN; ZT(h)=24) (Figure 1B). Per biological replicate sample, material of 10 plants was pooled and total RNA was extracted and sequenced. The mRNA sequence reads were mapped to the TAIR10 Arabidopsis genome and quantified in order to identify differentially expressed genes (DEGs; false discovery rate Q-value<0.05). A list of DEGs for all possible sample comparisons can be found in Supplemental File S1.

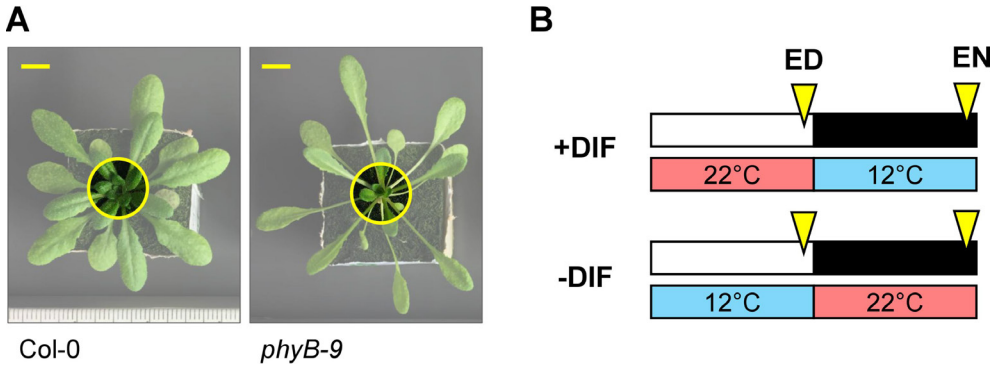


Figure 1: Sampling timepoints and tissue. A: Representative images of 32-day-old Arabidopsis Col-0 WT (left) and *phyB-9* mutant plants (right) grown under +DIF conditions. Area within yellow circle shows plant material that was sampled and used for RNA isolation. Yellow scale bar=1cm. **B**: Schematic representation of treatments and sampling timepoints. Arabidopsis Col-0 WT or *phyB-9* mutant plants were grown under +DIF conditions (12hL 22°C/12hD 12°C) for 32 days or they were grown for 28 days under +DIF conditions and then transferred to -DIF conditions (12hL 12°C/12hD 22°C). At 32 days after sowing, plant material was sampled during the last hour of the photoperiod (ZT(h)=12; ED), and during the last hour of the photoperiod (ZT(h)=24). White bars represent photoperiod, black bars represent dark period, blue bars represent 12°C and red bars represent 22°C. Yellow triangles represent sampling timepoints.

Differential gene expression in WT Col-0 under -DIF

Comparison of mRNA transcript levels in Col-0 WT plants under +DIF and -DIF revealed that, over the two timepoints, in total 2706 genes are differentially expressed under -DIF, of which 1631 genes are differentially expressed at ED and 1677 genes are differentially expressed at EN (Figure 2). For the genes that show a strong temperature dependency, independent of the timing of the temperature treatment, we predict an opposite direction of the change in gene expression level under -DIF at ED and EN. Results show that 352 genes are upregulated under -DIF at ED which are downregulated under -DIF at EN (Figure 2), indicating that expression of these genes is upregulated at 12°C, independent of the time of day. In contrast, only 198 genes are downregulated under -DIF at ED and

upregulated under -DIF at EN, defining the set of genes of which expression is downregulated at low temperature. However, most of the DEGs are up- or downregulated at EN or ED only, indicating that their transcriptional response to -DIF depends on the timing of the low temperature treatment. The temperature response of these genes may thus be gated by the circadian clock. At ED 644 genes show upregulation and 385 show downregulation under -DIF, while at EN 573 genes show upregulation and 502 show downregulation under -DIF (Figure 2). Only very few genes are upregulated (29) or downregulated (23) at both timepoints.

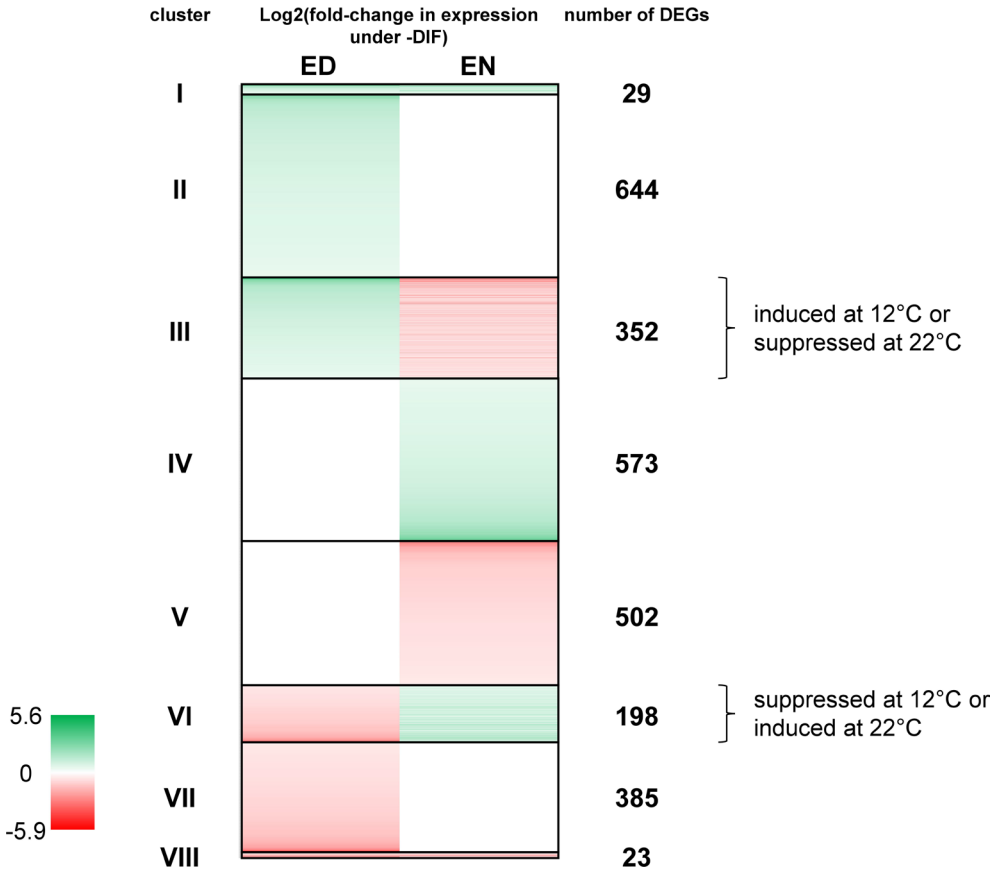


Figure 2: Heatmap of genes that are differentially expressed under -DIF. Heatmap shows log2(fold change in expression) of differentially expressed genes (false discovery rate Q-value<0.05) in shoot material of Arabidopsis Col-0 WT under -DIF at end of day (ED; left column) and end of night (EN; right column). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). DEGs are clustered (thick borders) based on similar regulation under -DIF. Numbers on the right indicate the number of DEGs belonging to these groups.

GO-enrichment analysis of genes affected by -DIF

To identify the main biological processes affected by -DIF, the set of DEGs were subjected to gene ontology (GO)-term enrichment analysis (Mi et al., 2013). Subsequently, semantic similarity analysis was performed with REVIGO (Supek et al., 2011) to remove redundant GO terms. The results of these analyses are shown in Supplemental Files S2-S7 and visualized in treemaps (Figure 3 and Figure 4). The genes in clusters III and VI show temperature-dependent expression, independent of the timing of the temperature treatment. Genes in cluster III, which are upregulated at 12°C, are mainly associated with processes related to protein translation, flavonoid biosynthesis and response to temperature stimulus (Figure 3A; Supplemental File S2). The genes in cluster VI, which are downregulated at 12°C, are only associated with the GO terms response to chemical and response to stimulus (Figure 3B; Supplemental File S3).

The genes in cluster II (uniquely upregulated at ED), like genes in cluster III, are also associated with processes related to protein translation and temperature responses (Figure 4A; Supplemental File S4). Therefore, low temperature seems to stimulate expression of genes related to protein translation and temperature responses. The regulation of some of these genes is independent of timing of the low temperature treatment (cluster III), while other genes related to these processes show gating by the clock and only respond to low temperature in the light (cluster II). Genes that are uniquely downregulated under -DIF at ED (cluster VII) are associated with fluid and oligopeptide transport, ion homeostasis and response to nutrient levels (Figure 4D; Supplemental File S5). Genes uniquely upregulated under -DIF at EN (cluster IV) are mainly associated with energy production (Figure 4B; Supplemental File S6), while gene uniquely downregulated at EN (cluster V) are associated with response to inorganic substances, carbohydrate metabolism and leaf development (Figure 4C; Supplemental File S7).

Enrichment analysis showed that the 29 genes that are upregulated at both timepoints (Supplemental Table S1) are mainly associated with processes related to photosynthesis, while no significantly enriched GO terms are identified within the list of genes downregulated at both timepoints (Supplemental Table S2).

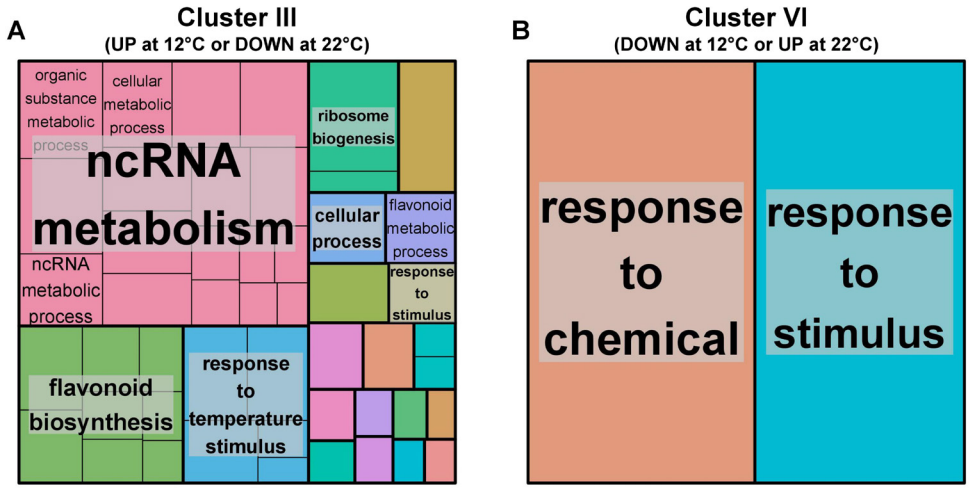


Figure 3: Treemaps of GO-term clusters for genes that are differentially expressed under -DIF in a temperature-dependent manner. A: Treemap showing clustered gene ontology (GO-)terms of biological processes that are over-represented in the list of genes that are both upregulated under -DIF at end of day (ED) and downregulated under -DIF at end of night (EN), and thus upregulated at 12°C at both timepoints (Cluster B). **B**: Treemap showing clustered GO-terms of biological processes that are over-represented in the list of genes that are both downregulated under -DIF at ED and upregulated under -DIF at EN, thus downregulated at 12°C at both timepoints (Cluster E). For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for gene clusters III and VI can be found in Supplemental Files S2 and S3, respectively. GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes are based on p-values (smaller p-value=bigger box).

PHYB-dependent and -independent gene regulation under -DIF

PHYB is an important temperature sensor in plants (Jung et al., 2016; Legris et al., 2016; Qiu et al., 2019) and comparison of the DEGs under -DIF in WT and the *phyB-9* mutant can reveal which of the DEGs are under control of PHYB. RNA was isolated from *phyB-9* mutant plants grown under +DIF and -DIF as described (Figure 1). In total 2938 genes are differentially expressed in *phyB-9* mutants grown under -DIF (Figure 5), compared to 2706 DEGs in WT under -DIF. The majority of the DEGs (2287) in the *phyB-9* mutant show differential expression under -DIF at ED (compared to 1631 DEGs at ED in WT). Also, in the *phyB-9* mutant fewer DEGs show unique temperature regulation: Only 70 genes are consistently upregulated at 12°C in the *phyB-9* mutant, compared to 352 DEGs in WT (compare cluster III of Figure 2 and Figure 5), and only 68 genes are consistently downregulated at 12°C in the *phyB-9* mutant, compared to 198 DEGs in WT (compare cluster VI of Figure 2 and Figure 5).

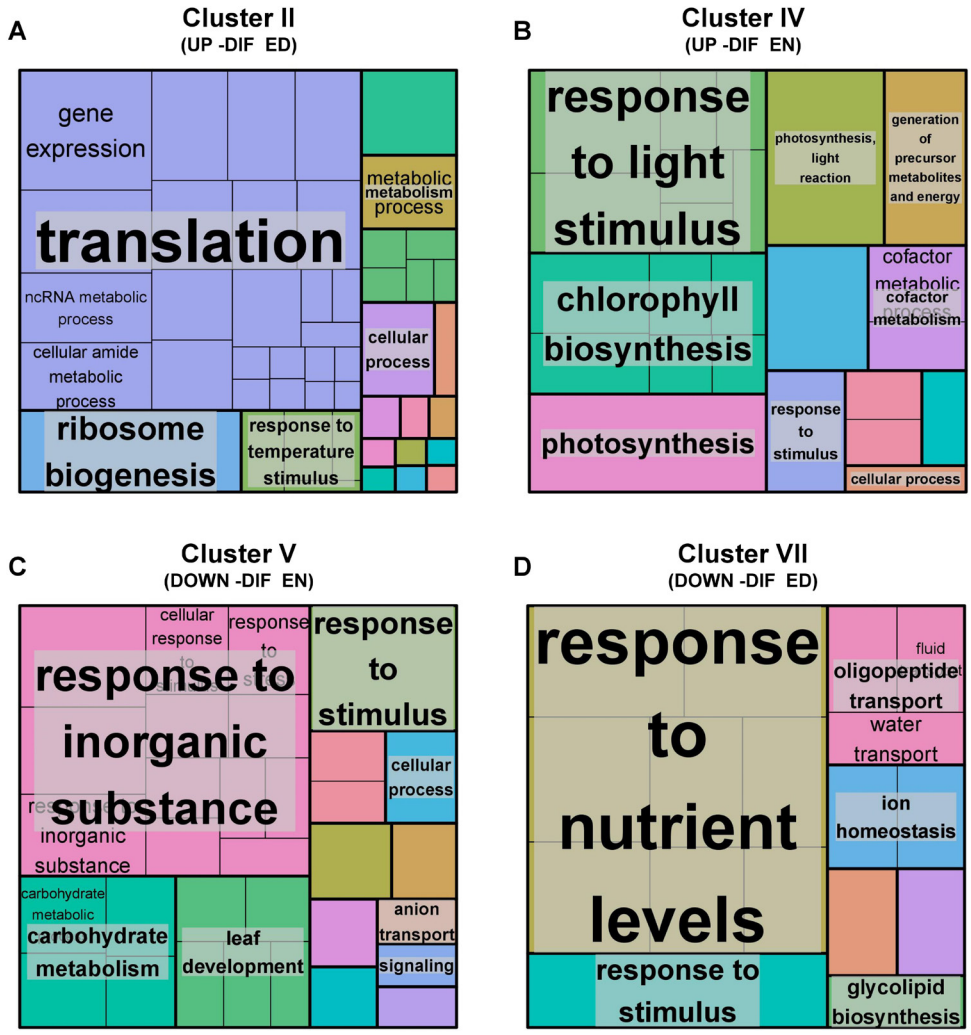


Figure 4: Treemaps of GO-term clusters for genes with unique differential expression (ED or EN) under -DIF. Treemaps showing clustered gene ontology (GO-)terms of biological processes that are over-represented in the list of genes that are upregulated under -DIF only at end of day (ED; **A**), upregulated under -DIF only at end of night (EN, **B**), downregulated under -DIF only at ED (**C**) or downregulated under -DIF only at EN (**D**). For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for gene clusters II, IV, V and VII can be found in Supplemental Files S4, S5, S6 and S7, respectively. GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes are based on p-values (smaller p-value=bigger box).

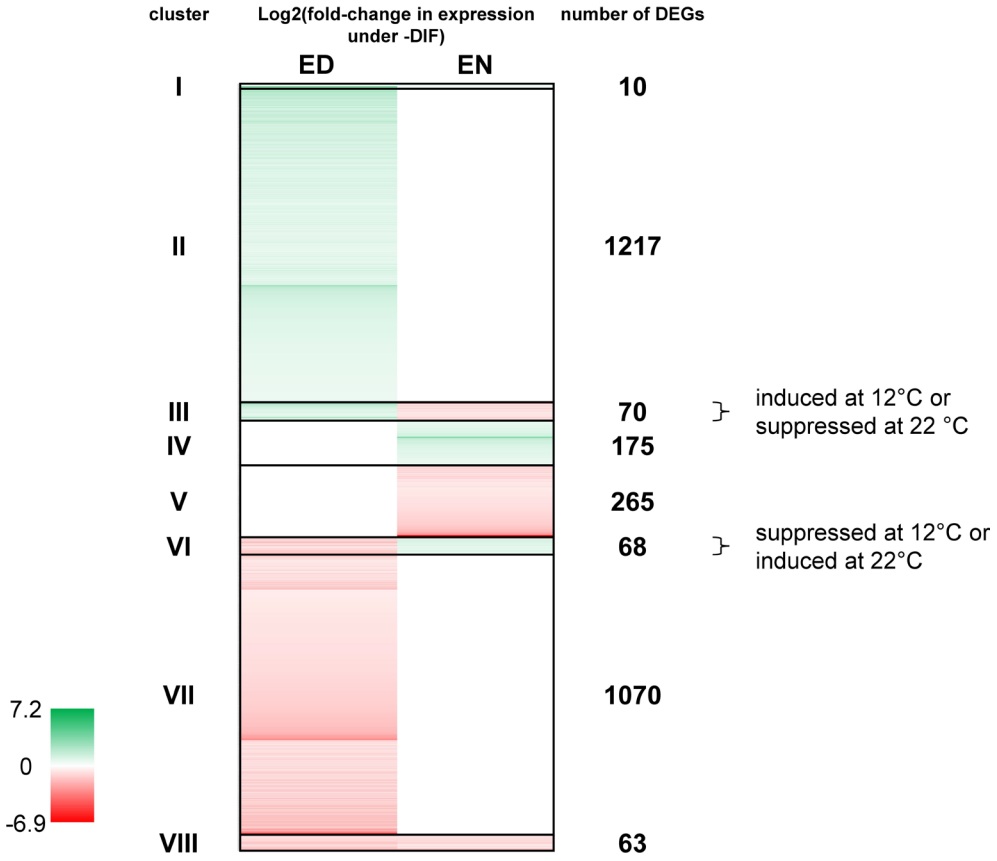


Figure 5: Heatmap of genes that are differentially expressed under -DIF in the *phyB-9* mutant. Heatmap shows log2(fold change in expression) of differentially expressed genes (DEGs; false discovery rate Q-value<0.05) in shoot material of the Arabidopsis *phyB-9* mutant under -DIF at end of day (ED; left column) and end of night (EN; right column). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). DEGs are clustered (thick borders) based on similar regulation under -DIF. Numbers on the right indicate the number of DEGs belonging to these groups.

The Venn diagram in Figure 6 shows the overlap of all DEGs under -DIF in WT and *phyB-9* mutant. Of the 2706 DEGs in WT, 1564 are also differentially expressed in the *phyB-9* mutant, indicating that the transcriptional regulation of these genes in response to -DIF does not depend on PHYB activity. GO enrichment analysis revealed that these genes are associated with processes related to protein translation and other processes such as water transport (Figure 6; Supplemental File S9). Regulation of the other 1142 DEGs in WT is PHYB-dependent, as these are not differentially expressed under -DIF in the *phyB-9* mutant. These DEGs are associated with processes linked to carbohydrate metabolism, photosynthesis and others (Figure 6; Supplemental File S8). In total, 1374 genes are differentially regulated in the *phyB-9* mutant, but not in WT. Possibly PHYB signaling in WT buffers against changes in expression of these genes at ED in response

to -DIF. Like the genes that are differentially expressed in WT and in the *phyB-9* mutant, these genes are associated with processes related to protein translation, but also with other processes such as response to stimulus and cell wall modification (Figure 6; Supplemental File S10).

The relatively small number of unique temperature-regulated genes in the *phyB-9* mutant (Figure 5) is in agreement with PHYB acting as a temperature sensor. A role for PHYB as a temperature sensor (in the -DIF response) is further supported by the observation that the majority (487) of the genes that show temperature regulation under -DIF in WT are not differentially expressed in the *phyB-9* mutant, suggesting their regulation is dependent on PHYB activity (Figure 7). GO enrichment analysis shows that these PHYB-dependent DEGs are mainly associated with processes that are stimulated at 12°C in WT (Figure 3A; Supplemental File S2), including processes related to protein translation and flavonoid metabolism (Figure 7; Supplemental File S11). These results indicate a role for PHYB in low temperature adaptation. The 63 genes that are differentially expressed in WT and the *phyB-9* mutant, and thus regulated in a PHYB-independent way, are mainly associated with response to (temperature) stimulus (Figure 7; Supplemental File S12), just like genes downregulated at 12°C in WT (Figure 3B; Supplemental File S3).

The Venn diagram in Figure 8 shows that, of the genes that are only differentially expressed under -DIF at ED, 372 are exclusively differentially expressed in WT, and thus regulated in a PHYB-dependent way. These DEGs are associated with processes such as organonitrogen compound biosynthesis and response to stress (Figure 8; Supplemental File S13). The 657 genes that are differentially expressed at ED in WT and the *phyB-9* mutant, and thus regulated in a PHYB-independent manner, are mainly associated with processes related to protein translation (Figure 8; Supplemental File S14). The majority (1630) of the genes that are differentially expressed in the *phyB-9* mutant at ED alone are not differentially expressed in WT. These genes are also associated with processes related to protein translation, but also with other processes such as pigment biosynthesis and processes linked to responses to other organisms (Figure 8; Supplemental File S15).

The Venn diagram in Figure 9 shows that most of the genes (974) that are differentially expressed in WT at EN alone are regulated by PHYB. These genes are mainly associated with processes linked to photosynthesis and response to (light) stimulus (Figure 9; Supplemental File S16). The 101 genes that are differentially expressed exclusively at EN in both WT and *phyB-9* mutant, and thus regulated in a PHYB-independent manner, are associated with calcium-mediated signaling and response to stimulus (Figure 9; Supplemental File S17). Compared to the 1630 genes at ED, only 339 genes are differentially expressed exclusively at EN in the *phyB-9* mutant, but not in WT. This suggests that PHYB signaling plays a bigger role in buffering gene expression in WT during the day than during the night under -DIF conditions.

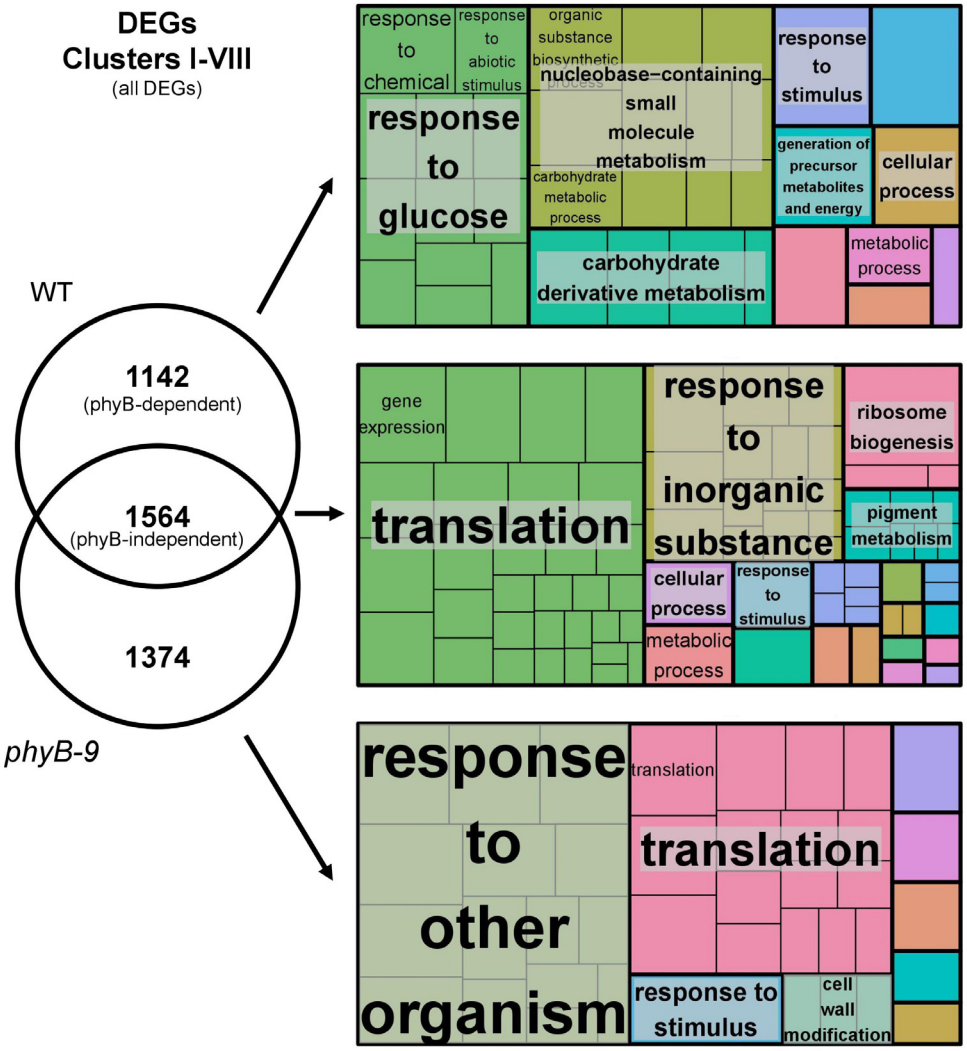


Figure 6: Overlap of genes that are differentially expressed under -DIF in WT and *phyB-9* mutants. Venn diagram on the left shows number of differentially expressed genes (DEGs) under -DIF (genes in clusters I to VIII of Figures 2 and 5) in Arabidopsis Col-0 WT only (1142), in the Arabidopsis *phyB-9* mutant only (1374) and in both lines (1564). Differential expression in WT only indicates transcriptional regulation of these genes is phytochrome B (PHYB)-dependent. Differential expression in WT and mutant (overlapping region) indicates transcriptional regulation of these genes is phytochrome B (PHYB)-independent. Treemaps on the right show gene ontology (GO-)terms of biological processes that are over-represented in the three groups of DEGs shown in the Venn diagram. For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for the three groups of DEGs can be found in Supplemental Files S8 (for PHYB-dependent DEGs), S9 (for PHYB-independent DEGs) and S10 (for *phyB-9* unique DEGs). GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes of treemaps are based on p-values (smaller p-value=bigger box).

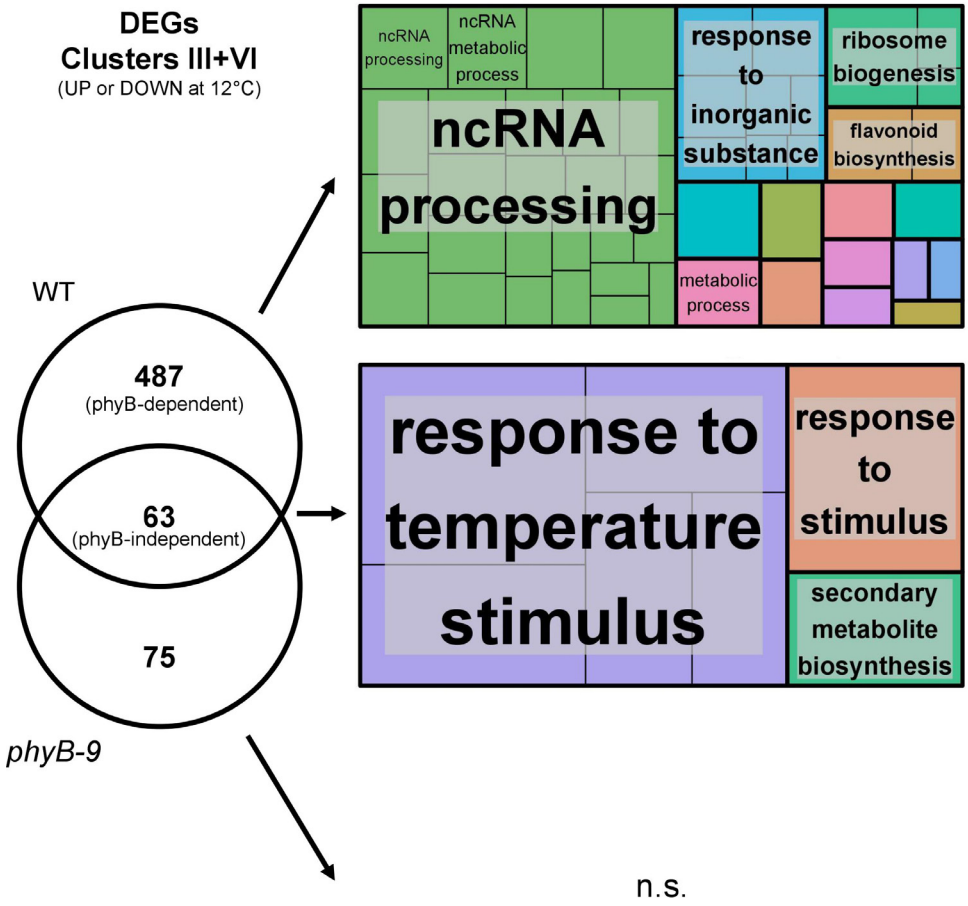


Figure 7: Overlap of genes that are differentially expressed under -DIF in a temperature-dependent manner in WT and *phyB-9* mutants. Venn diagram on the left shows number of differentially expressed genes (DEGs) under -DIF, in a temperature-dependent manner (genes in clusters III and IV of Figures 2 and 5), in Arabidopsis Col-0 WT only (487), in the Arabidopsis *phyB-9* mutant only (75) and in both lines (63). Differential expression in WT only indicates transcriptional regulation of these genes is phytochrome B (PHYB)-dependent. Differential expression in WT and mutant (overlapping region) indicates transcriptional regulation of these genes is phytochrome B (PHYB)-independent. Treemaps on the right show gene ontology (GO-)terms of biological processes that are over-represented in the three groups of DEGs shown in the Venn diagram. No significantly enriched GO terms were identified for the list of DEGs in Arabidopsis phyB-9 mutant only (n.s.). For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for two of the three groups of DEGs can be found in Supplemental Files S11 (for PHYB-dependent DEGs) and S12 (for PHYB-independent DEGs). GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes of treemaps are based on p-values (smaller p-value=bigger box).

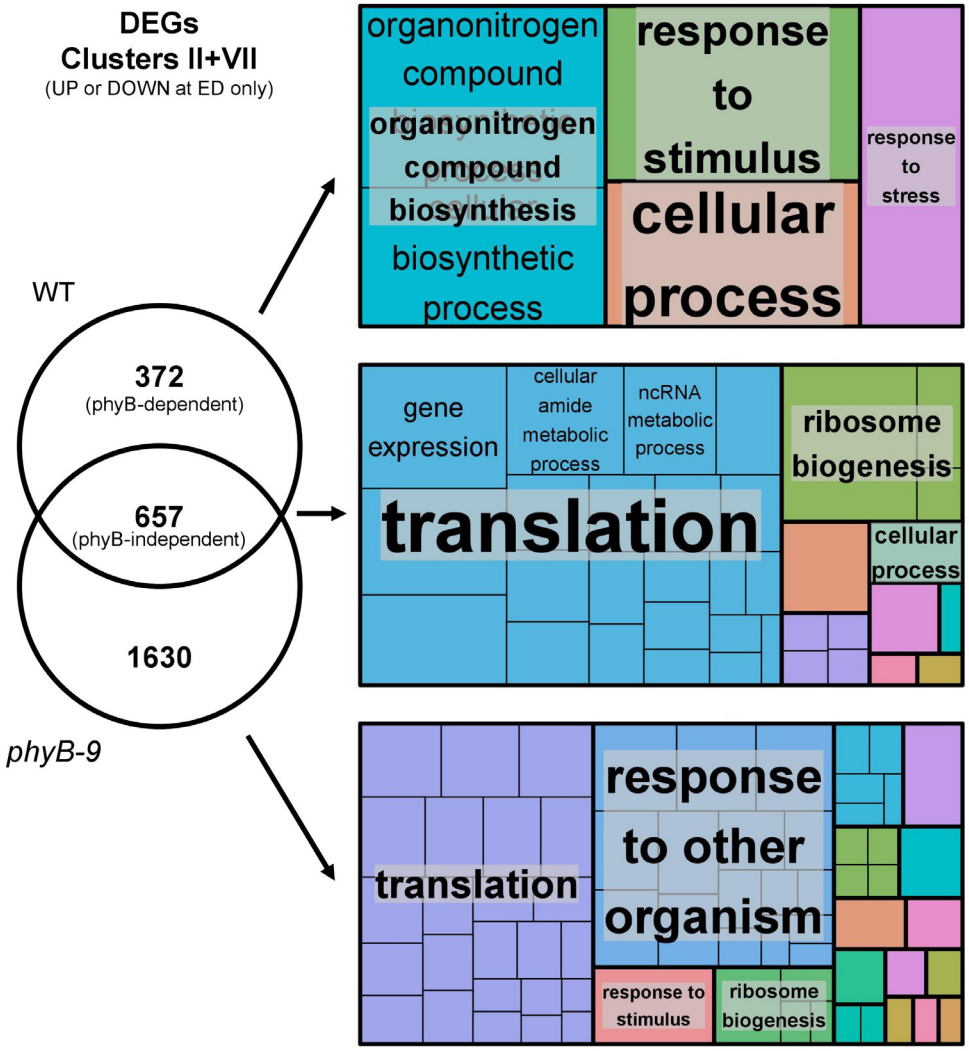


Figure 8: Overlap of genes that are differentially expressed under -DIF at ED only in WT and *phyB-9* mutants. Venn diagram on the left shows number of differentially expressed genes (DEGs) under -DIF, at end of day (ED) only (genes in clusters II and VII of Figures 2 and 5), in Arabidopsis Col-0 WT only (372), in the Arabidopsis *phyB-9* mutant only (1630) and in both lines (657). Differential expression in WT only indicates transcriptional regulation of these genes is phytochrome B (PHYB)-dependent. Differential expression in WT and mutant (overlapping region) indicates transcriptional regulation of these genes is phytochrome B (PHYB)-independent. Treemaps on the right show gene ontology (GO-)terms of biological processes that are over-represented in the three groups of DEGs shown in the Venn diagram. For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for the three groups of DEGs can be found in Supplemental Files S13 (for PHYB-dependent DEGs), S14 (for PHYB-independent DEGs) and S15 (for *phyB-9* unique DEGs). GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes of treemaps are based on p-values (smaller p-value=bigger box).

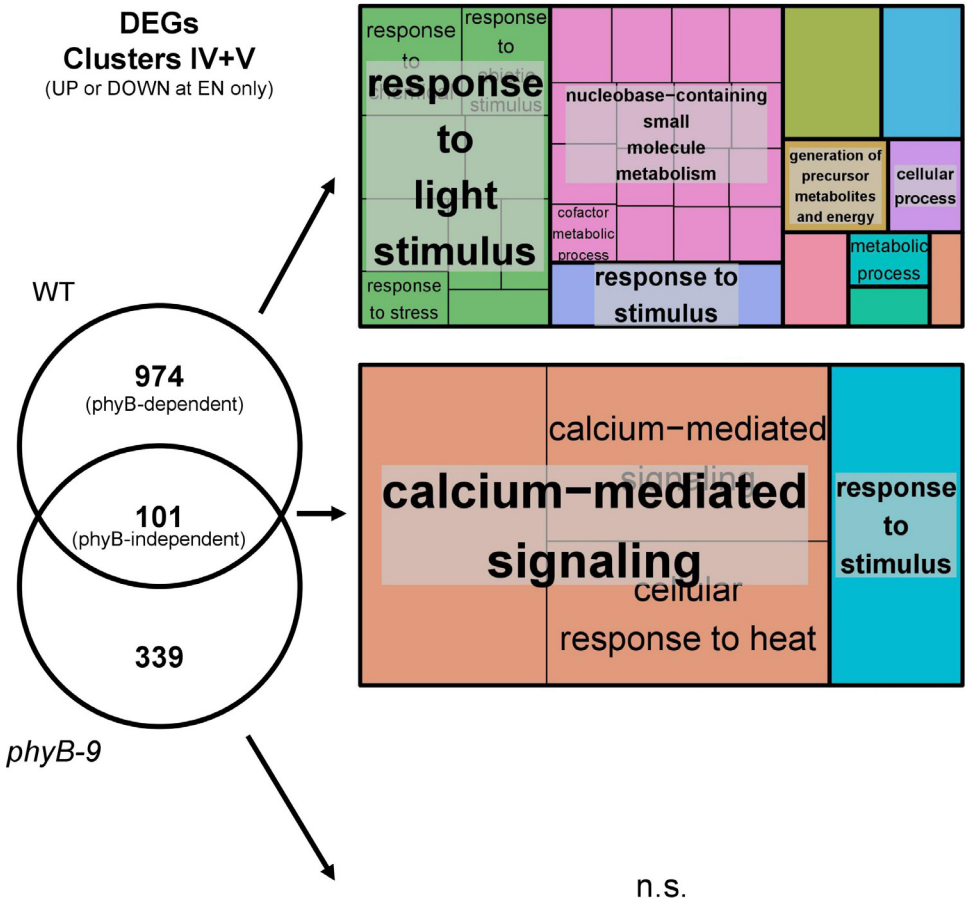


Figure 9: Overlap of genes that are differentially expressed under -DIF at EN only in WT and *phyB-9* mutants. Venn diagram on the left shows number of differentially expressed genes (DEGs) under -DIF, at end of night (EN) only (genes in clusters IV and V of Figures 2 and 5), in Arabidopsis Col-0 WT only (974), in the Arabidopsis *phyB-9* mutant only (339) and in both lines (101). Differential expression in WT only indicates transcriptional regulation of these genes is phytochrome B (PHYB)-dependent. Differential expression in WT and mutant (overlapping region) indicates transcriptional regulation of these genes is phytochrome B (PHYB)-independent. Treemaps on the right show gene ontology (GO-)terms of biological processes that are over-represented in the three groups of DEGs shown in the Venn diagram. No significantly enriched GO terms were identified for the list of DEGs in Arabidopsis phyB-9 mutant only (n.s.). For clarity reasons not all GO terms are shown in treemaps. The full list of enriched GO terms for two of the three groups of DEGs can be found in Supplemental Files S16 (for PHYB-dependent DEGs) and S17 (for PHYB-independent DEGs). GO enrichment analysis was done with the webtool of the PANTHER classification system (Mi et al., 2013) and treemaps were created with the REVIGO webtool (Supek et al., 2011). Size of boxes of treemaps are based on p-values (smaller p-value=bigger box). n.s.=no significant results

Changes in mRNA transcript levels under -DIF associated with changes in miRNA?

It has been shown that miRNAs play a role in the regulation of plant growth and adaptation to temperature through targeting specific mRNA transcripts for degradation (Alonso-Peral et al., 2010; May et al., 2013; Shriram et al., 2016). Therefore, samples harvested at ED and EN under +DIF and -DIF were also analyzed for miRNA content in order to determine whether the effect of -DIF on expression of some genes (in terms of mRNA transcript levels) may be explained by changes in levels of specific miRNAs. Small RNAs were isolated from the same tissue samples that were used for mRNA isolation (Figure 1), and miRNA sequence reads were aligned to the Arabidopsis microRNA sequences of miRbase (Kozomara and Griffiths-Jones, 2014). However, replicate samples showed high variability in miRNA read counts. Therefore, counts of miRNAs in all three replicate samples were added, and only used as initial indication of differential miRNA expression (Supplemental File S18). In the final analysis only miRNAs with ≥100 miRNA read counts were considered and an indication for potential differential expression was set at ≥2-fold-difference in miRNA read counts between samples. Using these criteria, a total of 96 miRNAs showed putative differential expression under -DIF compared to +DIF (Supplemental Figure S2A). Of these differentially expressed miRNAs (DEMs), 90 are differentially expressed at ED, while only 16 are differentially expressed at EN. Of the 90 DEMs at ED, the majority (84) showed upregulation. Expression of 9 miRNAs appears to be temperature-dependent. Of these, 3 miRNAs are upregulated at ED and downregulated at EN under -DIF, suggesting they are induced at 12°C. Six miRNAs are downregulated at ED and upregulated at EN under -DIF, suggesting they are downregulated at 12°C.

Table 1 lists the miRNAs with a putative differential expression under -DIF at ED or EN, for which at least one of their predicted target mRNAs shows a matching expression profile (miRNA up, target mRNA down or miRNA down with target mRNA up). Of these, 2 miRNAs are differentially expressed at both timepoints: miR396a and miR156d are upregulated at ED and downregulated at EN, and expression of these miRNAs thus appears to be temperature-dependent. The differential expression of miR156d is associated with opposite changes in its putative target mRNA levels for the gene *AT5G44020*, which encodes a HAD superfamily, subfamily IIIB acid phosphatase, both for ED and for EN. This strongly suggests that expression of this HAD gene is at least in part post-transcriptionally regulated by temperature through miRNA processing. The upregulation of miR396a at ED is associated with downregulation of mRNA levels for *AT3G19400*, which encodes a cysteine proteinase (Richau et al., 2012). However, downregulation of the same miR396a at EN is not associated with upregulation of these mRNAs levels, but with upregulation of mRNA transcript levels for *AT1G60140* (*TPS10*). *TPS10* encodes a trehalose6-phosphate (T6P) synthase (Ramon et al., 2009), which may function in sensing of the carbohydrate status of the plant. Because the putative differential expression of mi396a is associated with differential changes in different target mRNA levels at different timepoints, this raises the question whether or how specific miRNAs can select specific mRNAs with similar potential target sequences.

The majority of the miRNAs that are differentially expressed at ED is upregulated under -DIF, and the downregulated putative target mRNAs are involved in plant biotic stress responses (to insects AT5G24770, to *Botrytis cinerea* AT4G08870), water transport (AT1G01620), auxin signaling (AT3G17185) or cell expansion (AT1G21270). Downregulated miRNAs at ED target a gene encoding a protein involved in sugar transport (AT3G28007) or a C2H2-like zinc finger protein, transcription factor (AT5G52010).

miRNAs were also quantified in samples harvested from the *phyB-9* mutant samples. Again, miRNA read count was not reproducible between replicate samples and only the total read count of samples harvested at ED or EN were used. Using the same criteria as for the WT samples, 77 are differentially expressed under -DIF in the *phyB-9* mutant (Supplemental Figure S2B). In contrast to WT plants, in which the majority of the DEMs was only differentially expressed at ED, most of the DEMs are differentially expressed at EN in the *phyB-9* mutant (Supplemental Figure S2B). Of the 22 DEMs found in WT under -DIF that associate with changes in target mRNA transcript abundance, three were also identified in the *phyB-9* mutant samples (Table 1). However, differential expression of these miRNAs in *phyB-9* is not associated with differential changes in putative target mRNA transcript levels. For three other DEMs in the *phyB-9* mutant their response under -DIF was opposite to the response found in WT. This suggests that differential expression of these miRNAs is dependent on PHYB. In addition, there are 10 DEMs that are only found in the *phyB-9* mutant, indicating that normally their expression is buffered against changes by PHYB signaling.

Table 1: -DIF differentially expressed MicroRNAs and matching differentially expressed putative target mRNAs. Fold change in expression of differentially expressed miRNAs and their predicted target mRNAs under -DIF at end of day (ED) and end of night (EN) in Col-0 WT or *phyB-9* mutant plant material. Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Cells with thick borders contain miRNAs that are differentially expressed at both timepoints. The miRNAs and mRNAs were extracted from the same samples and considered differentially expressed if read count was ≥100 in at least one of the samples and fold change was ≥2. Prediction of Arabidopsis miRNA targets was done with psRNATarget (Dai and Zhao, 2011). Target genes were only included in table when differentially expressed (false discovery rate Q-value<0.05).

	miRNA	log2(fc in transcript level)		target gene		log2(fc in transcript level)	
	name	WT	*phyB-9*	TAIR id	name	WT	*phyB-9*
ED	miR396a-5p	3.19	-1.32	AT3G19400	Cyst-Prot	-0.72	.
				AT5G24660	LSU2	.	0.79
	miR156d-3p	2.35	.	AT5G44020	HAD	-1.05	.
	miR396b-5p	1.93	.	AT3G19400	Cyst-Prot	-0.72	.
	miR156c-3p	2.50	.	AT2G39420	MAGL8	-1.13	.
	miR167c-3p	2.45	.	AT5G24770	VSP2	-3.15	.
	miR2938	1.55	.	AT4G08870	ARGAH2	-0.96	.
	miR157c-3p	1.51	.	AT1G01620	PIP1	-0.52	.
	miR390a-5p	1.36	.	AT3G17185	TAS3	-0.79	.
	miR390b-5p	1.41	.	AT3G17185	TAS3	-0.79	.
	miR849	1.34	.	AT4G37410	CYP81F4	-1.43	.
	miR399b	1.00	.	AT1G21270	WAK2	-0.79	.
	miR850	-3.79	1.84	AT3G28007	SWEET4	1.20	.
	miR2111a-5p	-5.64	.	AT5G52010	C2H2-l	0.74	.
	miR169f-3p	2.28	2.47	AT1G13470	unknown	.	-0.64
	miR1886.2	1.37	-1.04	AT2G38770	MAC7	.	0.61
				AT4G37080	unknown	.	0.73
				AT5G15640	Mito-substrate carrier protein	.	1.16
EN	miR396a-5p	-1.08	1.01	AT1G60140	TPS10	0.90	.
	miR156d-3p	-1.05	1.37	AT5G44020	HAD	0.60	.
	miR396b-5p	.	1.07	AT3G19400	Cyst-Prot	.	-0.73
	miR847	.	2.04	AT5G60890	MYB34	.	-0.81
	miR157b-3p	.	2.44	AT1G12460	LRRK	.	-0.76
	miR157a-3p	.	2.30	AT1G12460	LRRK	.	-0.76
	miR858a	.	2.77	AT5G60890	MYB34	.	-0.81

Expression of *PIF4* and PIF-target genes is affected by -DIF

PIFs are transcription factors that induce transcription of genes involved in skotomorphogenesis (e.g. cell elongation) and repress genes that are involved in photomorphogenesis (e.g. synthesis of photosynthetic machinery components) (Leivar and Quail, 2011; Leivar and Monte, 2014; Xu et al., 2015; Pham et al., 2018). They are regulated on the protein level by PHYB, of which the activated Pfr form is able to enter the nucleus where it targets PIFs for degradation. Bours *et al*. previously showed suppression of cell elongation in hypocotyls of Arabidopsis seedlings is through downregulation of *PIF3*, *4* and *5* (Bours et al., 2015). Moreover, leaves of *phyB-9* mutant rosettes show reduced response to -DIF, which may be explained by increased stability of PIF proteins in the *phyB-9* mutant background, and suggests that PIFs also play a role in the leaf elongation response to -DIF in adult Arabidopsis plants. Besides regulation of PIFs at the protein level, some PIF genes are also regulated at the transcriptional level (Nozue et al., 2007; Nusinow et al., 2011; Mizuno et al., 2014; Zhu et al., 2016). For example, *PIF4* transcription in seedlings is downregulated in response to low temperatures and upregulated by high temperatures (Mizuno et al., 2014; Zhu et al., 2016). Downregulation of *PIF4* transcript levels is through the evening complex and the light/temperature-responsive transcription factor LONG HYPOCOTYL (HY5) (Catala et al., 2011; Nusinow et al., 2011; Delker et al., 2014; Nieto et al., 2015). Upregulation of *PIF4* transcript levels by high ambient is through the transcription factors BRASSINAZOLE RESISTANT 1 (BZR1) and TEOSINTEBRANCHED1/CYCLOIDEA/PCF 5 (TCP5) (Ibanez et al., 2018; Han et al., 2019).

Our RNAseq data shows that, for the selected timepoints, -DIF leads to downregulation of *PIF*4 transcript levels at ED, but no differential expression at EN (Supplemental Table S3). Reduced expression of *PIF4* in the light may have limited effects on PIF4 protein activity due to interaction between PIF4 and PHYB Pfr. To determine how -DIF affects expression of PIF target genes, the list of DEGs under -DIF at ED and EN was compared with the list of genes that are induced or suppressed by PIFs (Leivar and Monte, 2014).

Figure 10A shows that a substantial part of the genes that are repressed by PIFs are upregulated under -DIF (172 of 582), especially at EN (135). This subset of 135 genes that are upregulated under -DIF, are mainly involved in photosynthesis (e.g. Chlorophyll a-b binding proteins and Photosystem reaction center proteins (Figure 4B)), which is consistent with GO enrichment analysis showing that several genes that are upregulated only at EN are associated with the GO term ‘photosynthesis’ (GO:0015979) and that of the 77 genes mapped to the photosynthesis pathway in Arabidopsis (KEGG mapper; (Kanehisa and Goto, 2000)), 17 are upregulated at EN only (Supplemental Table S4). Upregulation of these genes under -DIF is not explained by any differential expression of *PIF4* at EN under -DIF. However, it could be explained by reduced stability of PIF4 protein acting as repressor under the warm night of -DIF.

Of the 462 PIF-induced genes, 22 are downregulated under -DIF at ED and 23 at EN (Figure 10B). PIF-induced genes that are downregulated under -DIF at ED included hormone-related genes (e.g. *INDOLE-3-ACETIC ACID INDUCIBLE 4*; *IAA4* and *GA-*

STIMULATED ARABIDOPSIS 6; *GASA6*) and *PHYTOCHROME A* (*PHYA*). Reduced expression of these PIF targets under -DIF at ED is now explained by reduced *PIF4* gene transcription under -DIF at ED. Targets positively regulated by PIFs that are downregulated under -DIF at EN included *IAA17* and *GROWTH REGULATING FACTOR 2* (*GRF2*). Indeed, activity of these PIF-regulated genes relate to growth and their lower expression under -DIF correlates with reduced elongation under -DIF. However, the expression of classic targets of PIF4 like *YUC8*, *IAA19*, *IAA29* and *SAUR15* (Kunihiro et al., 2011; Oh et al., 2012; Sun et al., 2012; Sun et al., 2013; Leivar and Monte, 2014) are not affected by -DIF, based on the selected time points. However, this could also indicate that some PIF targets are more sensitive to -DIF than others.

In contrast to WT, in the *phyB-9* mutant, -DIF did not affect *PIF4* transcript levels at ED (Supplemental Table S3). This suggests that reduced *PIF4* expression is the result of increased PHYB Pfr activity during the cold day. While in WT *PIF4* expression is not affected under -DIF at EN, *PIF4* expression is upregulated in the *phyB-9* mutant background at EN compared to that in WT (Supplemental Table S3). This suggests a feedback of PHYB on *PIF4* expression which dampens expression of *PIF4* during the warm night under -DIF conditions. Of the 135 PIF-repressed genes that are upregulated in WT under -DIF at EN, only 23 are also upregulated in the *phyB-9* mutant (Supplemental Figure S4B). Of the 21 PIF-induced genes that are downregulated in WT under -DIF at EN, only 2 are also downregulated in the *phyB-9* mutant (Supplemental Figure S4D). At ED however, still 44 out of 53 PIF-repressed genes that are upregulated in WT under -DIF are also upregulated in the *phyB-9* mutant (Supplemental Figure S4A), and of the 22 PIF-induced genes that are downregulated in WT under -DIF, 19 are also downregulated in the *phyB-9* mutant (Supplemental Figure S4C). Combined, results suggest that the reduced transcriptional activity of PIF proteins under -DIF at EN, is mainly dependent on PHYB.

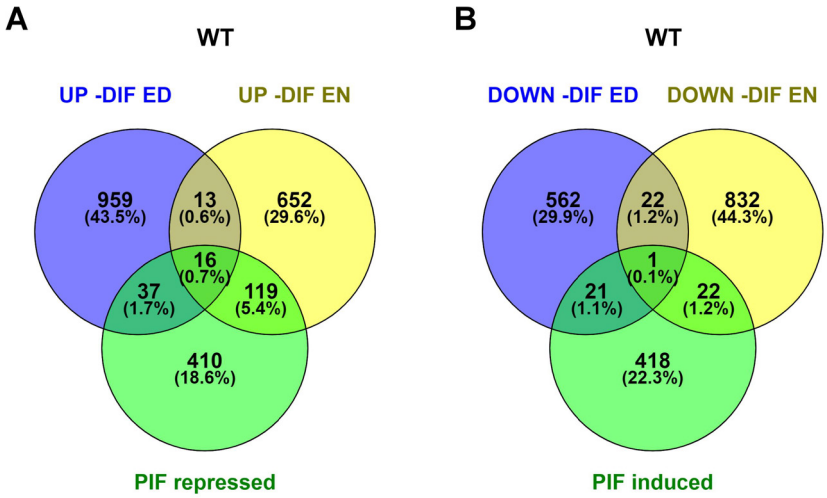


Figure 10: Genes that are differentially expressed under -DIF and/or regulated by PIFs. A: Venn diagram shows overlap in lists of genes that are upregulated under -DIF at end of day (ED) or end of night (EN) in Col-0 WT and genes that are PIF-repressed. **B**: Venn diagram shows overlap in lists of genes that are downregulated under -DIF at ED or EN in Col-0 WT and genes that are PIF-induced. Genes are significantly expressed under -DIF if false discovery rate Q-value<0.05. Lists of genes that are PIF-repressed/induced are from Supplemental Data Set 1 of (Leivar and Monte, 2014). Venn diagrams were created using the Venny 2.1 webtool (Oliveros, 2007-2015).

Discussion

Molecular processes affected by -DIF link to reduced growth under -DIF

-DIF affects multiple processes in Arabidopsis

Here we have characterized the transcriptional response to a -DIF treatment at two timepoints of the day (ED and EN). The growth response to -DIF for seedlings and rosette plants is a reduced elongation of hypocotyl (Bours et al., 2015) or leaf (petiole), respectively ((Thingnaes et al., 2008; Bours et al., 2013); Supplemental Figure S1). The GO enrichment analysis of genes that are differentially expressed under -DIF shows that -DIF affects several processes that link to plant growth, indicating that multiple processes contribute to the reduced growth under -DIF.

Cell wall biosynthesis: Plant cell elongation requires sequential steps in cell wall loosening, followed by increased water uptake to facilitate turgor-driven cell expansion, and finally cell wall crosslinking (reviewed in (Braidwood et al., 2014)). We showed that genes involved in plant-type cell wall organization and biogenesis are downregulated under -DIF, mostly at EN. Out of the 282 genes that are annotated to the GO-term 'plant-type cell wall organization or biogenesis' (GO:0071669), 23 are downregulated under -DIF at EN in WT (Supplemental Table S5), including several genes encoding expansins, which are cell wall modifying enzymes involved in plant cell growth (Cho and Cosgrove, 2000; Cosgrove, 2000; Cho and Cosgrove, 2002; Goh et al., 2012).

Water transport and uptake: Increased water uptake by leaf/petiole cells during the day requires transport of water from roots to sink leaves through transport in the xylem. Several genes downregulated under -DIF at ED alone are associated with water transport. Indeed, out of the 21 genes that are directly annotated to 'water transport' (GO:0006833), 7 are downregulated under -DIF at ED in WT (Supplemental Table S6). These genes mainly encode aquaporins such as PLASMA MEBRANE INTRINSIC PROTEINs (PIPs) and TONOPLAST INTRINSIC PROTEINs (TIPs). These aquaporins facilitate hydraulic permeability of plasma membranes and tonoplasts, and their expression is positively correlated with cell expansion in various plant tissues and organs (reviewed in (Chaumont and Tyerman, 2014)). Since genes involved in the facilitation of water transport are only suppressed at ED, our data suggests that cell expansion under -DIF is mainly suppressed during the day-period. This is in agreement with previous results showing that leaf elongation is mainly reduced during the cold photoperiod under -DIF (Bours et al., 2013).

Reduced PIF gene expression: Results show that -DIF reduces expression of the master growth regulator *PIF4* at ED (Supplemental Table S3). This is also reflected in altered expression of PIF target genes that relate to cell expansion (Figure 10) (Leivar and Monte, 2014). Although *PIF4* expression at EN was not significantly affected by -DIF, most of the PIF-repressed genes that are upregulated under -DIF are upregulated only at EN (Figure 10). In addition, the enhanced photosynthesis gene expression at EN under -DIF (Figure 4B) suggests lower PIF4 protein activity at EN under -DIF, since PIF4 acts as a suppressor of these genes. This could be due to reduced translation of the *PIF4* mRNA or increased PIF4 protein turnover under –DIF. Indeed, many of the genes that are downregulated by -DIF at EN (in a temperature-dependent manner) are involved in translational control (Figure 3A).

Energy status low at EN under -DIF: The upregulation of genes involved in photosynthesis reflect the low energy status under -DIF at EN (Figure 4B). GO enrichment analysis revealed that 3 out of 4 genes that are associated with the GO term 'cellular response to sucrose starvation' (GO:0043617) are upregulated under -DIF at EN. In addition, -DIF lead to EN upregulation of several genes that were identified in previous studies as markers for sugar availability (Supplemental Table S7; (Graf et al., 2010; Pilkington et al., 2015; Flis et al., 2016)). Sugar starvation can lead to a period of reduced growth and developmental arrest that extends into the photoperiod (Gibon et al., 2004; Graf et al., 2010). This happens through activation of the master growth regulator SnRK1 in response to low levels of sucrose and glucose (Baena-Gonzalez et al., 2007). The KIN10 subunit of SnRK1 negatively regulates expression of genes involved in growth and development, while stimulating expression of genes involved in energy production (Hardie, 2007; Tome et al., 2014). Cellular levels of soluble sugars are reflected by the energy signaling molecule trehalose-6-phosphate (T6P), which suppresses KIN10 activity (Zhang et al., 2009; Figueroa and Lunn, 2016). In Arabidopsis, 21 T6P biosynthesis genes have been identified (Ramon et al., 2009). Transcription of four of these genes (*TPS8-11*) responds strongly to sugar availability (up upon sugar starvation, down when sugars are supplied) (Price et al., 2004; Osuna et al., 2007; Usadel et al., 2008). Consistent with upregulation of sugar starvation marker genes under -DIF at EN,

TPS8, *10* and *11* are upregulated under -DIF at EN (Supplemental File S1). Interestingly, the miRNA miR396a, which has *TPS10* as a putative target, is downregulated under -DIF (Table 1), suggesting that *TPS10* mRNA levels may be regulated at the post-transcriptional level.

-DIF results in upregulation of cold acclimation processes at ED

Our results reveal a subset of genes for which transcription previously has been shown to be cold-induced. For many of these genes their induction of expression is not gated by the clock (Figure 3). As expected, most of these genes function various physiological, chemical and metabolic changes that help the plant to cope with cold stress (Hannah et al., 2005; Chinnusamy et al., 2007; Maruyama et al., 2009; Knight and Knight, 2012).

Flavonoid biosynthesis: A well-known cold adaptation process is the biosynthesis of flavonoids. Flavonoids are a large class of secondary metabolites that play a role in many (a)biotic stress responses (reviewed in (Di Ferdinando et al., 2012)). Under -DIF, many genes that map to the flavonoid biosynthesis pathway in Arabidopsis (KEGG mapper; (Kanehisa and Goto, 2000)) are upregulated at ED and downregulated at EN (Supplemental Table S8). This is consistent with low temperature-induced accumulation of flavonoids through upregulation of biosynthetic pathway genes (Schulz et al., 2015). In the light, flavonoids may function in suppression of accumulation of Reactive Oxygen Species (ROS), or in scavenging of ROS in order to protect photosystems from photoinhibition under cold (Havaux and Kloppstech, 2001). However, in WT flavonoid biosynthesis is reduced during the night under -DIF, compared to +DIF. If flavonoid biosynthesis is linked to ROS stress, this suggests that ROS stress is reduced during the night under -DIF, compared to that under +DIF.

Ribosome biogenesis: Other processes involved in cold-acclimation affected by -DIF, especially at ED, are ribosome biogenesis (Zhang et al., 2016) and carbohydrate metabolism (Tarkowski and Van den Ende, 2015). Out of the 364 genes that encode ribosome components (KEGG mapper (Kanehisa and Goto, 2000), 105 are upregulated under -DIF at ED, of which a small portion (13) are downregulated at EN (Supplemental Figure S5). In line with these results, we found that, of the 101 genes that can be mapped to the ribosome biogenesis pathway in Arabidopsis (KEGG mapper (Kanehisa and Goto, 2000)), 38 are upregulated under -DIF at ED, of which 16 genes are downregulated at EN (Supplemental Figure S6). In contrast to genes involved in flavonoid metabolism, most of the genes involved in ribosome biogenesis/protein translation are only differentially expressed under -DIF at ED, which may suggest that low temperature induction of ribosome biogenesis genes is for a large part gated towards the day-period.

Carbohydrate metabolism: In Arabidopsis, carbohydrate metabolism is affected by low temperatures to stimulate the accumulation of soluble sugars, which play a key role in acquiring cold tolerance through membrane stabilization, ROS scavenging and photosystem protection (Tarkowski and Van den Ende, 2015). The accumulation of soluble sugars upon cold stress is in part associated with starch degradation. The α-GLUCAN WATER DIKINASE (GWD) and β-AMYLASE 3 (BAM3) are major starch-

degrading enzymes induced under cold stress (Thalmann and Santelia, 2017). The genes encoding these enzymes were both shown to be upregulated upon cold stress and mutants deficient in GWD or BAM3 showed reduced sugar accumulation under cold stress (Kaplan and Guy, 2004, 2005; Yano et al., 2005; Sicher, 2011). In agreement with this, expression of these genes was upregulated under -DIF at ED (Supplemental Table S9). Genes upregulated under -DIF at ED alone relate to 'response to sucrose' (GO:0009744), suggesting higher than normal accumulation of soluble sugars under -DIF during the day (Supplemental File S4). Next to breakdown of starch to achieve increased sugar accumulation to protect against cold during the day under -DIF, increased starch accumulation in Arabidopsis in response to low temperatures has also been reported (Table 1 in (Thalmann and Santelia, 2017). In agreement with this, several genes encoding starch biosynthesis enzymes are also upregulated under -DIF at ED (Supplemental Table S9). It needs further investigation whether these opposite responses in CH metabolism relate to source petiole and sink leaf tissue. The regulation of carbohydrate metabolism is source and sink leaves in response to -DIF will therefore be target of our next studies on -DIF.

The role of PHYB in adaptation to -DIF

No reduced cell wall biosynthesis activity in phyB-9

The *phyB-9* mutant shows a reduced response to -DIF (Bours et al., 2013), and transcriptomics analysis of the *phyB-9* mutant can reveal which growth-related genes require PHYB. None of the DEGs associated with cell wall organization or biogenesis downregulated in WT at EN under -DIF are differentially expressed in the *phyB-9* mutant (Supplemental Table S5), confirming the reduced inhibition of elongation in *phyB-9*. In contrast, the genes associated with water transport that are downregulated under -DIF at ED are also downregulated in the *phyB-9* mutant (Supplemental Table S6), indicating that regulation of these genes is PHYB-independent. However, this also indicates that downregulation of these water transport genes is not limiting for elongation under -DIF.

Increased PIF and PIF target activity in phyB-9 under -DIF

A lack of functional PHYB also results in higher *PIF4* expression at EN (Supplemental Table S3), indicating feedback of PHYB on expression of *PIF4*. A higher *PIF* expression, combined with and absence of PHYB Pfr results in overall higher PIF protein activity. This is reflected in fewer PIF-induced genes that are downregulated under -DIF at EN and fewer PIF-repressed genes that are upregulated under -DIF at EN in the *phyB-9* mutant compared to in the WT (Supplemental Figure S4B+D).

PHYB regulates temperature-dependent transcription

The majority of the genes that show temperature-dependent expression under -DIF is dependent on PHYB activity (Figure 7). This is in agreement with PHYB acting as a temperature sensor (Jung et al., 2016; Legris et al., 2016; Qiu et al., 2019). Genes stimulated at 12°C (or suppressed at 22°C) that depend on PHYB are associated with cold acclimation processes such as ribosome biogenesis, CH metabolism and flavonoid metabolism (Figure 7). Looking in more detail at expression of genes involved in these pathways under -DIF at ED and EN shows that the transcriptional response in the WT and *phyB-9* mutant is very similar at ED, but not at EN: Most of the genes that are differentially expressed under -DIF at EN in WT are not differentially expressed in the *phyB-9* mutant (Supplemental Figures S3-S5 and S8; Supplemental Table S8). These results suggest that under -DIF, PHYB plays a key role in conveying temperature information at night, but not so much during the day.

Limited sugar starvation status in phyB-9

-DIF leads to increased expression of CH starvation marker genes in WT, which is linked to growth suppression (Gibon et al., 2004; Graf et al., 2010). In the *phyB-9* mutant however, only two starvation markers genes (which show strongest upregulation in WT) are also upregulated in the *phyB-9*. Also, the upregulation of *TPS8*, *10* and *11* under -DIF at EN in WT is absent in the *phyB-9* mutant background, while expression of miR396a (with putative target TPS10) is actually down in *phyB-9* (Table 1). Overall, this suggests that CH starvation at EN under -DIF is limited in *phyB-9* compared to that in WT (Supplemental Table S7). Consistent with this, only one gene involved in photosynthesis is upregulated at EN in the *phyB-9* mutant (compared to 18 in the WT) (Supplemental Table S8). The reduced sugar starvation status reached in *phyB-9* explains reduced response to -DIF for suppression of leaf elongation in *phyB-9*.

Temperature compensation by PHYB stronger during day under -DIF

Comparison of DEGs under -DIF WT and *phyB-9* indicates 1630 DEGs in *phyB-9* at ED but only 339 DEGs at EN for which expression is buffered by PHYB in WT (Figure 8 and 9). This indicates that PHYB has a bigger role in buffering gene expression under -DIF during day than during the night. This fits with reduced dark reversion of PHYB Pfr during the day under -DIF and prolonged interaction of PHYB Pfr with PIF proteins. PIF4 is a key regulator of elongation responses in plants and PIF4 is regulated on the protein level by PHYB, as the light-activated Pfr form of PHYB is able to trigger PIF4 degradation (Leivar and Quail, 2011; Leivar and Monte, 2014; Xu et al., 2015; Pham et al., 2018). Therefore, in addition to reduced *PIF4* gene transcription at ED under -DIF, effective PIF protein activity is also limited during the cold day under -DIF, due to increased PHYB Pfr action, as low temperature during day reduces the rate of dark reversion of Pfr to Pr (Jung et al., 2016; Legris et al., 2016). This also explains the differential expression of 75 PIF target genes at ED (Figure 10).

Although *PIF4* expression is not significantly affected at EN under -DIF, 158 PIF target genes show differential expression at EN under -DIF (Figure 10). Of these, 135 PIF-repressed genes are upregulated in WT under -DIF at EN, indicating reduced PIF protein activity under -DIF during the night. The reduced PIF protein activity under -DIF during the night is linked to PHYB as the majority (23) of these 135 DEGs are not upregulated in the *phyB-9* mutant (Supplemental Figure S4B). This is difficult to explain as the warm night under -DIF results in increased dark reversion of PHYB Pfr, and thus would be expected to mimic a *phyB* mutant background.

Concluding remarks

Consistent with the growth phenotype observed in response to -DIF, our RNAseq data analysis shows that in Arabidopsis a -DIF treatment affects expression of genes associated with processes linked to plant growth such as cell wall modification, water uptake, PIF expression and energy status. In addition, the low day-time temperature under -DIF leads to upregulation of genes associated with cold acclimation processes, including flavonoid- and CH-metabolism. Sequencing of miRNAs in the same samples used for sequencing mRNA indicates that some of the genes associated with the processes affected by -DIF could be regulated through targeting by miRNAs. Analysis of the *phyB-9* mutant, which does not show reduced elongation in response to -DIF, suggest a causal link between most of the processes linked to growth that are affected at EN, since associated genes are often not differentially expressed under -DIF in the *phyB-9* mutant. On the other hand, more genes show differential expression in the *phyB-9* mutant than in WT under -DIF at ED, suggesting that in WT PHYB plays a role in buffering changes in gene expression under -DIF during the day, but in inducing gene expression changes during the night.

Although our data presented here provide a general overview of processes affected under -DIF, there are some limitations to this study that have to be taken into account. First of all, to check the quality of our data, validating differential expression of mRNA and miRNAs by qRT-PCR on similar samples is needed. Second, only two timepoints were included here: ED and EN. Hence, any putative short-term responses to changes in light/temperature are not visible in our dataset. Furthermore, many components that regulate growth (including PIFs) are regulated by the clock. By looking at only two timepoints, it is difficult to capture any possible phase changes in expression of clock(-regulated) components. Third, as temperature affects enzymatic activity directly, the observed changes in expression of genes related to metabolic pathways do not have to lead to actual changes in metabolism in response to -DIF. Therefore, future studies on the effect on -DIF will focus on the effect on enzyme activity and metabolite levels. Finally, although our data on WT and the *phyB-9* mutant suggests a causal link between processes such as altered PIF expression and energy status with growth suppression under -DIF, this needs to be confirmed in further studies using appropriate mutants.

Supplemental Data

Supplemental Table S1: Differentially expressed genes that are upregulated under -DIF at end of day and end of night. Log2(fold change in expression) of genes that are significantly higher expressed (false discovery rate Q-value<0.05) in Col-0 WT under -DIF compared to +DIF at end of day (ED) and end of night (EN). Color indicates strength of change in expression (dark green=strong upregulation, light green=weak upregulation). First column from the left shows TAIR identifiers and second column shows name/description of genes.

TAIR id	name/description	ED	EN
AT3G28220	unknown	2.84	1.01
AT2G26010	Defensin-like protein 14; PDF1.3	2.17	1.14
AT2G05380	Glycine-rich protein 3 short isoform; GRP3S	1.87	1.41
AT5G54190	Protochlorophyllide reductase A, chloroplastic; PORA	1.86	3.11
AT1G29910	Chlorophyll a-b binding protein 3, chloroplastic; LHCB1.2	1.77	1.53
AT1G29920	Chlorophyll a-b binding protein 2, chloroplastic; LHCB1.1	1.77	1.53
AT1G58290	Glutamyl-tRNA reductase 1, chloroplastic; HEMA1	1.57	1.37
AT1G32540	LOL1	1.47	0.70
AT5G13630	Magnesium-chelatase subunit ChlH, chloroplastic; CHLH	1.38	0.95
AT4G16260	Probable glucan endo-1,3-beta-glucosidase	1.16	1.25
AT1G64900	Cytochrome P450 89A2; CYP89A2	1.15	0.82
AT2G47880	Glutaredoxin-C13; GRXC13	1.08	1.33
AT5G35970	P-loop containing nucleoside triphosphate hydrolases superfamily protein; MEE13.8	1.04	0.98
AT1G74470	Geranylgeranyl diphosphate reductase, chloroplastic; CHLP	0.97	1.09
AT5G67030	Zeaxanthin epoxidase, chloroplastic; ZEP	0.93	0.58
AT4G34350	4-hydroxy-3-methylbut-2-enyl diphosphate reductase, chloroplastic; ISPH	0.92	0.67
AT3G27690	Chlorophyll a-b binding protein 2.4, chloroplastic; LHCB2.4	0.86	2.11
AT5G54270	Chlorophyll a-b binding protein 3, chloroplastic; LHCB3	0.83	2.13
AT3G54500	LNK2	0.81	0.76
AT1G68520	Zinc finger protein CONSTANS-LIKE 6; COL6	0.76	1.43
AT5G57345	unknown	0.76	1.77
AT5G08050	Wiskott-aldrich syndrome family protein	0.70	0.49
AT2G29650	Sodium-dependent phosphate transport protein 1, chloroplastic; ANTR1	0.66	0.59
AT3G59400	Tetrapyrrole-binding protein, chloroplastic; GUN4	0.58	0.50
AT5G64040	Photosystem I reaction center subunit N, chloroplastic; PSAN	0.58	1.12
AT1G30880	unknown	0.56	0.50
AT5G61410	D-ribulose-5-phosphate-3-epimerase; RPE	0.52	0.52
AT3G19720	Dynamin-like protein ARC5	0.51	0.54
AT1G08005	Serine/threonine-protein phosphatase 6 regulatory subunit	inf	inf

Supplemental Table S2: Differentially expressed genes that are downregulated under -DIF at end of day and end of night. Log2(fold change in expression) of genes that are significantly lower expressed (false discovery rate Q-value<0.05) in Col-0 WT under -DIF compared to +DIF at end of day (ED) and end of night (EN). Color indicates strength of change in expression (dark red=strong downregulation, light red=weak downregulation). First column from the left shows TAIR identifiers and second column shows name/description of genes.

TAIR id	name/description	ED	EN
AT2G14247	Expressed protein	-4.79	-2.34
AT1G13609	Defensin-like protein 287	-3.47	-3.35
AT2G46880	Probable inactive purple acid phosphatase 14; PAP14	-2.92	-1.96
AT5G53450	Probable plastid-lipid-associated protein 14, chloroplastic; PAP14	-2.92	-1.96
AT1G73330	Dr4 protein; Dr4	-2.29	-1.35
AT5G67370	CONSERVED IN THE GREEN LINEAGE AND DIATOMS 27, chloroplastic; CGLD27	-2.29	-0.93
AT1G03870	Fasciclin-like arabinogalactan protein 9; FLA9	-2.24	-0.63
AT5G48850	Protein SULFUR DEFICIENCY-INDUCED 1; SDI1	-2.11	-1.69
AT5G03545	unknown	-1.75	-2.19
AT5G55350	Probable long-chain-alcohol O-fatty-acyltransferase 4; AT4	-1.75	-2.19
AT2G30766	Uncharacterized protein	-1.66	-1.70
AT4G15210	Beta-amylase 5; BAM5	-1.39	-1.61
AT1G64980	CDI	-1.08	-0.91
AT3G55710	UDP-glycosyltransferase 76F2; UGT76F2	-0.97	-0.78
AT2G30150	UDP-glycosyltransferase 87A1; UGT87A1	-0.81	-0.75
AT5G52882	P-loop containing nucleoside triphosphate hydrolases superfamily protein	-0.72	-0.68
AT5G67330	Metal transporter Nramp4; NRAMP4	-0.69	-0.75
AT5G37260	Protein REVEILLE 2; RVE2	-0.68	-0.50
AT1G12110	Protein NRT1/ PTR FAMILY 6.3; NPF6.3	-0.67	-0.61
AT4G25700	Beta-carotene 3-hydroxylase 1, chloroplastic; BETA-OHASE 1	-0.65	-0.61
AT4G10120	Probable sucrose-phosphate synthase 4; SPS4	-0.52	-0.78
AT5G22580	Stress-response A/B barrel domain-containing protein	-0.52	-1.08
AT4G12880	unknown	-0.51	-0.85

Supplemental Table S3: Regulation of PIF4 transcription under -DIF in *phyB-9* mutant and WT. Log2(fold change in expression) of *PIF4* in Col-0 WT and *phyB-9* mutants under -DIF at end of day (ED) or and of night (EN). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Expression is significantly different if false discovery rate Q-value<0.05.

		ED		EN	
TAIR ID	name	WT	*phyB-9*	WT	*phyB-9*
AT2G43010	PIF4	-1.15	0	0	2.62

Supplemental Table S4: Differentially expressed genes under -DIF that are involved in photosynthesis. Log2(fold change in expression) of genes that are differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of day (ED) or end of night (EN) and map to the photosynthesis pathway in Arabidopsis (KEGG mapper; (Kanehisa and Goto, 2000)). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Cells with thick borders contain Log2(fold change in expression) values of genes that are upregulated under -DIF in WT at ED and downregulated at EN. First column from the left shows TAIR identifiers and last column shows gene names and/or descriptions of gene function.

	ED		EN		
TAIR id	**WT**	***phyB-9***	**WT**	***phyB-9***	**name; description**
AT1G60950	1.22	1.00	0	0	ferredoxin
AT4G28660	0.69	0.74	0	-0.56	PSB28; photosystem II reaction center PSB28 protein
AT5G64040	0.58	0	1.12	0	PSAN; photosystem I reaction center subunit PSI-N
AT1G03600	0	0	0.98	0	PSB27; photosystem II family protein
AT1G08380	0	0	1.52	0	PSAO; photosystem I subunit O
AT1G30380	0	0	1.31	0	PSAK; photosystem I subunit K
AT1G30510	0	0	-0.73	0	RFNR2; root FNR 2
AT1G31330	0	0	1.44	0	PSAF; photosystem I subunit F
AT1G52230	0	0	1.35	0.63	PSAH2; photosystem I subunit H2
AT1G55670	0	0	1.33	0	PSAG; photosystem I subunit G
AT1G67740	0	0	0.73	0	PSBY; photosystem II BY
AT1G79040	0	0	0.83	0	PSBR; photosystem II subunit R
AT2G20260	0	0	1.22	0	PSAE-2; photosystem I subunit E-2
AT3G16140	0	0	0.95	0	PSAH-1; photosystem I subunit H-1
AT3G50820	0	0	0.64	0	PSBO2; photosystem II subunit O-
AT4G05180	0	0	1.35	0	PSBQ-2; photosystem II subunit Q
AT4G05390	0	0	-0.66	0	RFNR1; root FNR 1
AT4G09650	0	0	0.73	0	ATPD; F-type H+-transporting ATPase su
AT4G12800	0	0	1.14	0	PSAL; photosystem I subunit l
AT4G21280	0	0	0.81	0	PSBQA; photosystem II subunit QA
AT4G28750	0	0	1.25	0	PSAE-1; Photosystem I reaction centre subunit IV / PsaE p
AT5G66570	0	0	0.93	0	PSBO1; PS II oxygen-evolving com
AT3G01440	-0.72	0	0	0	PNSL3; PsbQ-like 1

Supplemental Table S5: Differentially expressed genes under -DIF that are associated with cell wall organization or biogenesis. Log2(fold change in expression) of genes that are differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of night (EN) and associated with plant-type cell wall organization or biogenesis (GO:0071669). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). First column from the left shows TAIR identifiers and second shows gene names.

TAIR id	name	EN_WT	EN_*phyB-9*
AT1G48100	unknown	-1.89	0
AT5G09870	MUM3	-1.29	0
AT1G26770	EXPA10	-1.10	0
AT3G29360	UGD2	-1.06	0
AT3G02230	RGP1	-1.00	0
AT5G15740	RRT1	-0.95	0
AT5G15490	unknown	-0.86	0
AT3G21190	MSR1	-0.85	0
AT2G36570	unknown	-0.84	0
AT3G29030	EXPA5	-0.83	0
AT2G04780	unknown	-0.75	0
AT2G03090	EXPA15	-0.68	0
AT5G15650	RGP2	-0.67	0
AT4G28250	EXPB3	-0.66	0
AT1G03870	unknown	-0.63	0
AT4G00740	unknown	-0.58	0
AT2G03820	NMD3	-0.55	0
AT1G75500	unknown	-0.50	0
AT5G60920	COBRA	-0.50	0
AT3G61130	LGT1	-0.50	0
AT5G64740	IXR2	-0.50	0
AT1G76670	unknown	-0.50	0
AT1G68060	MAP70-1	-0.48	0
AT4G17030	EXPR	0	-0.65
AT1G21310	RSH	0	-1.19
AT5G16600	MYB43	0	-1.79
AT1G76930	ORG5	0	-1.24
AT4G08920	BLU1	0.54	0
AT2G46710	ROPGAP3	0.59	0
AT1G69530	EXPA1	1.14	0
AT5G63800	BGAL6	1.32	1.51
AT2G40610	EXPA8	2.80	0

Supplemental Table S6: Differentially expressed genes under -DIF that are associated with water transport. Log2(fold change in expression) of genes that are differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of day (ED) and associated with water transport (GO:0006833). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). First column from the left shows TAIR identifiers and second column shows names of genes.

TAIR id	name	ED_WT	ED_*phyB-9*
AT3G16240	TIP2;1	-1.65	-2.14
AT3G53420	PIP2	-1.55	-1.40
AT4G23400	PIP1D	-1.32	-1.77
AT2G36830	TIP1;1	-1.31	-1.03
AT2G45960	TMP-A	-0.96	-0.96
AT3G61430	PIP1	-0.82	-1.20
AT1G01620	PIP1;3	-0.52	-1.15
AT2G37170	PIP2;2	0	-0.68
AT2G39010	PIP2;6	0	-0.81
AT4G35100	PIP3A	0	-0.60

Supplemental Table S7: Differentially expressed carbohydrate starvation marker genes under -DIF. Log2(fold change in expression) of carbohydrate starvation marker genes selected from literature (Graf et al., 2010; Pilkington et al., 2015; Flis et al., 2016) and differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of day (ED) or end of night (EN). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Cells with thick borders contain Log2(fold change in expression) values of genes that are upregulated under -DIF in WT at ED and downregulated at EN. First column from the left shows TAIR identifiers and last column shows gene names and/or descriptions of gene function.

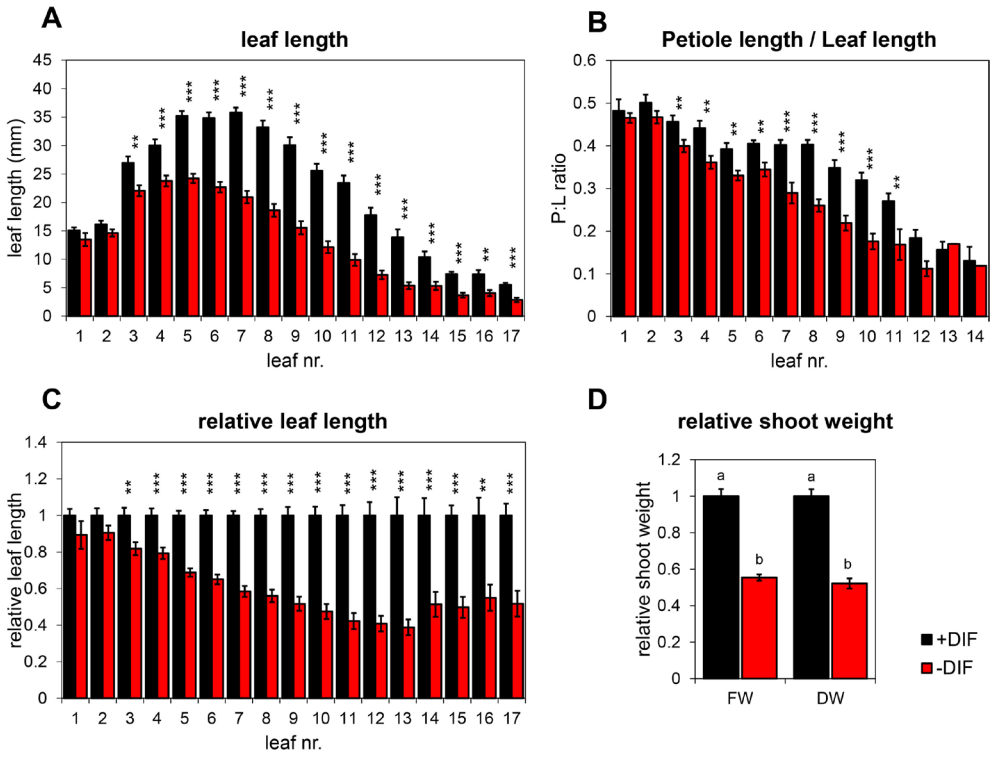
	ED		EN		
TAIR id	WT	*phyB-9*	WT	*phyB-9*	name/description
AT2G45170	-1.93	-1.62	1.17	0	ATG8E
AT5G18170	-1.18	-1.03	1.84	0	GDH1
AT3G47340	0	0	3.24	1.45	DIN6/ASN1
AT5G56100	0	0	2.57	1.25	glycine-rich protein
AT3G59940	0	-0.89	1.50	0	KMD4
AT5G07030	0	0	-0.63	0	aspartyl protease protein

Supplemental Table S8: Differentially expressed genes under -DIF that are involved in flavonoid biosynthesis. Log2(fold change in expression) of genes that are differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of day (ED) or end of night (EN) and map to the flavonoid biosynthesis pathway in Arabidopsis (KEGG mapper; (Kanehisa and Goto, 2000)). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Cells with thick borders contain Log2(fold change in expression) values of genes that are upregulated under -DIF in WT at ED and downregulated at EN. First column from the left shows TAIR identifiers and last column shows gene names and/or descriptions of gene function.

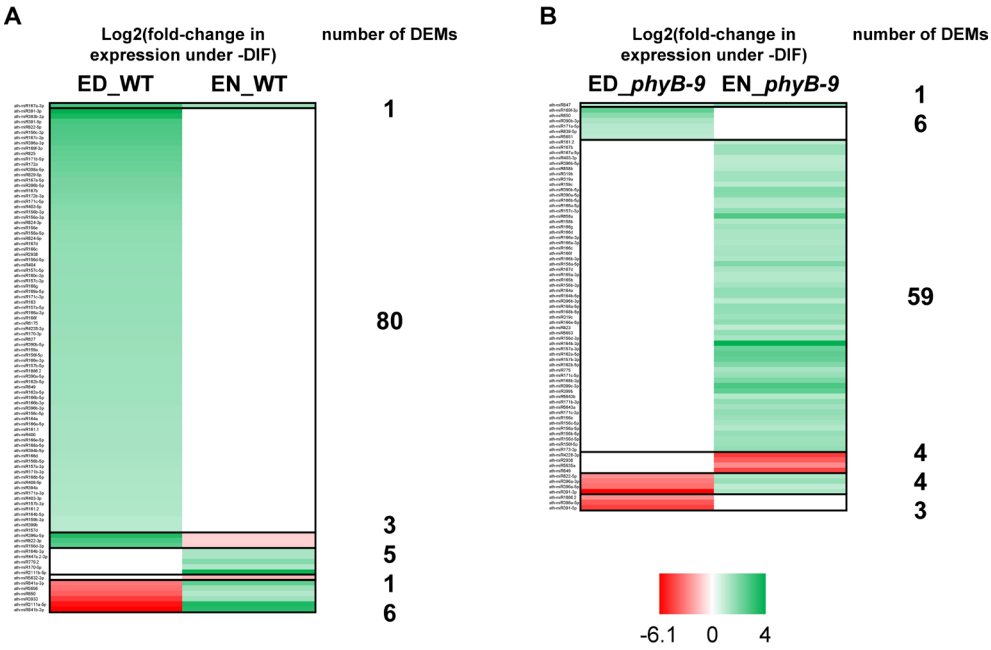
	ED		EN		
TAIR id	**WT**	***phyB-9***	**WT**	***phyB-9***	**name; description**
AT5G07990	2.47	0	-2.08	0	flavonoid 3'-monooxygenase
AT3G51240	2.35	3.46	-3.12	0	F3H; flavanone 3-hydroxylase
AT5G13930	2.02	2.91	-2.02	-0.97	TT4; Chalcone and stilbene synthase family protein
AT5G08640	1.62	0	-2.19	0	FLS1; flavonol synthase 1
AT3G55120	1.55	2.26	-1.79	0	Chalcone-flavanone isomerase family protein
AT5G42800	1.44	2.18	-1.02	0	DFR; bifunctional dihydroflavonol 4-reductase
AT5G05270	1.38	2.54	-2.31	-1.06	CHIL; Chalcone-flavanone isomerase family protein
AT4G22880	1.27	2.52	-1.54	0	anthocyanidin synthase
AT4G34050	0	0	-0.66	0	caffeoyl-CoA O-methyltransferase

Supplemental Table S9: Differentially expressed genes under -DIF that are involved in starch metabolism. Log2(fold change in expression) of genes that are differentially expressed (false discovery rate Q-value<0.05) in Col-0 WT and/or *phyB-9* mutants under -DIF at end of day (ED) or end of night (EN) and are associated with the Gene Ontology (GO) term starch metabolic process (GO:0005982). Genes are grouped based on whether they are involved in starch biosynthesis (upper part table) or starch breakdown (lower part table). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). Cells with thick borders contain Log2(fold change in expression) values of genes that are upregulated under -DIF in WT at ED and downregulated at EN. Second column from the left shows TAIR identifiers and last column shows gene names.

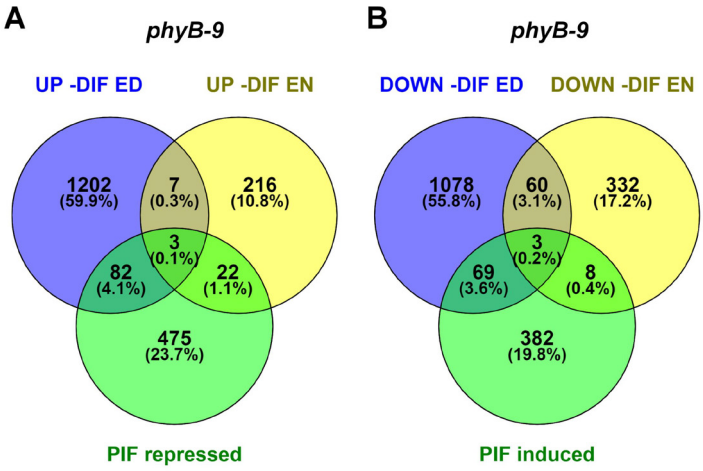
		ED		EN		
	TAIR id	**WT**	***phyB-9***	**WT**	***phyB-9***	**name**
biosynthesis	AT1G32900	1.33	2.05	-1.02	0	GBSS1
	AT2G36390	0.66	0.64	-0.67	0	SBE2.1
	AT1G27680	0.71	0	-0.93	0	APL2
	AT3G55760	1.43	0.99	0	0	LESV
	AT3G20440	0.68	1.92	0	0	BE1
	AT3G16000	0.52	1.06	0	0	MFP1
	AT2G21590	0	0	-0.99	0	APL4
	AT5G51830	0	0	-0.72	0	FRK1
breakdown	AT4G17090	1.23	1.39	-1.21	-0.93	BAM3
	AT4G09020	0.94	0.54	-1.02	0	ISA3
	AT1G10760	0.88	0.98	0	0	GWD1
	AT5G37780	0	0	-0.61	-0.49	CAM1
	AT1G69830	0	0	-1.16	0	AMY3
	AT3G10940	-0.63	-0.78	0	0	LSF2
	AT4G15210	-1.39	0	-1.61	0	BAM5
	AT3G30720	-1.71	0	0	0	QQS



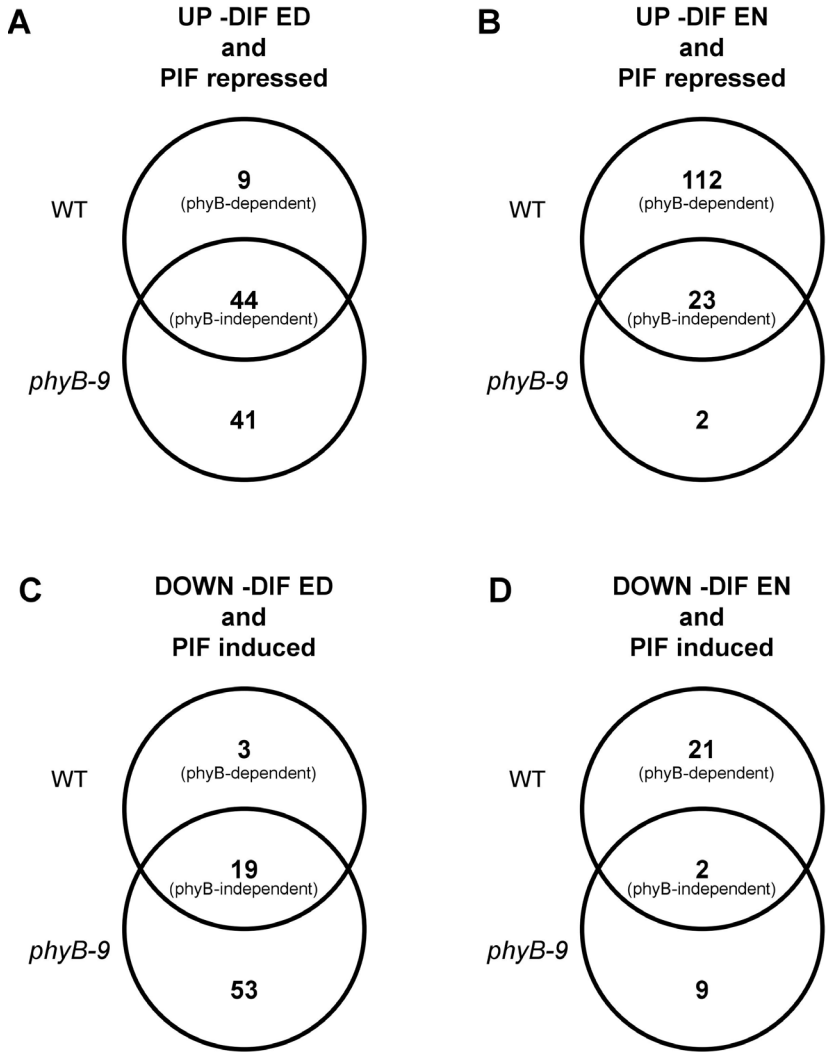
Supplemental Figure S1: Effect of -DIF on leaf length and shoot weight of adult Arabidopsis plants. Leaf length (**A**), ratio between lengths of petiole and leaf (P:L ratio; **B**), relative leaf length (**C**) and relative shoot weight (**D**) of Arabidopsis Col-0 WT plants grown under +DIF for 31 days (black bars) or under +DIF for 21 days and then transferred to -DIF for 10 days (red bars). The P:L ratio was calculated by dividing the petiole length of each leaf by total its total leaf length (petiole+blade). Relative leaf length was calculated by normalizing leaf length in mm to the average leaf length under +DIF. Relative shoot weight was calculated by normalizing shoot fresh weight (FW) or dry weight (DW) in grams to average shoot FW or DW under +DIF. Data are mean leaf length in mm, P:L ratio and relative leaf length ±SE (n=15 plants) and mean relative shoot weight ±SE (n=6 replicates of 5 pooled plants). Leaf numbers are based on order of emergence. Asterisks in A-C indicate significantly different from values under +DIF (*p<0.05, **p<0.01, ***p<0.001, Student's t-test). Different letters in D indicate significant differences in relative DW or FW (p<0.05, Student's t-test).



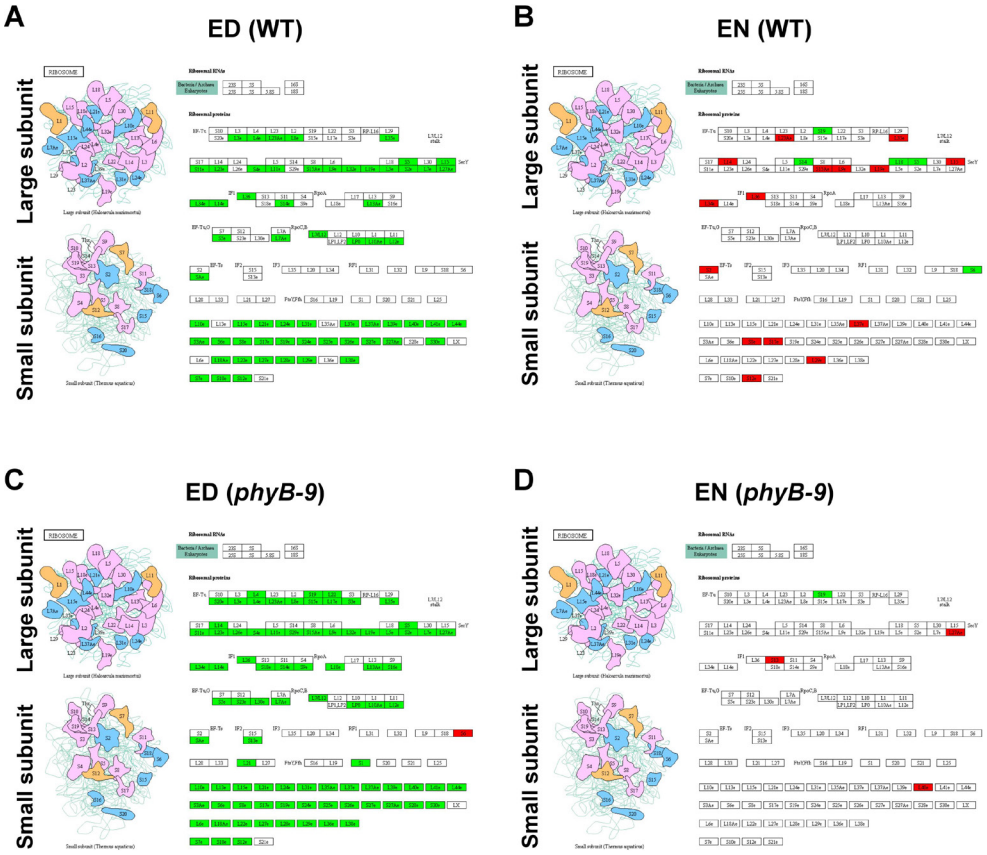
Supplemental Figure S2: Heatmaps of miRNAs that are differentially expressed under -DIF. Heatmap shows log2(fold change in expression) of differentially expressed miRNAs (DEMs; false discovery rate Q-value<0.05) in shoot material of Arabidopsis Col-0 WT (**A**) and the *phyB-9* mutant (**B**) under -DIF at end of day (ED; left columns) and end of night (EN; right columns). Colors indicate direction of change in expression (green=upregulation, red=downregulation, white=not significantly different). DEMs are clustered (thick borders) based on similar regulation under -DIF. Numbers on the right indicate the number of DEMs belonging to these groups.



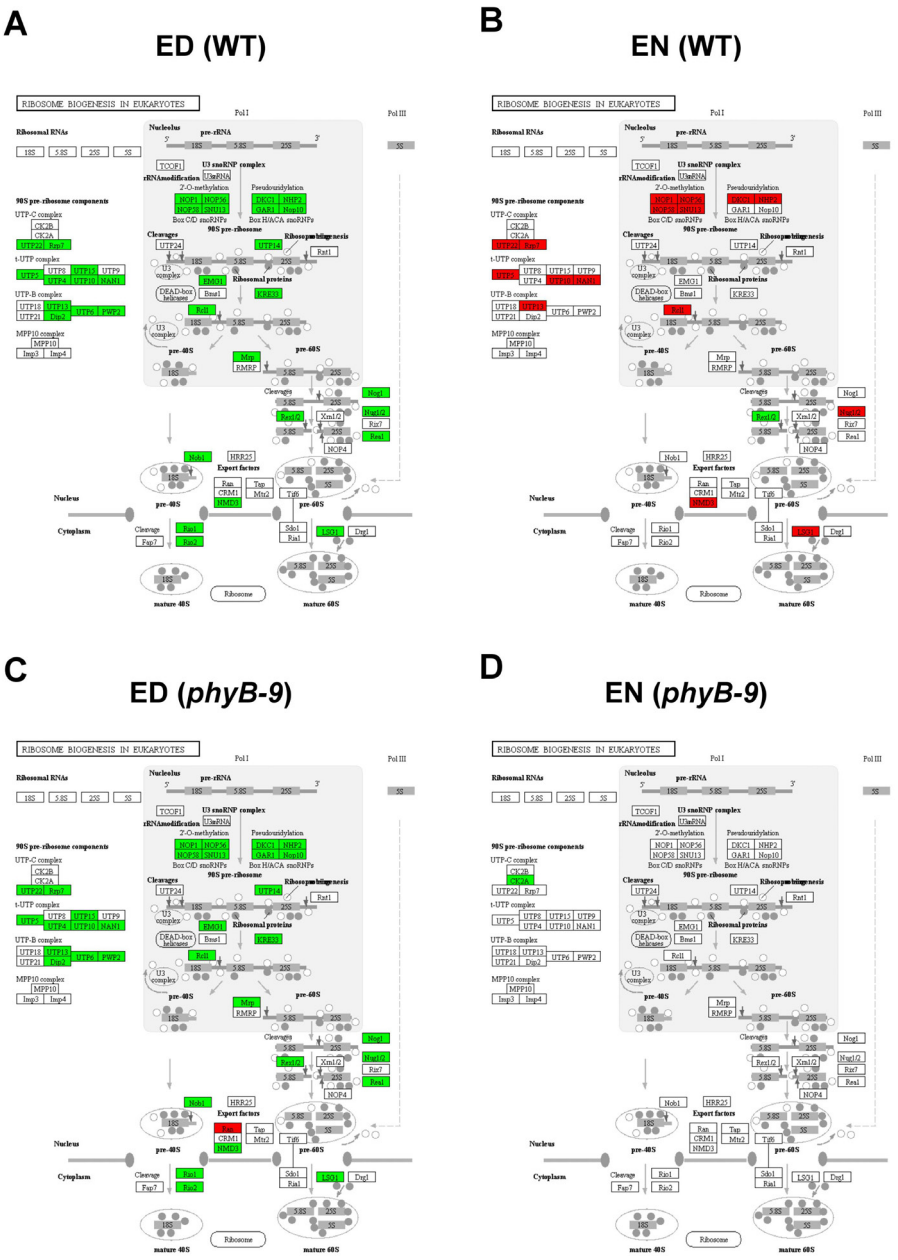
Supplemental Figure S3: Genes that are differentially expressed under -DIF in the *phyB-9* mutant and/or regulated by PIFs. A: Venn diagram shows overlap in lists of genes that are upregulated under -DIF at end of day (ED) or end of night (EN) in the Arabidopsis *phyB-9* mutant and genes that are PIF repressed. **B**: Venn diagram shows overlap in lists of genes that are downregulated under -DIF at ED or EN in the Arabidopsis *phyB-9* mutant and genes that are PIF-induced. Genes are significantly expressed under -DIF if false discovery rate Q-value<0.05. Lists of genes that are PIF-repressed/induced are from Supplemental Data Set 1 of (Leivar and Monte, 2014). Venn diagrams were created using the Venny 2.1 webtool (Oliveros, 2007-2015).



Supplemental Figure S4: Overlap between PIF-target genes that are differentially expressed under -DIF in WT and *phyB-9* mutants. A: Venn diagram shows overlap between genes that are upregulated under -DIF at end of day (ED) and transcriptionally suppressed by PIFs. **B:** Venn diagram shows overlap between genes that are upregulated under -DIF at end of night (EN) and transcriptionally suppressed by PIFs. **C:** Venn diagram shows overlap between genes that are downregulated under -DIF at ED and transcriptionally activated by PIFs. **D:** Venn diagram shows overlap between genes that are downregulated under -DIF at EN and transcriptionally activated by PIFs. Genes are significantly expressed under -DIF if false discovery rate Q-value<0.05. Lists of genes that are PIF-repressed/induced are from Supplemental Data Set 1 of (Leivar and Monte, 2014). Differential expression in WT only indicates transcriptional regulation of these genes is phytochrome B (PHYB)-dependent. Differential expression in WT and mutant (overlapping region) indicates transcriptional regulation of these genes is phytochrome B (PHYB)-independent.



Supplemental Figure S5: KEGG pathway maps for ribosome, indicating genes that are differentially expressed under -DIF. Schematic representation of the different ribosome components in Arabidopsis, indicating ribosome component-encoding genes that are differentially expressed under -DIF (false discovery rate Q-value<0.05) in Col-0 WT at end of day (ED; **A**) and end of night (EN; **B**) and in the *phyB-9* mutant at ED (**C**) and EN (**D**). Boxes represent gene products (proteins/RNA). Colors indicate direction of change in expression of encoding gene(s) under -DIF (green=upregulation, red=downregulation, white=not significantly different). Pathway maps are constructed and colored with KEGG mapper (Kanehisa and Goto, 2000).



Supplemental Figure S6: KEGG pathway maps for ribosome biogenesis, indicating genes that are differentially expressed under -DIF. Schematic representation of ribosome biogenesis pathway in Arabidopsis, indicating pathway component-encoding genes that are differentially expressed under -DIF (false discovery rate Q-value<0.05) in Col-0 WT at end of day (ED; **A**) and end of night (EN; **B**) and in the *phyB-9* mutant at ED (**C**) and EN (**D**). Boxes represent gene products (proteins/RNA). Colors indicate direction of change in expression of encoding gene(s) under -DIF (green=upregulation, red=downregulation, white=not significantly different). Pathway maps are constructed and colored with KEGG mapper (Kanehisa and Goto, 2000).

Supplemental File S1: List of differentially expressed genes (DEGs) for all possible sample comparisons in this study. (Microsoft Excel File)

Supplemental File S2: Results of GO-enrichment analysis for genes that are both upregulated under -DIF at end-of-day (ED) and downregulated at end-of-night (EN) in Col-0 WT. (Microsoft Excel File)

Supplemental File S3: Results of GO-enrichment analysis for genes that are both downregulated under -DIF at end-of-day (ED) and upregulated at end-of-night (EN) in Col-0 WT. (Microsoft Excel File)

Supplemental File S4: Results of GO-enrichment analysis for genes that are upregulated under -DIF in Col-0 WT, only at end-of-day (ED). (Microsoft Excel File)

Supplemental File S5: Results of GO-enrichment analysis for genes that are upregulated under -DIF in Col-0 WT, only at end-of-night (EN). (Microsoft Excel File)

Supplemental File S6: Results of GO-enrichment analysis for genes that are downregulated under -DIF in Col-0 WT, only at end-of-night (EN). (Microsoft Excel File)

Supplemental File S7: Results of GO-enrichment analysis for genes that are downregulated under -DIF in Col-0 WT, only at end-of-day (ED). (Microsoft Excel File)

Supplemental File S8: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF in Col-0 WT but not in the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S9: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF in both Col-0 WT and the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S10: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF in the *phyB-9* mutant but not in Col-0 WT. (Microsoft Excel File)

Supplemental File S11: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF in a temperature-dependent and timepoint-independent manner in Col-0 WT but not in the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S12: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF in a temperature-dependent and timepoint-independent manner in both Col-0 WT and the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S13: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF, only at end-of-day (ED), in Col-0 WT but not in the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S14: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF, only at end-of-day (ED), in both Col-0 WT and the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S15: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF, only at end-of-day (ED), in the *phyB-9* mutant but not in Col-0 WT. (Microsoft Excel File)

Supplemental File S16: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF, only at end-of-night (EN), in Col-0 WT but not in the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S17: Results of GO-enrichment analysis for genes that are differentially expressed under -DIF, only at end-of-night (EN), in both Col-0 WT and the *phyB-9* mutant. (Microsoft Excel File)

Supplemental File S18: List of differentially expressed microRNAs (DEMs) for all possible sample comparisons in this study. (Microsoft Excel File)

Chapter 4

Physiological disruption of photoperiod-controlled starch mobilization reduces growth of sink leaves in Arabidopsis

Mark van Hoogdalem

Umidjon Shapulatov

Lidiya Sergeeva

Jacqueline Busscher-Lange

Marielle Schreuder

Diaan Jamar

Xiao Han

Alexander R. van der Krol

In preparation for publication

Abstract

The circadian clock in plants coordinates the timing of growth-related processes to ensure optimal plant growth under fluctuating environmental conditions. On the other hand, expression of core clock components is regulated by environmental conditions such as light and temperature. Most studies on regulation of clock components are done in plants grown under constant light and/or temperature. However, in nature plants are subjected to cyclic changes in both light and temperature because temperatures are higher during the day than during the night (referred to as +DIF). Previously, it was shown that growing Arabidopsis rosettes under an inversed light/temperature regime (cold days/warm nights; -DIF) suppresses growth and clock-controlled leaf movement in young developing sink leaves, which suggests that growth suppression under -DIF could act through modulation of the clock.

Here we show that phase and amplitude of rhythmic clock gene expression is affected under -DIF in both source and sink leaves. For example, the phase of the morning clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) is delayed under -DIF. This suggests that -DIF affects growth through altered regulation of core clock components. Similar to seedlings grown on low sucrose, the delayed phase of *CCA1* under -DIF was associated with upregulation of several carbohydrate (CH) starvation marker genes at the end of the night (EN) in source and sink leaves. Although upregulation of these marker genes is linked to low sugar availability, only sucrose levels in sink leaves were lower under -DIF compared to +DIF, suggesting that transport of sucrose from source to sink leaves was suppressed. The induction of CH starvation at EN under -DIF was linked to altered starch metabolism, leading to increased starch breakdown during the night under -DIF, only in source leaves, while starch accumulation during the day was slowed down. Suppression of leaf elongation in response to -DIF or other physiological treatments that may induce CH starvation (e.g. intermittent extended nights or spraying leaves with trehalose) support a link between CH starvation status and growth suppression under -DIF.

Taken together, our results suggest that -DIF affects both the circadian clock and starch metabolism, leading to CH starvation at EN and reduced growth of sink leaves.

Introduction

Optimal plant growth requires sequential timing of different steps needed for growth, including cell wall loosening, water uptake and cell wand crosslinking (Braidwood et al., 2014). The proper coordination of gene expression encoding the proteins effectuating these steps needs to be maintained under different diurnal fluctuations in light and temperature resulting from earth's rotation and growth at different latitudes (Harmer et al., 2000; de Montaigu et al., 2010; Braidwood et al., 2014). The plant circadian clock plays a central role in adapting the timing and coordination of these cyclic gene expression programs towards growth as function of the local photoperiod. Local changes in environmental signaling towards the endogenous clock of plants may therefore be used to affect growth of plants directly or indirectly through modulation of the clock (de Montaigu et al., 2010; Fung-Uceda et al., 2018). In Arabidopsis, the central oscillator of the circadian clock is formed by a set of transcription factors that through different feedback interactions generate endogenous rhythms of gene activity with a period of approximately 24 hours. These circadian rhythms are sustained under constant light and temperature (free running conditions) (de Montaigu et al., 2010; Hsu and Harmer, 2014; Webb et al., 2019). Moreover, the clock can be re-set by environmental signals such as light and temperature through a process called entrainment (McClung et al., 2002; Kusakina et al., 2014; Oakenfull and Davis, 2017). Studies on light entrainment signals are usually under constant temperature, while the effect of temperature entrainment is studied under conditions of constant light (McClung et al., 2002; Kusakina et al., 2014; Oakenfull and Davis, 2017; Johansson and Koster, 2019; Webb et al., 2019). However, under natural growth conditions these entrainment signals are applied in diurnal light/temperature regimes, with high temperature associated with the light of the day and low temperature associated with the dark of the night.

The natural combination of light and temperature (warm day, cold night) is here referred to as +DIF (Erwin et al., 1989). It has been shown that an inversed light/temperature regime (warm temperature during night, cold temperature during day: called -DIF (Erwin et al., 1989)) leads to growth suppression in many plant species, including Arabidopsis. For this reason, -DIF is commonly used in horticulture to control plant growth (Myster and Moe, 1995; Moe and Heins, 2000; Carvalho et al., 2008; Bours et al., 2013; Bours et al., 2015). Growth suppression under -DIF conditions of young leaves of Arabidopsis rosettes coincided with an altered phase and amplitude of clock-controlled leaf movement (Bours et al., 2013). The effect of -DIF on the phase and amplitude of clock-controlled leaf movement suggests that growth suppression under -DIF may be linked to an altered functioning of the circadian clock. However, expression of the central components of the circadian clock has not been studied under -DIF. Here the expression of selected clock genes was studied under +DIF and -DIF treatment using firefly luciferase reporter plants (Millar et al., 1992). These studies confirm that, compared to +DIF, a -DIF regime causes shifts in the phase of different key clock components, resulting in an altered coordination and expression of clock-regulated genes. Some clock-regulated genes are involved in hormone biosynthesis and signaling or carbohydrate (CH) metabolism, while hormones and CHs may also signal feedback to the circadian clock (Hanano et al., 2006; Haydon et al., 2013; Haydon et al., 2017; Frank

et al., 2018). The effect of -DIF on the clock may thus be direct (clock gene expression) or indirect (e.g. feedback regulation of clock gene expression by altered hormone and/or CH signaling). Therefore, the studies on -DIF presented here are focused on the effects of -DIF on the clock, and how these link to carbohydrate status of the plant and carbohydrate status control of growth.

Previously it has been shown that endogenous sugar levels directly affect the rhythmic expression of several core clock components under free running conditions, including that of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Haydon et al., 2013). Low endogenous sugar levels are linked to a delay in *CCA1* peak activity, while the effect of added sugar on the phase of *CCA1* activity is strongest when exogenous sugars are provided during the subjective morning. This indicates that responses of the clock gene *CCA1* to sugars are gated by the clock itself to that period of the day (Haydon et al., 2013). Changes in *CCA1* expression are linked to changes in expression of another clock component *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), which is a negative regulator of *CCA1* activity (Haydon et al., 2013). In turn, the change in *PRR7* transcription in response to sugar availability is dependent on the BASIC LEUCINE ZIPPER 63 (bZIP63) transcription factor, which can bind directly to the *PRR7* promoter and stimulate its expression (Frank et al., 2018). Activity of bZIP63 is stimulated upon sugar starvation through activation of the KIN10 (SnRK1.1) subunit of SUCROSE NON-FERMENTATION1-RELATED KINASE 1 (SnRK1) complex, which acts as a key energy sensor (Baena-Gonzalez et al., 2007; Mair et al., 2015; Frank et al., 2018). The low CH status that activates KIN10 can be mimicked by overexpression of *KIN10* (Shin et al., 2017). Combined, these results indicate a direct link between the energy status of the plant, as sensed by the energy-sensor SnRK1 complex and transmitted through KIN10, bZIP63, PRR7 and CCA1, the circadian clock, and ultimately clock-regulated growth. Sugars such as sucrose, fructose and glucose, which affect the phase of circadian rhythms, most likely affect the level of the signaling sugar trehalose-6-phosphate (T6P). T6P is a marker for sugar availability, and high T6P levels negatively regulate KIN10 activity of the SnRK1 complex, resulting in low bZIP63 activity (Zhang et al., 2009; Mair et al., 2015; Figueroa and Lunn, 2016). Indeed, the modulation of circadian rhythms by sugars through PRR7, bZIP63 and KIN10 requires TREHALOSE-6-PHOSPHATESYNTHASE1 (TPS1), which synthesizes T6P from glucose-6-phosphate and UDP-glucose (Ponnu et al., 2011; Frank et al., 2018).

Current insight into how circadian rhythms are modulated by CH status are mostly based on experiments using seedlings. However, rosette-stage Arabidopsis plants have multiple true leaves at different developmental stages. The young small sink leaves initially do not have enough photosynthetic capacity to produce sufficient CHs to support their growth potential. Sink leaves therefore import sugars (in the form of sucrose) from older larger source leaves, which are able to produce an excess amount of sugars (Turgeon, 1989). Thus, the local CH status may differ between source and sink leaves, depending on differential regulation of carbohydrate metabolism and the efficiency and timing of CH transport from source to sink (Turgeon, 1989; Kolling et al., 2015). Effects of -DIF on the clock and overall physiology may therefore also be different for source and sink tissues, and these need to be studied separately to understand the effect of -DIF on

growth. Here we determined how the shifts in the phase of key clock components in Arabidopsis rosettes under -DIF is associated with changes in CH status, and whether the link between CH status and circadian rhythms (as previously shown for seedlings) holds for both source and sink leaves in adult Arabidopsis plants. For this purpose, the diurnal expression of clock genes and carbohydrate status in 4-week-old plants grown under +DIF or -DIF was analyzed using firefly luciferase (ffLUC) reporter plants, in which ffLUC reporter gene activities in sink and source were quantified separately.

The interpretation of ffLUC activity under diurnal temperature cycles is not without problems and needs validation by qPCR for key time points. However, combined results indicate that -DIF causes a delay in peak activity of *CCA1* and other morning-expressed clock genes, both in source and sink leaves. In contrast, the phase of evening-expressed genes like *GIGANTEA (GI)* was more advanced under -DIF. In agreement with findings in seedlings, the delayed *CCA1* expression relates to a CH carbohydrate starvation status, as indicated by induction of sugar starvation marker genes at the end-of-night (EN), including *DARK INDUCIBLE 6* (*DIN6/ASN1)* (Baena-Gonzalez et al., 2007; Mair et al., 2015). However, measurement of sugar levels in source and sink tissues indicate that EN CH starvation status under -DIF only coincides with low sucrose levels in sink leaves, but not in source leaves. This indicates that sugar levels in source leaves per-se are not directly responsible for the phase shifts in clock gene expression in source leaves. Further analysis of CH metabolism shows that EN carbohydrate starvation under -DIF may be explained by over-utilization of starch during the night under -DIF. We demonstrate that CH starvation status is correlated with reduced growth, not only under -DIF, but also under other regimes that affect CH status. The importance of these findings for the physiological control of plant growth in greenhouses is discussed.

Material and Methods

Plant material

Arabidopsis thaliana seeds were either obtained from the Nottingham Arabidopsis Stock Centre (NASC, number in parentheses) or were donated by the authors who first described the line. Lines used in this study are: Col-0 (N1092), *PPR9::LUC* (N9962; (Edwards et al., 2010)), *LHY::LUC* (N9963; (McWatters et al., 2007)), *GI::LUC* (N9961; (Edwards et al., 2010)), *TOC1::LUC* (N9960; (McWatters et al., 2007)), *BCAT2::LUC* (*pAt1g10070::LUC*; (Graf et al., 2010)), *gi-100* (Huq et al., 2000), *35S::otsA* and *35S::otsB* (Schluepmann et al., 2003), *ntrc* knockout mutant line (Lepistö et al., 2009), *OE-NTRC* line (Toivola et al., 2013), *AOX1a::LUC* line (Ng et al., 2013). All transgenic lines used in this study were in Col-0 background.

Growth conditions and treatments

Arabidopsis seeds were stratified in the dark for three days at 5°C, after which they were sown on 4x4x4cm rockwool blocks (Grodan, Roermond, The Netherlands) that were pre-soaked in Hyponex nutrient solution (Unifarm, Wageningen, The Netherlands). Plants

were pre-grown in a climate chamber (12hL 22°C/12hD 17°C; relative humidity (RH) at 65%) where light was provided by fluorescent tubes (150 µmole m^{-2} s^{-1}). Plants were watered 2x a week with Hyponex solution.

After 26 days, reporter plants for obtaining diurnal ffLUC activity profiles were transferred to a custom-made climate cabinet fitted with a CCD camera for semi-continuous imaging of ffLUC reporter plants under controlled light and temperature conditions (LUMINATOR; Chapter 2). Here plants were grown under +DIF (12hL 22°C/12hD 12°C; RH70%) or -DIF conditions (12hL 12°C/12hD 22°C; RH70%). Temperature changed gradually over a period of approximately one hour, right after light-dark/dark-light transitions. Light during both conditions was a combination of blue, red and far red LED light, and intensity of blue and red light was lower during the first and last hour of the photoperiod to mimic natural changes in light conditions during dusk and dawn as described before (Chapter 2).

Plants used for analyzing gene expression (qRT-PCR and RNAseq experiment), enzyme activity, sugar and starch levels and leaf elongation were transferred to a Weiss Technik climate cabinet (described in (Bours et al., 2012)) after 3 weeks. In the Weiss cabinet plants were grown under +DIF conditions (12hL 22°C/12hD 12°C; RH65%). After 1 week, half of the plants were transferred to a second Weiss cabinet in the morning and grown under -DIF conditions (12hL 22°C/12hD 12°C; RH65 %). Plants (growing under +DIF or -DIF conditions) were harvested 3 days later or imaged during 1 week. Temperature in both cabinets changed gradually over a period of approximately one hour, right after light-dark/dark-light transitions. Light in both cabinets was provided by fluorescent tubes (180 µmole m^{-2} s^{-1}) and during the first and last hour of the photoperiod light intensity gradually increased or decreased, respectively, to simulate dawn/dusk. Plants were watered every other day with half-strength Hyponex.

Intermittent extended night and trehalose/sorbitol spray experiments were done in the growth chamber used to pre-grow plants for other experiments. Growth conditions and watering were not changed. To extend the night every 1st and 4th day of the week, plants were covered by a light-tight box just before lights were switched on, which was removed again 3h after the lights were switched on.

RNA extraction and qRT-PCR for gene expression analysis

Shoots of 32-day-old Arabidopsis Col-0 WT rosette plants were harvested at indicated time points. Sink and source leaves were immediately separated by placing the shoot on a flat surface and use a punch (2 mm diameter) to cut out the center part of the rosette. The center (sink) and outer (source) parts of the rosette were put in separate containers and immediately frozen in liquid nitrogen. For each time point, sink and source parts of 6 individual plants were pooled in one container. Frozen source/sink material was homogenized manually using a mortar and pestle in liquid nitrogen. Part of the homogenized material was used for RNA extraction; the remainder was used for starch and sugar extractions and enzymatic assays (see below). Total RNA was isolated using the InviTrap® Spin Plant RNA Mini kit (Stratec, Birkenfeld, Germany) according to manufacturer's instructions. Subsequently, genomic DNA was removed with the TURBO

DNA-freeTM kit (Invitrogen, Carlsbad, CA, USA). Quality of the RNA was checked by running 2µl on agarose gel and concentration and purity was determined by NanonDropTM spectrophotometer (Thermo Scientific, Waltham, MA, USA). For each sample 1µg RNA was used to synthesize cDNA using iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR reactions were performed on the CFX ConnectTM Real-Time PCR Detection System using SYBR® Green qPCR mix and CFX MaestroTM software was used to analyze the data (all Bio-Rad, Hercules, CA, USA). Expression levels were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), relative to expression levels of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780). Primers used are listed in Supplemental Table S1.

Diurnal luciferase profiles

Imaging of ffLUC activity and obtaining relative luminescence profiles was done as described before (Chapter 2). Luminescence in sink leaves was obtained by measuring luminescence within the center part of the rosette (circle with 2 cm diameter). Luminescence in source leaves was obtained by subtracting luminescence in the center part from luminescence in the total rosette.

Starch and soluble sugar extraction and quantification

Soluble sugars were extracted from the source and sink material that was harvested and homogenized in liquid nitrogen as described above. Homogenized source/sink material was freeze-dried and weighed. From 10-15mg plant material Soluble sugars were extracted for 15 minutes at 76°C in 1ml 80% MeOH, containing 0.4g/l melezitose as internal standard. Next, MeOH was evaporated and samples were diluted in 1ml MQ and centrifuged at 13,000 rpm for 3 minutes. Supernatant was removed and diluted 10x and analyzed for soluble sugars using high-pressure liquid chromatography (Dionex, Carbopac PA1 column, electrochemical detection) as described in (Sergeeva et al., 2000). Sugar levels were corrected using internal standard and amount of sugars (mg) per gram dry material was calculated.

The pellet was used for starch quantification, using the starch assay kit SA20 (Sigma-Aldrich, St. Louis, MO, USA). Pellets were washed 2x times with 80% EtOH, and after evaporation of the EtOH, 1ml MQ was added and samples were boiled for 10 minutes to gelatinize starch granules. From that point onwards, procedures as described in the technical bulletin of the starch assay kit were followed, with the exception that volumes were adjusted for measuring absorbance at 340nm using a 96-well plate reader (Molecular Devices, San Jose, CA, USA).

Enzymatic assays

Enzymes were extracted from the source and sink material that was harvested and homogenized in liquid nitrogen as described above. Soluble Invertase, AGPase (ADP-glucose pyrophosphorylase) and PGM (plastidial phosphoglucomutase) were extracted from the same material according to the extraction procedure for PGM as described in (Sergeeva et al., 2004). Extraction was done at 0°C-4°C in 0.6 mL of 50 mM HEPES (pH 7.4), 5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (w/v) bovine serum albumin (BSA) and 5 mM dithiothreitol (DTT), with addition of 10 mg of insoluble polyvinylpyrrolidone. The homogenate was centrifuged for 5 min. at 15,000g. The supernatant was split in small portions and stored at -80°C for measurement of different enzymes later.

Soluble Invertase activity was determined spectrophotometrically as described in (Sergeeva et al., 2006). In a microtiter plate, 50 µL of enzyme extract was added to 200 µL of 25 mM citrate-phosphate buffer (pH 5.2) containing 25 mM sucrose. After 1h of incubation at 30°C, the reaction was stopped by heating in a boiling water bath for 4 min. The glucose content in reaction mixtures was determined enzymatically by using a glucose test kit (Glucose (HK) Assay Kit, GAHK-20, Sigma, USA) and a 96-well plate reader (Molecular Devices, San Jose, CA, USA). Blank incubations were done with heat-denaturized extracts.

AGPase activity was determined spectrophotometrically as described in (Sergeeva et al., 2012). In a microtiter plate, 30 µL of enzyme extract was added to 250 µL reaction mixture containing 75 mM Hepes-NaOH (pH 8.0), 0.44 mM ethylenediamine tetra-acetic acid (EDTA), 5 mM MgCl2, 3 mM dithiothreitol (DTT), 2 mM ADP-glucose (ADPGlc), 2.7 U PGM (Boehringer, Mannheim, Germany), 8.0 U glucose-6-phosphate dehydrogenase (G6PDH; Leuconostoc, Boehringer), 1 mM nicodinamide-adenide dinucleotide (NAD), 20 mM glucose-1,6-bisphosphate, 0.1% bovine serum albumin (BSA), 2 mM 3-phosphoglycerate (3PGA) and 10 mM NaF. After mixing, substrate (1.5 mM pyrophosphate (PPi)) was added and absorption at 340 nm was measured during 10 min at 30°C with a 96-well plate reader (Molecular Devices, San Jose, CA, USA). Blank incubations, in which the substrate was omitted, were done for each extract.

PGM activity was determined spectrophotometrically as described in (Sergeeva et al., 2004). In a microtiter plate, 20 µL of enzyme extract was added to 210 µL of 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 5 mM DTT, 0.1% (w/v) BSA, 1 unit mL-1 Glc-6-phosphate dehydrogenase, 1 mM NAD, and 0.02 mM Glc-1,6-bisphosphate. After mixing and waiting for 3 min, substrate (20 µL of 50 mM Glc1P) was added, and absorption at 340 nm was measured during 15 min at 25°C with a 96-well plate reader (Molecular Devices, San Jose, CA, USA). Blank incubations in which the substrate was omitted were done for each extract.

β-amylase and α-amylase were extracted according to assay procedure from ‘Betamyl-3 method’ kit (Megazyme, Ireland) with some modifications. In brief, 1 ml of Tris/HCl buffer (1 M) with disodium EDTA (20 mM) and sodiumazide (0.02% w/v) was added to 10 mg of freeze-dried powdered plant material. Immediately before use, cysteine HCl

(final concentration 100 mM, Sigma) was added to the buffer and pH was adjusted to 8.0 by 4 M NaOH. After one-hour extraction at room temperature and 5-6 times stirring on a vortex mixer over the 1 h period, samples were centrifuged at 10,000 g for 10 min. The supernatants were divided into portions (0.2 mL), frozen in liquid nitrogen, and stored at –80°C until required.

β-amylase activity was determined spectrophotometrically using 'Betamyl-3 method' kit (Megazyme, Ireland) according to manufacturer's instructions with some modifications. Prior to the measurements, enzyme extracts were diluted 5-10-fold in dilution buffer (MES buffer (1 M, pH 6.2) with disodium EDTA (20 mM) and sodiumazide (0.1 % w/v) and pre-incubated at 40° C for 5 min. Also, betamyl-3 substrate solution was pre-incubated at 40° C for 5 min. Betamyl-3 substrate solution (40 µl) was added to diluted extract (40 µl), stirred on a vortex and incubate at 40° C for 10 min. At the end of incubation period 0.6 ml of stopping reagent (1 % (w/v) Trizma base (pH 8.5) was added and stirred. The absorbance of the reaction solutions and the reagent blank were read at 400 nm against distilled water with a 96-well plate reader (Molecular Devices, San Jose, CA, USA). For reagent blank, stopping reagent was added at the beginning to Betamyl-3 substrate solution, after which diluted extract was added.

α-amylase activity was determined by 'Ceralpha method' kit (Megazyme, Ireland) with some modifications. Prior to the measurements, enzyme extracts were diluted 5-10-fold in Ceralpha dilution buffer and pre-incubated at 40° C for 5 min. Also, Amylase HR reagent was pre-incubated at 40° C for 5 min. Amylase HR reagent (40 µl) was added to diluted extract (40 µl), stirred on a vortex and incubated at 40° C for 10 min. At the end of incubation period 0.6 ml of stopping reagent (1% (w/v) Trizma base (pH 8.5) was added and stirred. The absorbance of the reaction solutions and the reagent blank were read at 400 nm against distilled water with a 96-well plate reader (Molecular Devices, San Jose, CA, USA). For reagent blank, stopping reagent was added at the beginning to Amylase HR reagent, after which diluted extract was added.

RNAseq experiment

Description of experimental design, RNA isolation and RNA sequencing analyses can be found in the Material and Methods section of Chapter 3.

Leaf growth measurements

Projected leaf lengths of Arabidopsis Col-0 WT and transgenic lines were measured as described in (Bours et al., 2012). Projected leaf lengths were used to calculate leaf elongation as described in the results. Graphs of leaf profiles of Arabidopsis Col-0 WT plants that were subjected to different treatments were made by harvesting the plants, separating the rosette leaves and ordering the leaves based on order of emergence. Then these leaf profiles were photographed and used to measure leaf lengths using ImageJ software (imagej.nih.gov/ij).

Results

Differential phase changes in clock gene expression under -DIF

Before, the effect of -DIF on the clock was studied indirectly by using leaf movement as an output of a clock-regulated process (Bours et al., 2013). These studies showed that -DIF causes a delay in phase of diurnal leaf movement, compared to under +DIF (Bours et al., 2013), indicating altered timing of the clock under -DIF. To study the effect of -DIF on the circadian clock in a more direct way, we used firefly luciferase (ffLUC) reporter plants, expressing different key clock ffLUC-reporter genes. For this we obtained ffLUC reporter plants for the morning-phased clock genes *PSEUDO RESPONSE REGULATOR 9* (*PRR9*) (Edwards et al., 2010), *LATE ELONGATED HYPOCOTYL* (*LHY*) (McWatters et al., 2007), which has partial redundant function with CCA1 (Mizoguchi et al., 2002; Lu et al., 2009; Yakir et al., 2009), and for evening-phased clock genes *GIGANTEA* (*GI*) (Edwards et al., 2010) and *TIMING OF CAB EXPRESSION 1* (*TOC1*) (McWatters et al., 2007). Arabidopsis reporter plants were pre-grown for four weeks under +DIF and subsequently the diurnal ffLUC activity of each clock reporter was imaged in rosette leaves for two days under +DIF or under -DIF. Since rhythmic clock gene expression has been shown to depend on leaf age/developmental stage (Kim et al., 2016), the activity of ffLUC reporters in source and sink leaves was analyzed separately.

Since temperature affects ffLUC enzymatic activity, we note that any differences between the ffLUC activity profiles under -DIF and +DIF conditions may be related to changes in promoter activity of the reporter and/or changes in ffLUC enzymatic activity (see Chapter 2). Still, after normalization of the ffLUC activity to that of start of the photoperiod (ZT(h)=0), the profiles can be used to get an initial impression of the effect of the two DIF treatments on circadian regulation of cock gene expression (e.g. phase-shifts or changes in amplitude). Results show in general a broadening of the peak in clock reporter activity during the day under -DIF in both source and sink leaves (Supplemental Figure S1). The morning clock reporter *PRR9::LUC* shows a delayed phase in source and sink, with a slightly stronger delay in sink (Table 1). Instead of a clear activity peak, the *LHY::LUC* activity shows a plateau in expression during the day under -DIF in source and sink. For both genes the amplitude in expression under -DIF is slightly higher compared to under +DIF. For the evening clock reporter *GI::LUC* the phase is advanced by 1.5 hour in both source and sink under -DIF, while the phase of *TOC1::LUC* expression is slightly delayed (Supplemental Figure S1 and Table 1). The amplitude in expression of *GI::LUC* is slightly lower under -DIF compared to under +DIF. In contrast, *TOC1::LUC* activity shows a strong increase in amplitude under -DIF (Supplemental Figure S1). Comparison of reporter activity in source and sink leaves shows that in general the phase in reporter activity is similar in sink and source. The amplitude in reporter activity is in general slightly higher in sink leaves compared to that in source leaves, but for *PRR9::LUC* the amplitude in expression in sink leaves is almost 2-fold higher compared to that in source leaves, both under +DIF and -DIF (Supplemental Figure S1).

Because of the complication of temperature effects on ffLUC reporter activity, the diurnal expression of the same set of clock genes was also analyzed by quantifying endogenous

mRNA transcript levels by qPCR. This was done every two hours during the day for source and sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF or DIF during the last 4 days before harvesting (see Material and Methods). In addition, the expression of *CCA1* and the clock gene *KELCH REPEAT, F-BOX 1* (*FKF1)* was analyzed (Figure 1). *FKF1* encodes a blue-light photoreceptor that interacts with GI in the regulation of photoperiodic flowering time and starch synthesis (Sawa et al., 2007; Song et al., 2012; Mugford et al., 2014) and its expression profile is similar to that of *GI* (Fowler et al., 1999; Tootle et al., 2003). Table 1 compares the average phase of clock gene expression under +DIF and -DIF determined by analysis of ffLUC reporter activity or mRNA transcript levels.

As expected, there are some discrepancies between ffLUC activity of clock reporters and mRNA transcript levels of clock genes. For instance, the mRNA measurements show higher amplitude in expression than the related ffLUC reporters (Figure 1). Endogenous transcript levels of clock genes confirm a delayed phase in expression of all morning-phased clock genes *CCA1*, *PRR9* and *LHY* (Figure 1). However, the delayed phase of *LHY* expression under -DIF is only observed in source leaves and not in sink leaves. The evening-phased clock genes *TOC1*, *GI* and *FKF1* all show an advanced phase under -DIF in source leaves, but in sink leaves this is only observed for *TOC1*. All measured clock genes, except for *LHY*, show higher amplitude in expression under -DIF. The peak amplitude of *PRR9* and *FKF1* expression is significantly lower in sink leaves compared to source leaves, both under +DIF and -DIF. Overall, both the clock ffLUC reporters and direct measurement of selected clock-gene mRNA levels indicate that the -DIF treatment affects clock genes in different ways, with some clock genes showing an advanced phase, while others show a delay in phase of expression. Furthermore, changes in phase of clock gene expression are not always observed in sink leaves, indicating that the effect of -DIF on clock phase is stronger in source leaves. Since previously it has been shown that sugars can affect the phase of clock genes (Haydon et al., 2013; Frank et al., 2018), we subsequently investigated whether the -DIF induced phase change in in clock gene expression relates to altered CH content under -DIF.

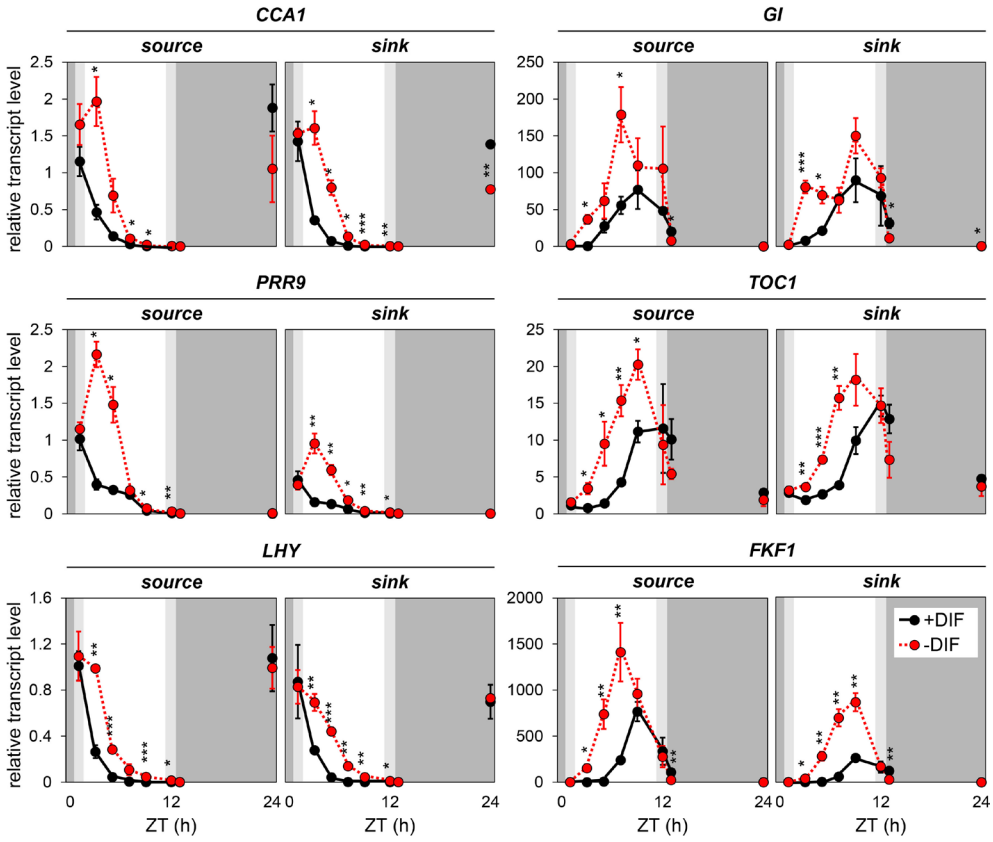


Figure 1: mRNA levels of clock genes in source or sink leaves under +DIF and -DIF. mRNA transcript levels of clock genes at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.

Table 1: Phase of clock gene expression determined by measuring ffLUC-reporter activity or mRNA transcript level. Table shows average phase (ZT(h)) of gene expression for several clock genes in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C) or -DIF conditions (12hL 12°C/12hD 22°C). Phase of *PRR9*, *LHY1*, *GI* and *TOC1* gene expression was determined by analyzing ffLUC activity of *PRR9::LUC*, *LHY::LUC*, *GI::LUC* and *TOC1::LUC* reporter plants (Supplemental Figure S1, day 2) or mRNA transcript levels of these genes (Figure 1). Phase of *CCA1* and *FKF1* gene expression was only determined by analyzing mRNA transcript levels (Figure 1). Phase changes (in hours) were calculated by subtracting phase under +DIF from phase under -DIF. Since changes in temperature may lead to changes in ffLUC signal that are not the result of changes in promoter activity (Chapter 2), the ffLUC activity peaks around temperature transitions are attributed to temperature effects and ignored for phase analysis. Because the *LHY:LUC* reporter did not show a clear peak in activity under -DIF (Supplemental Figure S1), the results on the phase of *LHY::LUC* activity (red numbers) should be interpreted with caution.

clock gene	source /sink	ffLUC phase +DIF (ZT(h))	ffLUC phase -DIF (ZT(h))	change (h)	qPCR phase +DIF (ZT(h))	qPCR phase -DIF (ZT(h))	change (h)
CCA1	source	n.d.	n.d	n.d.	0	3	+3
	sink				1	3	+2
PRR9	source	3.5	5.5	+2	1	3	+2
	sink	3	6	+3	1	3	+2
LHY	source	3	4	+1	0	1	+1
	sink	3	4	+1	1	1	0
GI	source	10.5	9	-1.5	9	7	-2
	sink	10.5	9	-1.5	9	9	0
TOC1	source	11	11.5	+0.5	12	9	-3
	sink	11	11.5	+0.5	12	9	-3
FKF1	source	n.d.	n.d.	n.d.	9	7	-2
	sink				9	9	0

CH starvation in sink leaves under -DIF at EN links to growth inhibition

In seedlings it has been shown that changes in the phase of rhythmic clock gene activity relates to sugar availability (Haydon et al., 2013; Frank et al., 2018). For example, in seedlings grown in continuous light, low sugar availability in the morning resulted in a delayed phase in *CCA1* expression, which is linked to activity of PRR7, bZIP63 and KIN10 (Haydon et al., 2013; Frank et al., 2018). Since -DIF also causes a delay in phase of *CCA1* expression in source and sink leaves of adult Arabidopsis plants, this raises the question whether this delay is also caused by reduced sugar availability in the early

morning. Several genes are induced under low endogenous sugar availability and are used as carbohydrate (CH) starvation marker (Graf et al., 2010; Pilkington et al., 2015; Flis et al., 2016). For instance, a higher activity at the end of the night (ZT(h)=24; EN) of *BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2* (*BCAT2; AT1G10070*) has been used as indicator of low sugar levels (Graf et al., 2010). Using *BCAT2::LUC* reporter plants grown under +DIF or -DIF we could show that -DIF indeed leads to higher expression of *BCAT2::LUC*, indicating a CH starvation status in the morning under -DIF (Supplemental Figure S2). To confirm the CH starvation status at EN, leaf tissues from adult Arabidopsis plants grown under +DIF and -DIF were harvested at ED and EN for RNA sequencing analysis of mRNA isolated from sink leaves (Chapter 3). Results show indeed significant upregulation of multiple CH starvation marker genes (DARK INDUCIBLE 6 (*DIN6/ASN1; AT3G47340*) (Flis et al., 2016; Frank et al., 2018), *BCAT2, GLUTAMATE DEHYDROGENASE 1* (*GDH1; AT5G18170*) (Flis et al., 2016) and *AT5G07030* (Pilkington et al., 2015)) under -DIF at EN. However, these genes were not upregulated under -DIF at end-of-day (ZT(h)=12; ED; Supplemental Table S7 of Chapter 3). Moreover, qRT-PCR on mRNA isolated from separate source and sink leaves shows that these CH starvation markers are induced in both source and sink leaves (Figure 2A, B and Supplemental Figure S3).

CH starvation at EN links with reduced sucrose levels in sink leaves but not in source leaves

So far, we have shown that the -DIF regime results in a delayed phase of the morning clock gene *CCA1* (Figure 1, Supplemental Figure S1 and Table 1), which may be explained by low sugar availability at early morning (Haydon et al., 2013). Indeed, several CH starvation marker genes are induced at EN under -DIF (Figure 2A+B and Supplemental Figure S3). Subsequently we analyzed soluble sugars (sucrose, fructose, glucose) directly in leaf tissues of 32-day-old plants grown under +DIF or -DIF (during the last 4 days) and harvested at ED and EN. Results show that sucrose levels are lower in sink leaves under -DIF at EN, compared to +DIF (Figure 2C), supporting the induction of CH starvation marker genes at EN. However, the sucrose levels in source leaves at EN are not affected by -DIF, while CH starvation marker genes are induced in source leaves at EN (Figure 2A-C and Supplemental Figure S3). This suggests that in source leaves the sugars may be at a sub-cellular location where there is no conversion or sensing of the signaling molecule T6P. The glucose and fructose levels are not affected by -DIF at EN in either source or sink leaves, compared to that in leaves grown under +DIF (Supplemental Figure S4). In contrast to EN under -DIF, at ED under -DIF the sucrose, glucose and fructose levels are actually higher compared to under +DIF, both for source and sink leaves (Figure 2C and Supplemental Figure S4). This could be due to a cold acclimation response during day under -DIF, which is supported by the transcriptional activation of several cold responsive genes at ED under -DIF (Chapter 3).

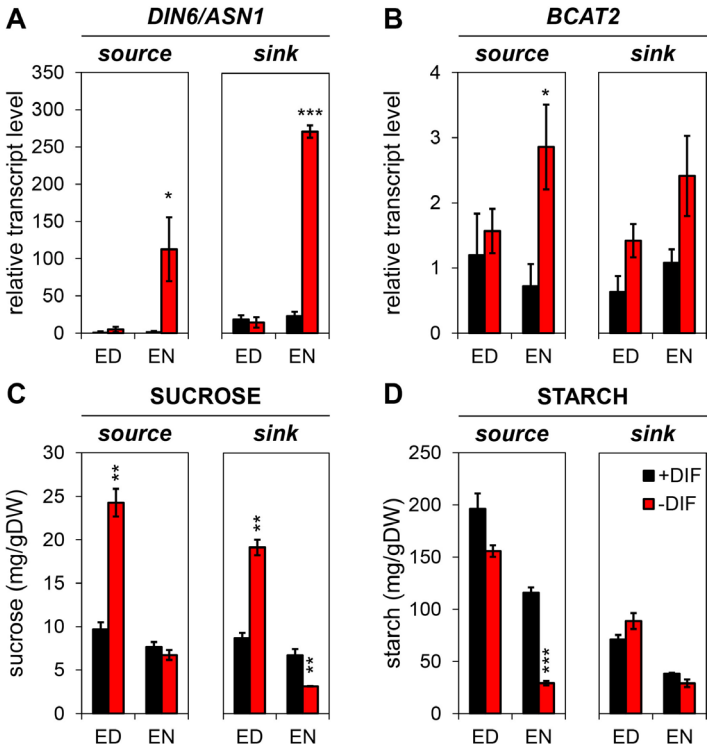


Figure 2: mRNA levels of carbohydrate starvation markers and associated levels of sucrose and starch in source or sink leaves under +DIF and -DIF. mRNA levels of carbohydrate starvation marker genes *DIN6/ASN1* (*AT3G47340*) (**A**; (Flis et al., 2016; Frank et al., 2018)) and *BCAT2* (*AT1G10070*) (**B**; (Graf et al., 2010)) and levels of sucrose (**C**) and starch (**D**) at end of day (ED; ZT(h)=12) and end of night (EN; ZT(h)=24) in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). Data are mean relative mRNA transcript levels ±SE, mean sucrose levels in mg per g dry material ±SE and mean starch levels in mg per g dry material ±SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ED. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.

Increased starch breakdown in source but not in sink leaves under -DIF

During the night, growth of plants depends on sugars released from leaf starch reserves, which are built-up during the day (Stitt and Zeeman, 2012; Streb and Zeeman, 2012). The larger starch reserves are in the source leaves, which no longer need all their sugars for growth at night. Therefore, source leaves export sucrose at night to the phloem, which transports sucrose to sink leaves to support growth (Turgeon, 1989). To determine how altered sugar levels under -DIF relate to starch levels, the starch content was measured during the day and night for plants grown under -DIF or +DIF. Starch levels were measured in source and sink leaf tissue of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF and -DIF during the last 4 days before harvesting. As expected, starch levels in source leaves are generally higher than in sink leaves, both under +DIF and -DIF (Figure 2D and Supplemental Figure S5). Under -DIF the build-up of starch reserves during the day is slower in source leaves, but at the onset of night starch levels in source leaves are not significantly different compared to +DIF. However, at the EN the starch levels in source leaves are severely reduced compared to under +DIF. This indicates an

increased starch turnover rate during the warm night under -DIF and mis-regulation of the clock-controlled starch utilization during the night and may linked to the sugar starvation status at EN in source leaves. In contrast, in sink leaves under -DIF, the build-up of starch reserves is slightly increased during the day, while starch reserves are not affected at EN in sink leaves under -DIF compared to under +DIF (Figure 2D and Supplemental Figure S5). The reduced sucrose levels in sink leaves at EN under -DIF are therefore not explained by a strongly altered starch metabolism in sink leaves under -DIF. Alternatively, the reduced sucrose levels in sink leaves could be related to a reduced import of sucrose from source leaves. Sucrose levels are not decreased in source leaves, while CH starvation markers are induced, suggesting that sucrose in source leaves may be loaded into the phloem but fail to be transported to sink tissues.

Altered regulation of CH metabolism related enzymes and genes under -DIF

Sucrose breakdown: To determine how the altered sugar and starch levels in leaves of plants grown under -DIF related to carbohydrate synthesis and breakdown capacity, the activity of different enzymes related to sugar breakdown (soluble/vacuolar invertase; VIN), starch biosynthesis, or starch breakdown was analyzed in leaf tissue of plants growth under +DIF or -DIF. Results show that VIN activity is generally lower in source and sink leaves under -DIF (Supplemental Figure S6), which suggests that the sucrose accumulation during the day under -DIF may be due to increased breakdown of sucrose. Reduced VIN activity under -DIF at EN indicates that reduced sucrose levels in sink leaves at this timepoint is not the result of increased sucrose breakdown by VIN activity (Ruan et al., 2010). However, high VIN activity is associated with increased cell expansion (Sergeeva et al., 2006; Ruan et al., 2010). Therefore, the lower VIN activity under -DIF is consistent with the reduced elongation in Arabidopsis under -DIF.

Regulation of starch synthesis: Starch metabolic processes are catalyzed by a wide range of different enzymes (overview in (Streb and Zeeman, 2012)). Regulation of these enzymes may be, depending on the enzyme, through transcriptional regulation of the encoding genes (Kaplan and Guy, 2004; Smith et al., 2004; Crevillen et al., 2005; Kaplan and Guy, 2005) and through post-translational regulatory mechanisms such as redox activation, protein phosphorylation and formation of protein-complexes (reviewed in (Kotting et al., 2010)). The altered starch levels under -DIF during the day, and especially EN, suggest that starch metabolism is affected by -DIF. Indeed, RNA sequencing data on sink leaves of adult Arabidopsis plants grown under +DIF and -DIF, shows differential expression of 16 genes involved in starch metabolism under -DIF at EN and/or ED (ZT(h)=12; ED; Supplemental Table S9 of Chapter 3). The majority of these genes that encode enzymes involved in starch biosynthesis are upregulated at ED and downregulated at EN under -DIF, compared to under +DIF (Supplemental Table S9 of Chapter 3, upper part). To study the transcriptional regulation of enzymes involved in starch biosynthesis under -DIF the endogenous gene expression of *PLASTIDIAL PHOSPHOGLUCOMUTASE* (*PGM*) and *STARCH BRANCHING ENZYME 2.1* (*SBE2.1*) was profiled by qRT-PCR in both source and sink leaves. Under -DIF *PGM* and *SBE2.1* expression was higher during the middle of the day than under +DIF, in both source and

sink leaves. However, expression of these genes already decreased before the end of the day under -DIF, while under +DIF expression increased until the end of the photoperiod (Figure 3). This early change in expression under -DIF during a time window in which light and temperature conditions are constant, indicates a control by endogenous signal(s) acting on expression of these genes.

Besides *PGM* mRNA levels, also PGM protein activity was measured at selected time points and in the same samples the ADP-glucose pyrophosphorylase (AGPase) enzyme activity was quantified. While *PGM* mRNA levels are already on the decline at the ED under -DIF (Figure 3), the enzymatic activity of PGM in source and sink leaves actually increases until the end of the day under -DIF compared to +DIF (Figure 4). The activity of AGPase, which is a major target for post-transcriptional regulation of starch biosynthesis, is lower during the day under -DIF in both source and sink leaves. At EN under -DIF AGPase activity is only significantly reduced compared to +DIF in source leaves. While the lower PGM and AGPase activity during the middle of the day under -DIF is correlated with lower starch levels during the middle of the day in source leaves, this is not the case for sink leaves (Figure 2D and Supplemental Figure S5). Combined with the observed opposite response of PGM and AGPase activity under -DIF at ED, these results suggest that altered starch accumulation under -DIF is not (solely) caused by differences in potential activity of enzymes involved in starch synthesis.

Altered redox regulation NTRC under -DIF. The activity of several starch metabolic enzymes is redox-activated, which for Arabidopsis AGPase is through NADP-dependent thioredoxin reductase C (NTRC) (Michalska et al., 2009). Reduced redox activation of AGPase in *ntrc* knock out mutants results in lower ED and EN starch levels compared to WT (Michalska et al., 2009). The reduced rate of starch accumulation and reduced AGPase activity during the day under -DIF may therefore be caused by a reduced redox activation through NTRC. One possibility is that *NTRC* expression is reduced during the day under -DIF. However, mRNA measurements show that endogenous expression of *NTRC* is the same or even slightly higher in sink leaves under -DIF (Supplemental Figure S7). However, Arabidopsis plants with altered *NTRC* expression (*NTRC* overexpression or *ntrc* knock-out mutant) do show reduced elongation in sink leaves compared to Col-0 WT, both under +DIF and -DIF (Supplemental Figure S8). This indicates that a normal redox regulation through NTRC is essential for elongation responses.

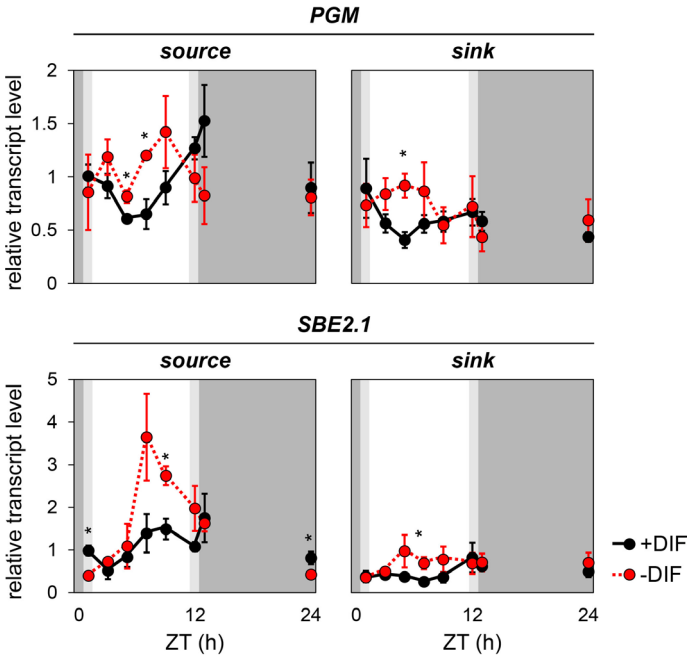


Figure 3: mRNA levels of genes involved in starch biosynthesis in source or sink leaves under +DIF and -DIF. mRNA levels of *PGM* and *SBE2.1* at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.

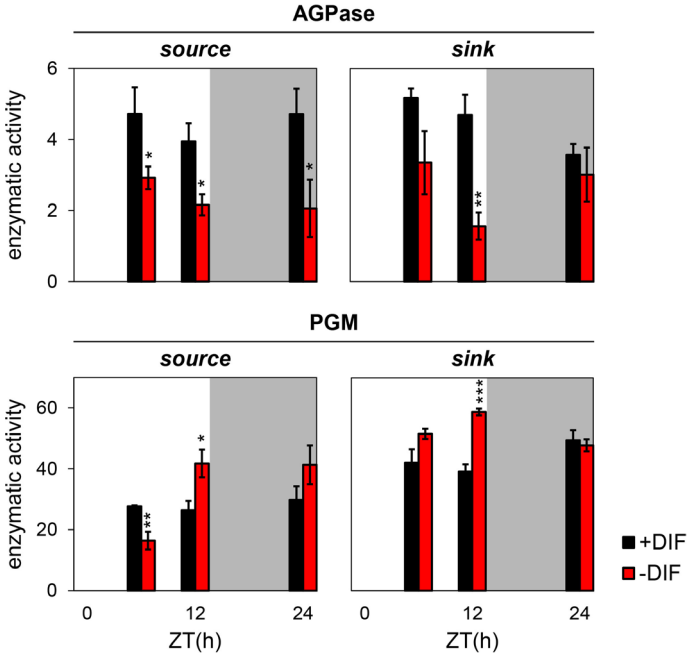


Figure 4: Activity of enzymes involved in starch biosynthesis in source or sink leaves under +DIF and -DIF. Enzymatic activity of ADP-glucose pyrophosphorylase (AGPase) and plastidial phosphoglucomutase (PGM) at ZT(h)=6, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}). Data are mean enzyme activity Units (amount of enzyme required to release one nmol NADH/minute) per mg dry material ± SE (n=3 biological replicates of 6 pooled plants). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from activity under +DIF.

Regulation of starch breakdown: The transcriptional regulation of enzymes involved in starch breakdown was studied by measuring gene expression of *BETA-AMYLASE 3* (*BAM3*), *GLUCAN WATER DIKINASE 1* (*GWD1*) and *ISO-AMYLASE 3 (ISA3)*. Under -DIF, transcript levels of all three genes were higher during the day (Figure 5). The higher expression of starch breakdown genes is in agreement with the reduced build-up of starch during the day under -DIF compared to under +DIF (Figure 2D and Supplemental Figure S5). With the exception of *GWD1*, expression of the genes is lower during the night compared to +DIF (Figure 5). Interestingly, *BAM3* expression shows an immediate strong response to the decrease in day-temperature under -DIF conditions, while the response of *GWD1* and *ISA3* to low temperature during the day is more delayed (Figure 5). This could indicate gating of the transcriptional response of *GWD1* and *ISA3* towards the afternoon. Besides the transcriptional response of genes related to starch breakdown, also the enzymatic activity towards starch breakdown was quantified by measuring α-amylase and β-amylase activity. Remarkably, the higher mRNA transcript levels of β-amylase during the day under -DIF in source and sink leaves are not translated to higher β-amylases enzymatic activity, except for at ED (Figure 6). Moreover, while the β-amylase gene shows lower expression at EN, the β-amylase enzyme activity is significantly higher at EN for source leaves (Figure 6). For sink leaves the β-amylase mRNA steady state levels are the same at EN, but again β-amylase enzyme activity is higher under -DIF compared to under +DIF (Figure 6). The activity of α-amylases, which form another major class of starch degrading enzymes, was also higher in source and sink leaves at ED under -DIF, but was only significantly higher in sink leaves at EN. Overall, the higher potential for starch breakdown and lower level of starch accumulation explains why plants may reach a CH starvation at the EN under -DIF. It was shown that when Arabidopsis plants are grown under 12hL/12hD cycles at constant 22°C, the rate of starch mobilization during the night is tightly regulated by the clock to prevent running out of starch near the end of the night under different photoperiods (Graf et al., 2010). Our results show that this is also the case under +DIF, as starch levels are still relatively high at EN (Figure 2D and Supplemental Figure S5). However, it seems that -DIF interferes with this tight clock regulation of night-time starch breakdown, resulting in a temporal CH starvation status in the morning. This CH starvation status may thus be causal for the reduced growth as observed under -DIF (Figure 8A+B).

Increased respiration in source leaves at night under -DIF

Although the initial rate of starch accumulation is reduced under -DIF, the ED starch levels in both source and sink leaves are not significantly lower compared to those under +DIF (Supplemental Figure S5). However, the starch turnover rate during the following night in source leaves (which contain the majority of starch reserves) appears to be much higher under -DIF, leading to significantly lower starch levels at EN in source leaves (Figure 2D and Supplemental Figure S5). This is likely a consequence of the increased night temperature, which may increase the demand for sugars for respiration and thereby the demand for starch breakdown (Pyl et al., 2012; Pilkington et al., 2015). Expression of the alternative oxidase (AOX) enzyme is a marker for elevated Cyanide-insensitive

respiration, which only occurs in plants and appears to play a role in various abiotic stress responses (Millenaar and Lambers, 2003; Arnholdt-Schmitt et al., 2006; Plaxton and Podestá, 2006; Millar et al., 2011; Xu et al., 2011). Indeed, *AOX1a* mRNA levels are strongly increased at the onset and end of the dark period under -DIF in source leaves, but not in sink leaves (Supplemental Figure S10).

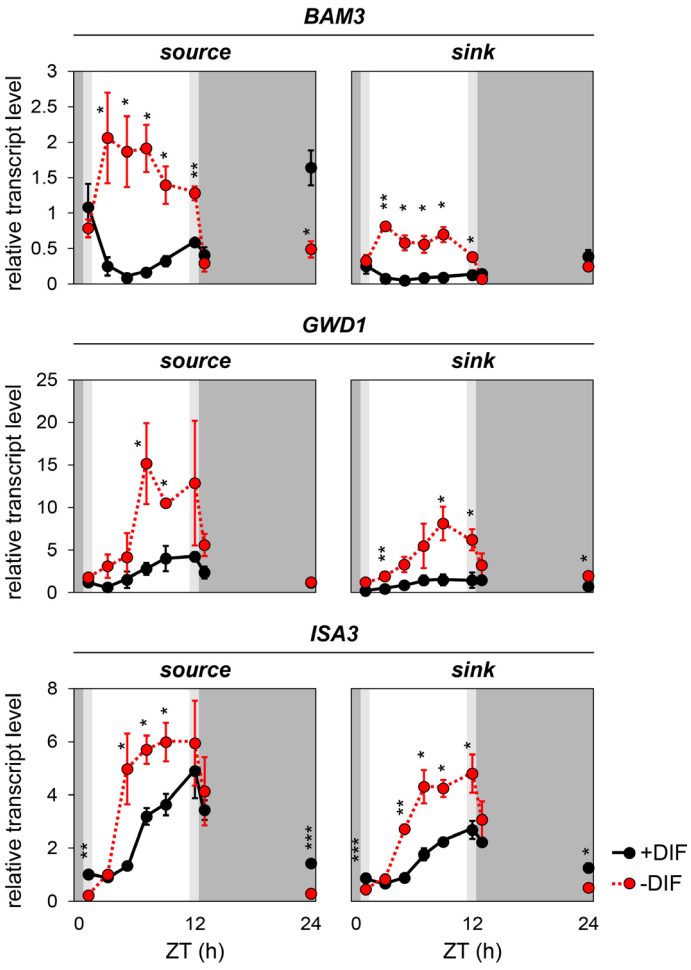


Figure 5: mRNA levels of genes involved in starch breakdown in source or sink leaves under +DIF and -DIF. mRNA levels of *BAM3* and *GWD1 and ISA3* at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.

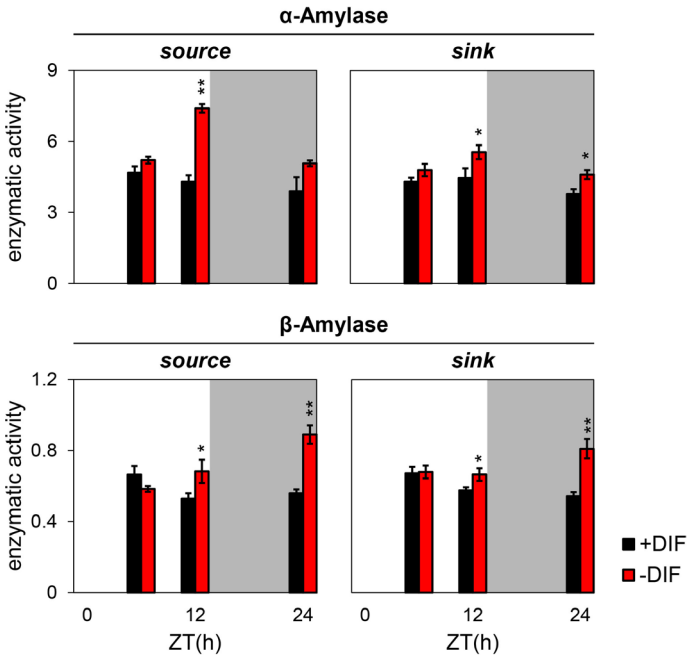


Figure 6: Activity of enzymes involved in starch breakdown in source or sink leaves under +DIF and -DIF. Enzymatic activity of α-amylase and β-amylase at ZT(h)=6, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}). Data are mean Ceralpha® (α-amylase activity) or Betamyl-3® (β-amylase activity) Units/mg dry material ± SE (n=3 biological replicates of 6 pooled plants). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from activity under +DIF.

Different ways of inducing CH starvation all link to reduced leaf elongation

The CH starvation status at EN in sink leaves could be causal to the reduced growth of sink leaves under -DIF (Bours et al., 2013). To strengthen the putative causal relationship between CH starvation status and reduced growth of sink leaves, different ways to affect CH status or CH sensing were tested for their effect on leaf expansion.

Artificially lowering endogenous T6P levels: The CH status is sensed through levels trehalose-6-phosphate (T6P) (Zhang et al., 2009; Mair et al., 2015; Figueroa and Lunn, 2016). To determine if CH sensing through T6P is linked to leaf expansion, the T6P levels in mature Arabidopsis rosette plants were artificially manipulated and the effect on leaf elongation was observed. The T6P levels can artificially be increased through expression of the bacterial T6P synthase gene *otsA* or decreased by over-expression of the bacterial T6P hydrolase gene *otsB* (Schluepmann et al., 2003). Arabidopsis plants with either *35S::otsA* (increased T6P levels; high sugar signal) or *35S::otsB* (decreased T6P levels; low sugar signal) were grown under +DIF and -DIF and growth of sink leaves was compared to that of untransformed Col-0 WT plants. The artificial reduced levels of T6P in *35S::otsB* plants induces a stronger CH starvation status, and already under +DIF sink leaves in the *35S::otsB* plants show reduced elongation compared to WT (Figure 7A). Moreover, in response to -DIF, the *35S::otsB* plants show an enhanced response to -DIF compared to Col-0 and *35S::otsA* plants, confirming that CH starvation, either induced by -DIF or by reducing T6P levels in *35S::otsB* plants, inhibits growth of sink leaves in adult Arabidopsis plants (Figure 7B).

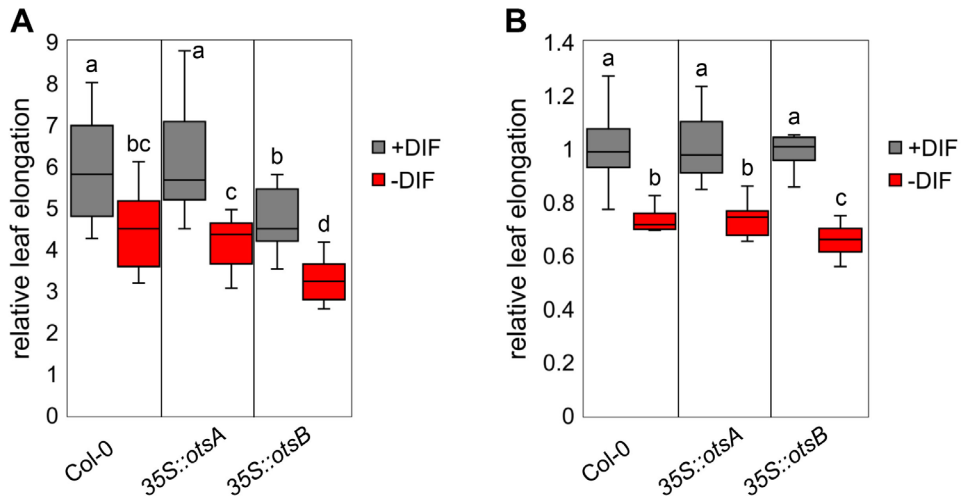


Figure 7: Elongation response of Col-0, *35S::otsA* and *35S::otsB* sink leaves to +DIF and -DIF. Relative leaf elongation of young developing sink leaves (start length 4-9mm) of 4-week-old Arabidopsis *35S::otsA*, *35S::otsB* (Schluepmann et al., 2003) and Col-0 WT plants during 1 week +DIF (12hL 22°C/12hD 12°C; grey boxes) or -DIF (12hL 12°C/12hD 22°C; red boxes). **A**: Relative leaf elongation calculated by dividing projected leaf length at start of the treatment by projected leaf length after 1 week of treatment. **B**: Relative leaf elongation calculated by normalizing leaf elongation during one week -DIF to leaf elongation under 1 week +DIF. Data are mean relative leaf elongation ±SE (n=10-23 leaves). Different letters indicate significant differences in leaf elongation during 1 week ($p<0.05$, Student's t-test).

Artificially increasing T6P levels: Although under -DIF CH starvation is linked to severely reduced levels of starch at EN in source leaves, induction of CH starvation may also be induced when starch mobilization is impaired. The *starch excess 1* (*sex1*) mutant lacks the activity of GWD1, and is therefore not able to produce sufficient amount of sugars during the night (Caspar et al., 1991; Streb and Zeeman, 2012). As a result, these mutants accumulate large amounts of starch but show signs of CH starvation during the night and reduced growth (Caspar et al., 1991; Streb and Zeeman, 2012; Paparelli et al., 2013). Arabidopsis seedlings grown on medium containing trehalose also accumulate large amounts of starch and show reduced growth (Wingler et al., 2000; Schluepmann et al., 2004). Accumulation of starch was also shown when feeding leaf discs of Arabidopsis with trehalose (Kolbe et al., 2005). This may be explained by very high increase in levels of endogenous T6P in response to the exogenous trehalose, since increased levels of T6P were shown to stimulate starch synthesis through redox activation of AGpase (Kolbe et al., 2005) and suppress starch breakdown (Martins et al., 2013). Apparently, such high levels of T6P are not reached in plants expressing otsA, as growth in not reduced in otsA overexpressing plants (Figure 7). We tested whether spraying with trehalose may be used to limit starch mobilization during the night, resulting in a CH starvation status at EN. For this, plants were sprayed with trehalose every 1st and 4th day of the week, using spraying with an equimolar solution of sorbitol as control. After four weeks of treatment leaves were harvested at EN and ED for starch analysis and leaf length was determined (Figure 8 E and F). Results show that starch levels are

indeed higher in both source and sink leaves in response to the trehalose treatment (Figure 8E). Moreover, leaf length on the Arabidopsis plants treated with trehalose is significantly reduced compared to that of control plants (Figure 8F).

Manipulating starch content at end night: At constant temperature the rate of mobilization of starch is tightly controlled by the clock to adjust to the given photoperiod (Graf et al., 2010), while our results under -DIF indicate that this clock control of starch utilization during the night is no longer in tune with the given photoperiod. To determine whether a shortage of starch near the end of night is linked to growth suppression in leaves of adult Arabidopsis plants, we used extended night as alternative for mis-regulation of starch turnover (Graf et al., 2010). For this, the effect of a three-hour extended night on growth and starch levels in of four-week old Arabidopsis plants was tested. For starch measurements, leaves were harvested at the end of the three-hour extended night. As expected, starch levels in both source and sink leaves are significantly lower in both source and sink leaves compared to starch levels at the normal EN (Figure 8C). The effect of extended nights on leaf growth can only be studied by prolonged treatment of extended nights. However, with regular repeated extended night the control of starch breakdown in the plants will adjust to the new photoperiod (Gibon et al., 2004), preventing induction of a CH starvation status. Therefore, the extended night treatment was only given intermittently by extending night by 3 hours every 1st and 4th day of the week. After four weeks leaf length was measured in plants grown under control conditions and grown under the extended night treatment. Results show that the regular induction of a CH starvation status by the extended night treatment did indeed result in a significant induction of leaf length (Figure 8D).

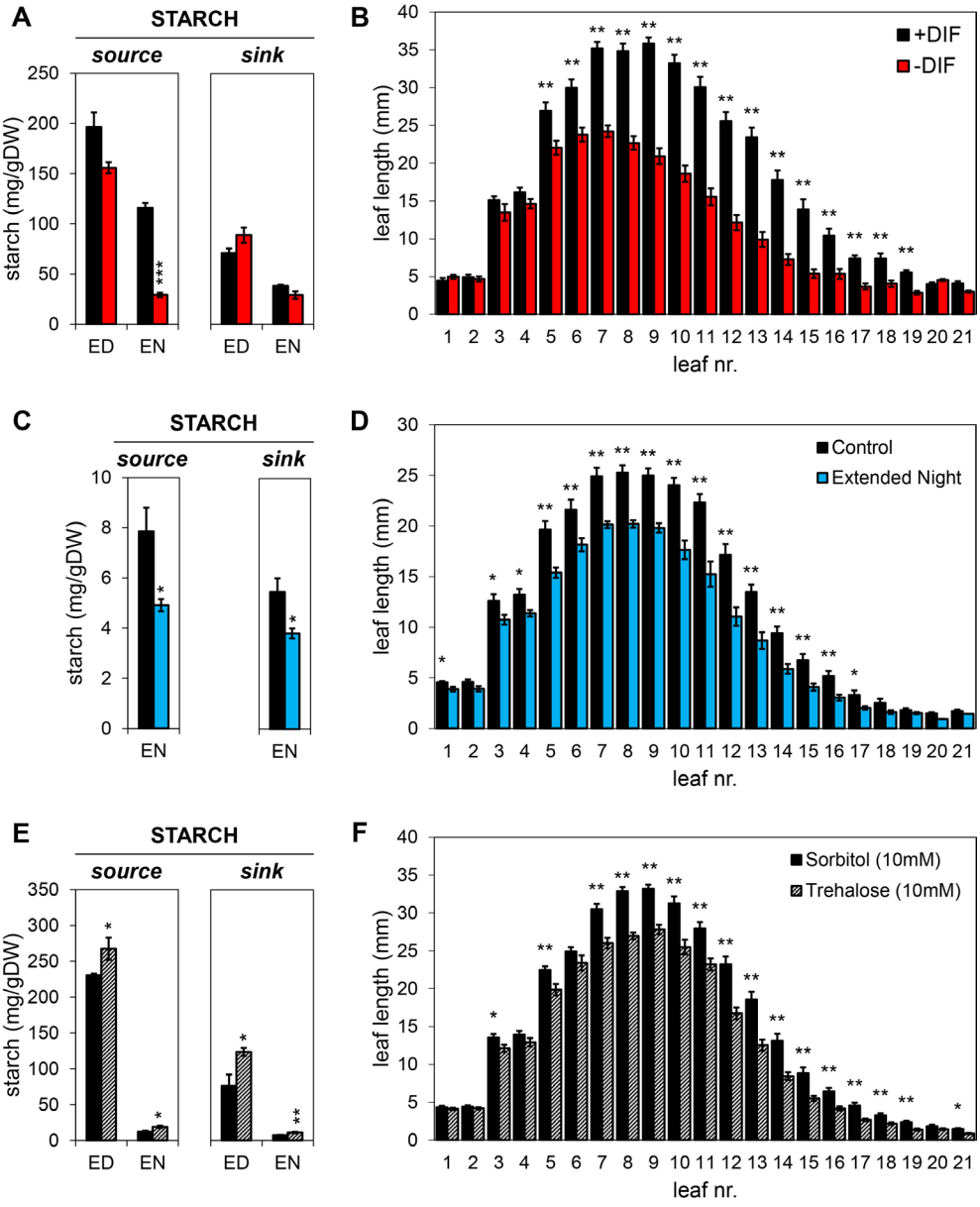


Figure 8: Effect of different treatments on starch levels and leaf growth of adult Arabidopsis plants. A: Starch levels at end of day (ED; ZT(h)=12) and end of night (EN; ZT(h)=24) in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). **B**: Leaf profiles of Arabidopsis Col-0 WT plants grown under +DIF (black bars) or under +DIF for 21 days and then transferred to -DIF for 10 days (red bars). **C**: Starch levels at the end of a 12h night (control; black bars) or at the end of a night prolonged by 3 hours (extended night; blue bars) in source or sink leaves of 4-week-old Arabidopsis Col-0 WT plants grown under 12hL 22°C/12hD 17°C. **D**: Leaf profiles of 4-week-old Arabidopsis Col-0 WT plants grown under control conditions (12hL 22°C/12hD 17°C; black bars) or subjected to an extended night of 3 hours every 1st and 4th day of the week (extended night; blue bars). **E**: Starch levels at ED and EN in source or

sink leaves of 4-week-old Arabidopsis Col-0 WT plants grown under 12hL 22°C/12hD 17°C and sprayed with either 10mM Sorbitol (black bars) or 10mM trehalose (black/white patterned bars) in the morning of every 1st and 4th day of the week. **F**: Leaf profiles of 4-week-old Arabidopsis Col-0 WT plants grown under 12hL 22°C/12hD 17°C and sprayed with either 10mM Sorbitol (black bars) or 10mM trehalose (black/white patterned bars) in the morning of every 1st and 4th day of the week. Leaf nr. based on order of emergence. Data are mean starch levels in mg per g dry material ±SE (n=3 biological replicates of 6 pooled plants) or mean leaf length ±SE (n=15 leaves). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from starch levels/leaf length under +DIF/control/Sorbitol 10mM.

Discussion

Differential effects on phase of clock genes under -DIF

Here we investigated the effects of an alternative diurnal day/night temperature regime (-DIF) on growth of Arabidopsis plants. These studies complement previous investigations into the effect of single entrainment signals which have shown how light cycles under constant temperature or temperature cycles under constant light can affect phase and amplitude of clock genes (McClung et al., 2002; Kusakina et al., 2014; Oakenfull and Davis, 2017; Johansson and Koster, 2019; Webb et al., 2019). Plants are subjected to different combinations of these two key entrainment signals under natural cyclic changes in the environment, or through artificial cyclic changes in greenhouses to control plant growth. Such cyclic entrainments lead to continuous and dynamic adjustment of the clock (reviewed in (Webb et al., 2019)). Moreover, in mature plants the different physiological state of source and sink leaves introduces an additional complexity in the growth response to a treatment like -DIF. Understanding how -DIF relates to plant elongation responses is of relevance in horticulture, where application of -DIF is common practice to control plant growth in greenhouses. Here we have shown that -DIF directly affects the phase of clock genes, resulting in a delayed phase for morning clock genes *CCA1*, *PRR9* and *LHY*, but an advanced phase for evening clock genes *GI*, *TOC1* and *FKF1*. These phase changes were slightly different in source and sink leaves of adult Arabidopsis plants (Table 1). The different phase changes for individual clock genes has consequences for the coordination of growth as the sequential steps required for growth (cell wall loosening, water influx, cell wall synthesis) are regulated by the clock. Steps related to growth that are under control of the clock (Harmer et al., 2000). Therefore, the window between gene expression of genes more under control of morning genes and others more under control of evening genes will enlarge under -DIF, while for other clock-regulated genes the window between peak expression may reduce under -DIF. This may in part explain the reduced growth under -DIF.

CH starvation status as indicated by marker genes explains phase-change in *CCA1* and *GI* expression under -DIF

Under -DIF the phase of *CCA1* expression is delayed in both source and sink leaves compared to +DIF (Figure 1 and Supplemental Figure S1). Previously it was shown that seedlings with low levels of endogenous sugars showed a delayed phase in *CCA1::LUC* activity under free-running conditions. Indeed, this delay is inhibited by exogenous

application of sugars in the morning (Haydon et al., 2013). Here, the delayed phase in *CCA1* expression under -DIF is also related to induction of a CH starvation status, both in source and sink leaves, as indicated by the CH starvation markers. (Figure 2A+B and Supplemental Figure S3). For sink leaves the CH starvation status under -DIF correlates with reduced sucrose levels. However, in source leaves the phase-delay in *CCA1* expression and CH starvation status does not correlate to reduced sucrose levels at EN under -DIF (Figure 2C). Glucose levels in source and sink leaves were not affected at EN under -DIF, while fructose levels were even higher in both types of leaves (Supplemental Figure S4). Thus, measured soluble sugar levels may not always be a good indication of CH status in leaf tissue, and induction of the CH starvation markers may therefore be a more reliable marker for what is experienced by the cells. We propose that the discrepancy between sucrose level and the indications for CH starvation (phase *CCA1*, expression starvation marker genes) can be explained by the sucrose in source leaves being in a different compartment than were CH levels are sensed. For instance, sucrose may be loaded into phloem, but not being transported to sink tissues when plants are grown under -DIF. Indeed, RNAseq data shows upregulation of *SWEET11* under -DIF at EN, while SWEET11 has been shown to be involved in sucrose phloem loading (Chen et al., 2012). This interpretation of the data is supported by the observation that a reduced sucrose transport to sink leaves is also observed in Arabidopsis in which CH starvation is induced by depletion of starch reserves due to an unexpected extended night (Kolling et al., 2015). Transport of sucrose in phloem is gradient driven (Lalonde et al., 2003) and the results thus suggest that unloading or sucrose processing is impaired in sink leaves at night under -DIF. As sucrose in phloem of source leaf cells does not contribute to the CH-status of the bulk leaf mesophyll cells, this could explain induction of CH starvation markers in these cells. Alternatively, or in addition, cells in source leaves may compete for sucrose as well by increased respiration activity during the night under -DIF as the higher AOX gene expression in source vs sink does suggest a higher respiration rate in source leaves (Fig S9 and S10).

CH status as sensed through T6P links to clock gene expression

The CH starvation status is sensed through T6P, the marker for sugar availability, and Frank *et al.*, (2018), proposed that T6P signaling adjusts the circadian clock to CH status through negative regulation of KIN10 activity (Zhang et al., 2009; Mair et al., 2015; Figueroa and Lunn, 2016; Frank et al., 2018). Regulation of the phase in *GI* expression is also linked to CH-status, as was demonstrated by overexpressing KIN10 (mimicking CH starvation signaling), which results in a delayed phase in *GI* activity under free running conditions (Shin et al., 2017). The delayed phase in *CCA1* expression thus correlates with a CH-starvation status in the morning, while the advanced phase in *GI* expression correlates with the increased sugar levels detected at the end of the day (Figures 1, 2, S1, S3 and S4).

Effect temperature on phase clock genes through CH sensing?

Temperature also affects the phase of clock genes (Salome and McClung, 2005; Gould et al., 2006; Salome et al., 2010; Kusakina et al., 2014; Hansen et al., 2017). For instance, seedlings grown at 12°C displayed a delayed phase in *CCA1* activity compared to seedlings grown at 22°C (Salome et al., 2010). Although sugars accumulate during cold acclimation, it could be that, similar to under -DIF, the sucrose at EN is not sensed, and that leaf cells are actually in a CH starvation status. It was shown that the accumulation of sugars in response to cold during the day is dependent on *GI* (Cao et al., 2005; Cao et al., 2007). This is consistent with the increased amplitude of *GI* expression during the cold day under -DIF (Figure 1). Thus, the increased sugar levels at ED under -DIF are explained by higher *GI* expression during day under -DIF, while the higher sugar levels during the day explain the advanced phase in *GI* activity (Figure 1). Future research may determine whether the CH status (sucrose and/or T6P) is the primary signal for the phase control of *CCA1* and *GI* activity or whether temperature affects phase-of-expression of these clock genes also independent of CH-status.

-DIF disrupts balanced clock regulation of starch utilization during the night

Combined, our results show that an unbalanced mobilization of starch during the night under -DIF leads to a CH starvation status, which reduces elongation growth. Thus, -DIF disrupts the strict balanced photoperiod-control of starch degradation during the night, resulting in an increase in starch mobilization during the night that is not in tune with the starch accumulation during the day. In this respect, the -DIF treatments mimics the effect of a long day photoperiod on starch mobilization during the night (depletion of starch as if under short night). In combination with the 12L/12D diurnal light cycle, this causes a virtual daily extended night effect by which starch at EN is depleted and CH starvation status is induced. In contrast to a real extended night treatment, to which plants rapidly adjust their rate of starch synthesis during the following day (which is increased) and mobilization during the following night (which is decreased) (Gibon et al., 2004), the repeated -DIF treatment does not result in increased starch synthesis rate during the day and/or a decreased night time starch mobilization rate. As a result, plants grown under -DIF consistently show CH starvation at EN (Supplemental Figure S2). Moreover, we have shown in multiple ways that there is a consistent link between induction of CH starvation and reduced growth of sink leaves in adult Arabidopsis plants, as we found such link between CH starvation status and reduced growth by the -DIF treatment, by the effect of intermittent extended nights (Figure 8C+D) and by the effect of trehalose treatment on growth (Figure 8E+F). A link between disrupted starch metabolism, induction of CH starvation status and reduced growth has also been shown for mutants unable to properly synthesize starch (e.g. *sex1*) or mobilize starch (e.g. *pgm1*) (Caspar et al., 1985; Caspar et al., 1991; Streb and Zeeman, 2012; Paparelli et al., 2013).

Growth suppression under -DIF possibly through clock regulation of PIF activity

Leaf elongation in Arabidopsis is stimulated by Phytochrome Interacting Factor (PIF) transcription factors. As the name suggests, PIF proteins interact with the light-activated form (Pfr) of Phytochrome (PHY) and this interaction results in a destruction of PIFs (Leivar and Quail, 2011; Leivar and Monte, 2014; Xu et al., 2015; Pham et al., 2018). It has been shown that both PIF4 and PHYB are involved in the growth response of Arabidopsis hypocotyls under +DIF and -DIF (Bours et al., 2015), while PHYB Pfr functions as temperature sensor for PIF activity during night and during day (Jung et al., 2016; Legris et al., 2016; Qiu et al., 2019). The warm night under -DIF reduces PHYB Pfr activity during the night, which may stabilize PIF protein during the night. However, recently it has been shown that clock components PRR5, 7, 9 and TOC1 negatively regulate PIF protein activity, resulting in negatively regulation of growth promoting targets of PIFs, like *CDF5* (Martin et al., 2018), *YUC8* and *IAA29* (Kunihiro et al., 2011; Sun et al., 2012; Pucciariello et al., 2018). PIF proteins progressively accumulate during the night, and while TOC1 directly interacts with PIFs and represses their transcriptional activity, this influence of TOC1 diminishes over the night. Near the end of the night the TOC1 levels decline, but PRR protein levels increase, extending the suppression of PIF activity towards growth (Soy et al., 2016). In addition, PIFs feedback on the clock by repressing *CAA1* and *LHY* expression (Shor et al., 2017). Since -DIF leads to increased expression of *PRR9* and higher expression of *TOC1* the putative increase in PRR9 and TOC1 protein levels during the warm night under -DIF may lead to stronger suppression of PIF protein activity towards growth and reduced repression of *CCA1*/*LHY* expression. Combined, this provides a link between the effect of -DIF on the clock and growth, as regulated by PIFs. Future analysis may need to focus on the relative strength of interactions between PIFs, CCA1 and PRR9 and the different targets of PIFs related to the clock, growth and carbohydrate metabolism.

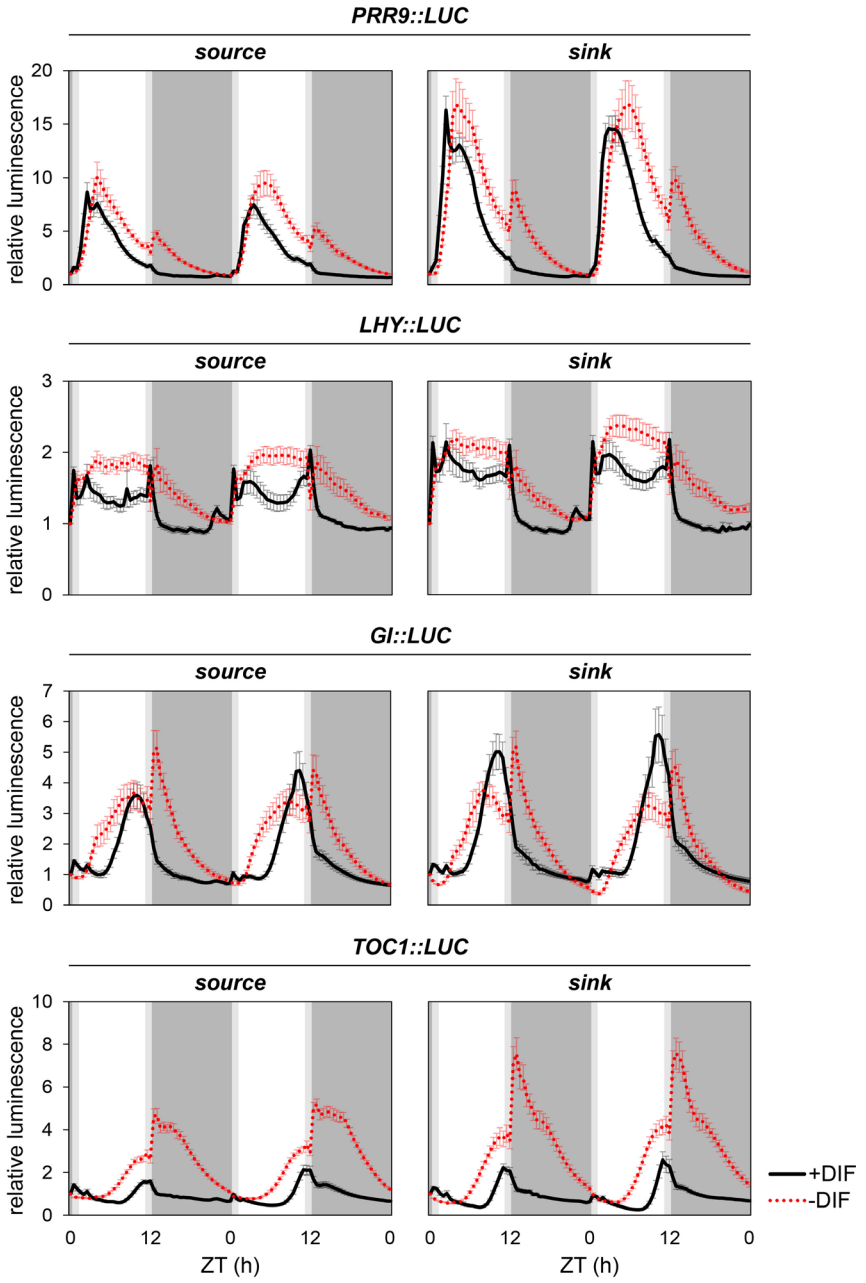
Supplemental Data

Supplemental Table S1: Primers used for qRT-PCR.

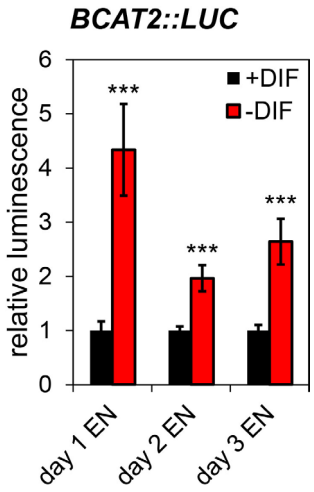
primer name	TAIR id	sequence
YLS8 fw	AT5G08290 (reference)	TTACTGTTTCGGTTGTTCTCCATTT
YLS8 rev	AT5G08290 (reference)	CACTGAATCATGTTCGAAGCAAGT
IPP2 fw	AT3G02780 (reference)	CATGCGACACACCAACACCA
IPP2 rev	AT3G02780 (reference)	TGAGGCGAATCAATGGGAGA
CCA1 fw	AT2G46830	TCAAGCTTCCACATGAGACTCTA
CCA1 rev	AT2G46830	GGAAACAAATACAAAGGCCTCA
PRR9 fw	AT2G46790	TGAGATACTGGGGCAACTTTT
PRR9 rev	AT2G46790	GCTTAGCCTGATCATTTGCAG
LHY fw	AT1G01060	ACAGCAACAACAATGCAACT
LHY rev	AT1G01060	GAGAGCCTGAAACGCTATAC
GI fw	AT1G22770	TTTCTCCGATATTCGTCGATCTC
GI rev	AT1G22770	CTGAATCAAACAGCTAAACCC
TOC1 fw	AT5G61380	GAAGATGTTGATCGACTGAC
TOC1 rev	AT5G61380	GAGCCAACATTGCCTTAGAG
FKF1 fw	AT1G68050	TTTGGCGGGCTTGCAAATAG
FKF1 rev	AT1G68050	AATGCGCTACACTCAAGCTC
DIN6/ASN1 fw	AT3G47340	AAGACGCGCCTTTGAAAACG
DIN6/ASN1 rev	AT3G47340	AGACCACCAGAGAGCAAAACTC
BCAT2 fw	AT1G10070	AGGCTTCTTCTTGCAACGTC
BCAT2 rev	AT1G10070	ACACTTTTCCGCGTAATCCC
GDH1 fw	AT5G18170	TATCAGGGCAGCGTTTTGTC
GDH1 rev	AT5G18170	TGTCACTCACGGCAACAATC
AT5G56100 fw	AT5G56100	AGCCGCTTTGGTATGTTGTG
AT5G56100 rev	AT5G56100	ATTCTCTCACCACCGACTCAAC
PGM fw	AT5G51820	TCGCAACAAGGACACGAAAC
PGM rev	AT5G51820	TTCCGTATGTCGCCCAATACTC
SBE2.1 fw	AT2G36390	ATGGCCATTCAAGAGCATGC
SBE2.1 rev	AT2G36390	TCATCAGGTGTTCCAAAGCG

Supplemental Table S1 (continued)

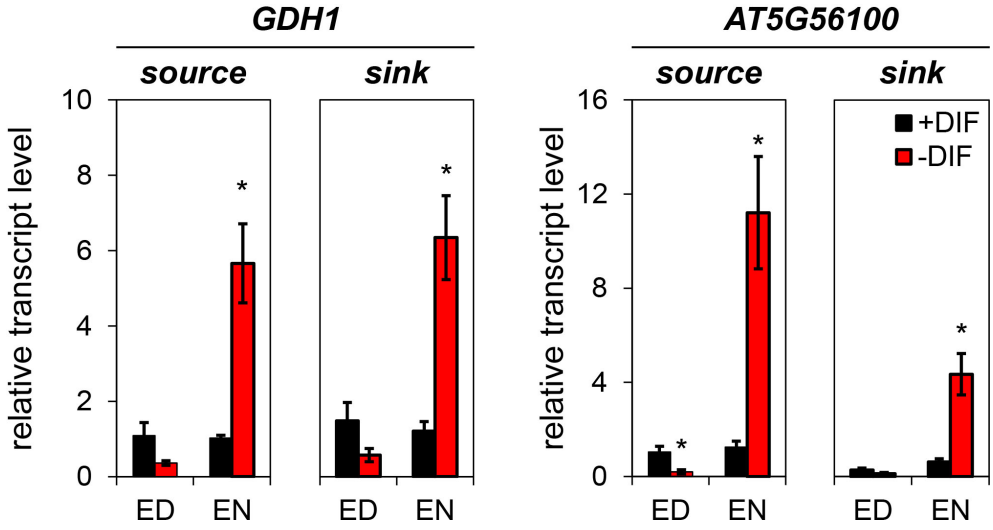
primer name	TAIR id	sequence
BAM3 fw	AT4G17090	TCCGTGAACGATTTGAAGGC
BAM3 rev	AT4G17090	ATTCTCCACAAGGTCCCATTCC
GWD1 fw	AT1G10760	AATTTGCCCTCGACATGTGC
GWD1 rev	AT1G10760	TTTGCTTGCCAGTGCTAGTC
ISA3 fw	AT4G09020	AGTCGCCGGATGATTTTGTG
ISA3 rev	AT4G09020	AAGAGAATGGGGCCACATTG
NTRC fw	AT2G41680	TACGGGTGAAGAAACTGAGCTG
NTRC rev	AT2G41680	ACTGACTGTTTGGCGAATGC
AOX fw	AT1G32350	TGCCCAATGTTCGGCTATTG
AOX rev	AT1G32350	AACTTTACCATCGGCCGTTC



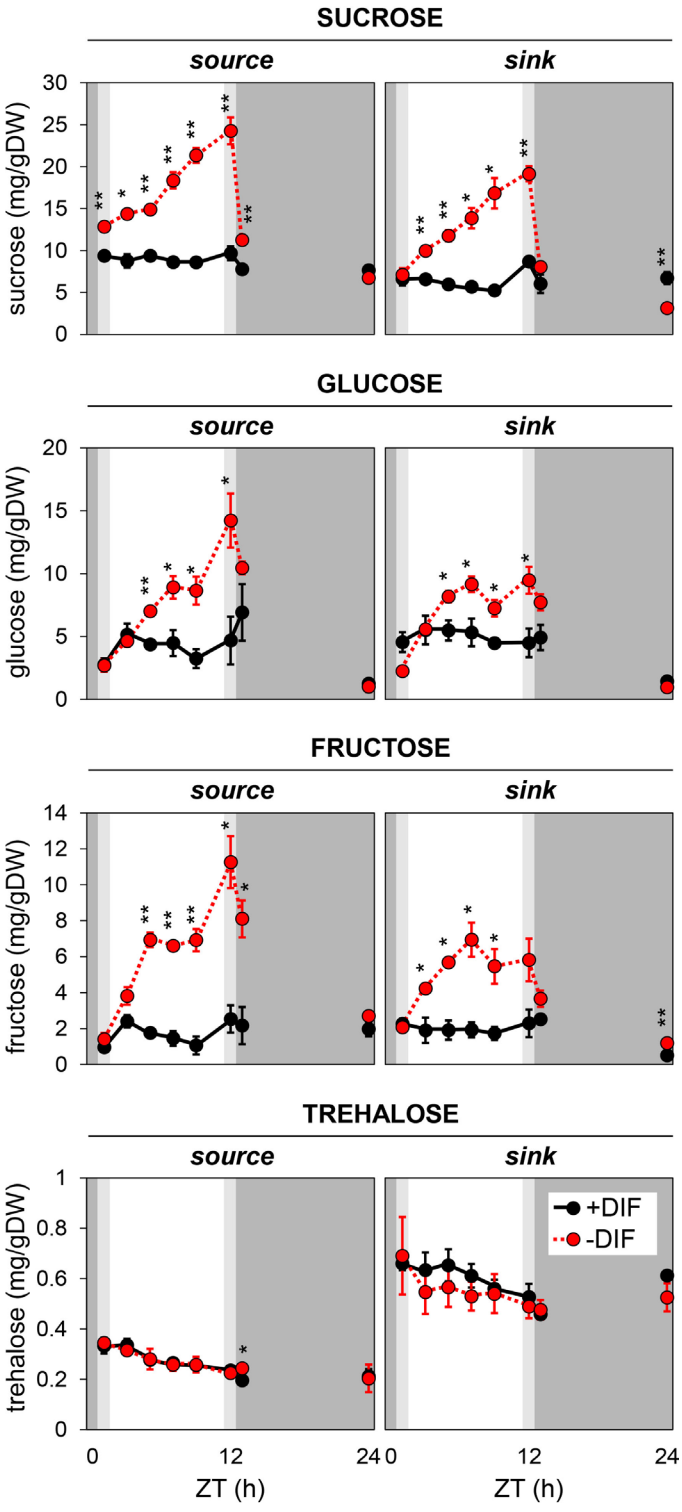
Supplemental Figure S1: Diurnal ffLUC activity of clock reporters in source or sink leaves under +DIF and -DIF. Diurnal *PRR9::LUC*, *LHY::LUC*, *GI::LUC* or *TOC1::LUC* activity in source and sink leaves of 4-week-old Arabidopsis reporter plants under +DIF (12hL 22°C/12hD 12°C; black solid line) or -DIF (12hL 12°C/12hD 22°C; red dashed line) during two subsequent days. Dark grey graph areas represent dark period, white areas represent day light (100 µmole m^{-2} s^{-1}) light grey areas represent dawn/dusk (30 µmole m^{-2} s^{-1}). Data are mean luminescence ± SE (n=6 biological replicates) normalized to luminescence at ZT(h)=0 on first day.



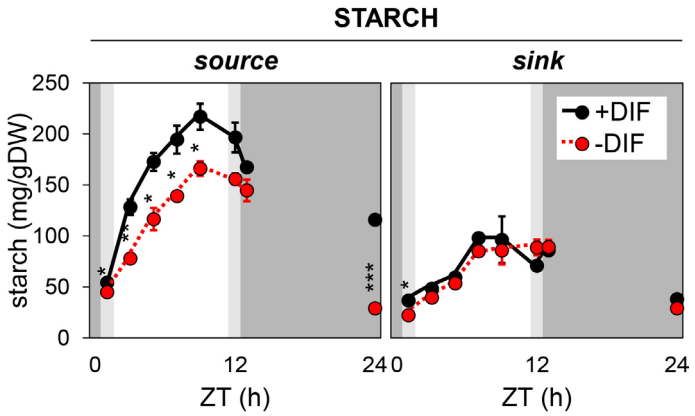
Supplemental Figure S2: ffLUC activity of carbohydrate starvation reporter under +DIF and –DIF during 3 subsequent days. Activity of carbohydrate starvation reporter *BCAT2::LUC* (Graf et al., 2010) at end of night (EN; ZT(h)=24) in the shoot of 4-week-old Arabidopsis reporter plants under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF (12hL 12°C/12hD 22°C; red bars) during three subsequent days. Data are mean luminescence ± SE (n=10 biological replicates) normalized to luminescence under +DIF on the same day. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from luminescence under +DIF.



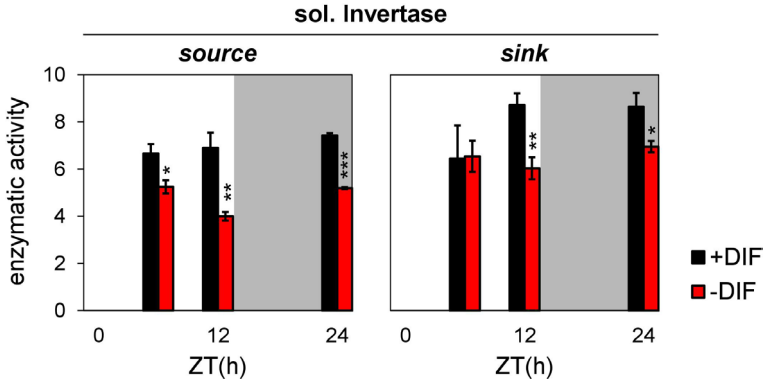
Supplemental Figure S3: mRNA levels of carbohydrate starvation markers in source or sink leaves under +DIF and -DIF. mRNA levels of carbohydrate starvation marker genes *GDH1* (*AT5G18170*) (Flis et al., 2016) and *AT5G56100* (Pilkington et al., 2015) at end of day (ED; ZT(h)=12) and end of night (EN; ZT(h)=24) in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA levels in source leaves under +DIF at ED. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.



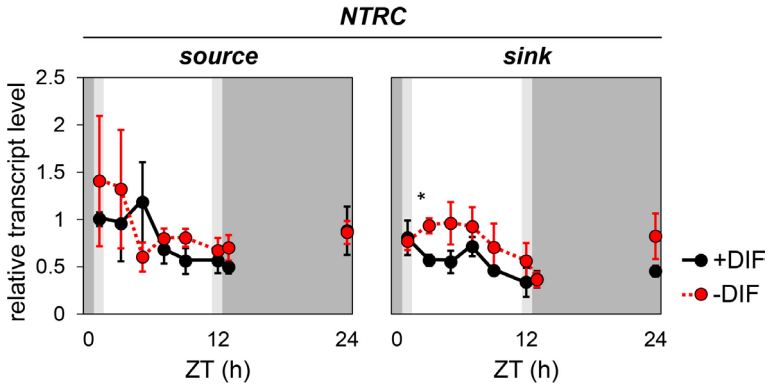
Supplemental Figure S4: Diurnal profile of soluble sugar levels in source or sink leaves under +DIF and -DIF. Levels of sucrose, fructose, glucose and trehalose at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean sugar levels in mg per g dry material ±SE (n=3 biological replicates of 6 pooled plants). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from sugar levels under +DIF.



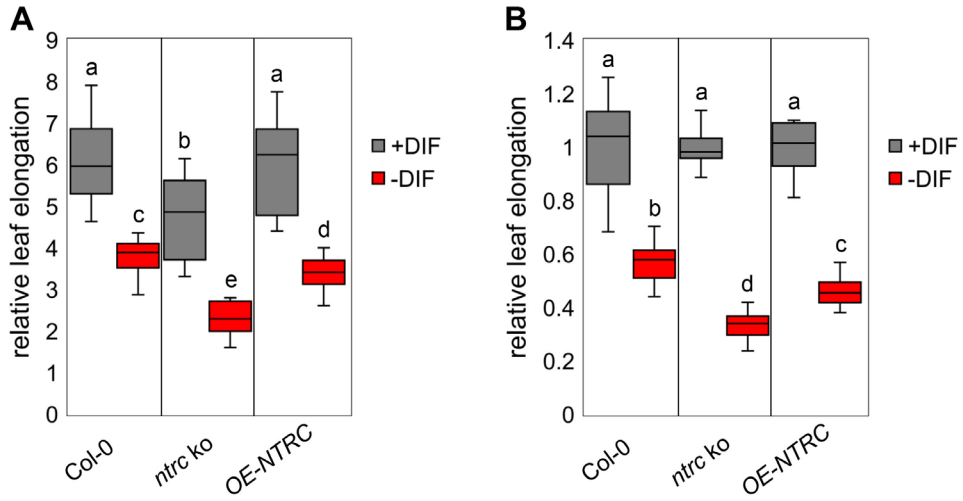
Supplemental Figure S5: Diurnal profile of starch levels in source or sink leaves under +DIF and -DIF. Starch levels at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean starch levels in mg per g dry material ±SE (n=3 biological replicates of 6 pooled plants). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from starch levels under +DIF.



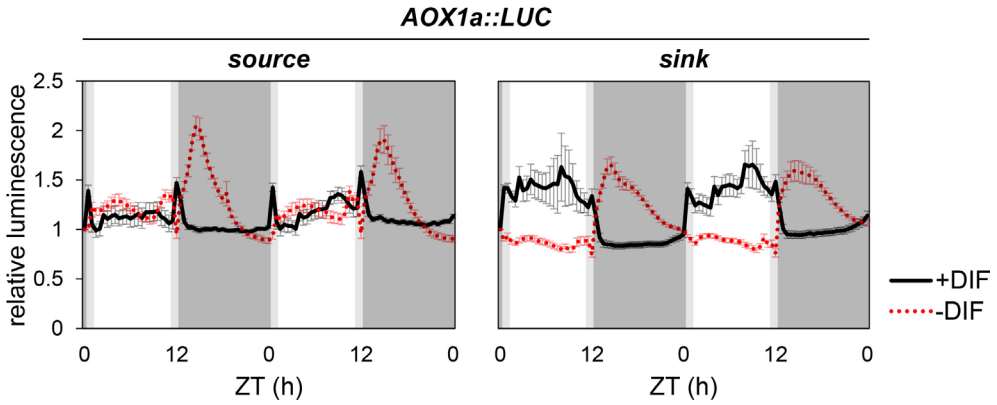
Supplemental Figure S6: Activity of soluble invertase in source or sink leaves under +DIF and -DIF. Enzymatic activity of soluble (vacuolar) invertase at ZT(h)=6, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black bars) or -DIF conditions (12hL 12°C/12hD 22°C; red bars). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}). Data are mean enzymatic activity in nmol NADH/mg dry material/min. ±SE (n=3 biological replicates of 6 pooled plants). *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from activity under +DIF.



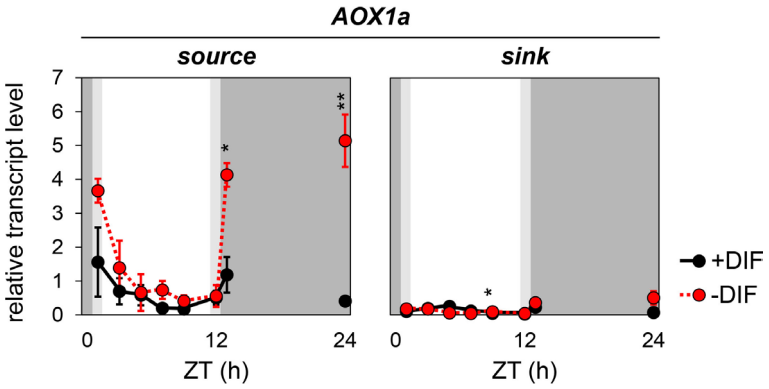
Supplemental Figure S7: mRNA levels of *NTRC* in source or sink leaves under +DIF and -DIF. mRNA levels of *NTRC* at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.



Supplemental Figure S8: Elongation response of Col-0, *ntrc ko* and *OE-NTRC* sink leaves to +DIF and -DIF. Relative leaf elongation of young developing sink leaves (start length 4-9mm) of 4-week-old Arabidopsis *ntrc ko*, *OE-NTRC* and Col-0 WT plants during 1 week +DIF (12hL 22°C/12hD 12°C; grey boxes) or -DIF (12hL 12°C/12hD 22°C; red boxes). **A**: Relative leaf elongation calculated by dividing projected leaf length at start of the treatment by projected leaf length after 1 week of treatment. **B**: Relative leaf elongation calculated by normalizing leaf elongation during one week -DIF to leaf elongation under 1 week +DIF. Data are mean relative leaf elongation ±SE (n=10-16 leaves). Different letters indicate significant differences in leaf elongation during 1 week (p<0.05, Student's t-test).



Supplemental Figure S9: Diurnal *AOX1a::LUC* activity in source or sink leaves under +DIF and -DIF. Diurnal *AOX1a::LUC* activity in source and sink leaves of 4-week-old Arabidopsis reporter plants under +DIF (12hL 22°C/12hD 12°C; black solid line) or -DIF (12hL 12°C/12hD 22°C; red dashed line) during two subsequent days. Dark grey graph areas represent dark period, white areas represent day light (100 µmole m^{-2} s^{-1}) light grey areas represent dawn/dusk (30 µmole m^{-2} s^{-1}). Data are mean luminescence ± SE (n=6 biological replicates) normalized to luminescence at ZT(h)=0 on first day.



Supplemental Figure S10: mRNA levels of *AOX1a* in source or sink leaves under +DIF and -DIF. mRNA levels of *AOX1a* at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF.

Chapter 5

From Lab to precision Horticulture: Predicting long-term plant growth from short-term transcriptional responses to LEDs

Mark van Hoogdalem

Jolien Verweij

Jacqueline Busscher-Lange

Umidjon Shapulatov

Maarten Wassenaar

Elias Kaiser

Habtamu Giday

Ana Quintanilla

Monique van Vegchel

Wim van Ieperen

Alexander R. van der Krol

Abstract

Plant growth is regulated by PHYTOCHROME INTERACTING FACTORs (PIFs), which stimulate cell elongation, and ELONGATED HYPOCOTYL 5 (HY5), which is involved in suppression of cell elongation. Activity of these transcription factors is regulated by light quality. Therefore, wavelength-specific additional light treatments using light-emitting diodes (LEDs) may be used to regulate PIF/HY5 activity and control plant growth in greenhouses. However, despite current knowledge, predicting the effect of added light treatments on the activity of PIFs/HY5 and plant growth is still difficult. Here we used our custom-built LUMINATOR system and Arabidopsis firefly-luciferase (ffLUC) reporter plants to study the transcriptional responses of *PIF3*/*4*/*5*, *HY5* and the PIF-induced target gene *IAA29* to one hour added red or blue LED light at dawn and dusk. We subsequently tested whether transcriptional responses of these genes are predictive for long-term plant growth responses. This was done by studying the effect of the added light on growth of tomato in SOLINATOR climate cabinets and greenhouses.

Although results show some discrepancies between responses of ffLUC reporter activity and endogenous gene expression, overall results indicate that the added light treatments trigger different transcriptional responses. Added red light leads to a strong reduction in integrated *IAA29* activity during the night, which suggests it may lead to suppression of elongation through reduced PIF protein activity. This prediction was confirmed by growth experiments in SOLINATOR. However, additional red light only suppressed growth in greenhouses during one of two independent experiments. Added blue light resulted in decreased *PIF4* activity and increased *HY5* activity integrated over the day, which suggests suppression of elongation. Added blue light did not significantly reduce growth of tomato in SOLINATOR, but it did in both greenhouse experiments.

Our results show that gene activity responses to added light treatments can be monitored using ffLUC reporter plants in LUMINATOR and that added light pulses can affect gene activity outside the time window during which the light pulse is provided. Depending on the growth conditions, the transcriptional responses of key-genes involved in growth regulation in Arabidopsis may be predictive for the effects of added light treatments on growth of tomato.

Introduction

In horticulture it is important to control elongation of stems and to grow compactly shaped plants, especially in the production of ornamental plants. However, achieving this year-round can be challenging during periods with low light levels or high temperatures, since both these conditions may induce elongation, resulting in spindly-shaped plants. Current growth control in greenhouses is mostly by application of chemical plant growth retardants (PGRs), which mainly inhibit growth through inhibition of GA biosynthesis (Carvalho et al., 2008). However, the use of chemical PGRs is increasingly limited by regulations and there is an increasing demand for more sustainable methods to control growth in greenhouses. One option, which is widely used in commercial plant production to control plant growth without the use of chemical PGRs, is realizing a negative day-night temperature difference (cold days/warm nights; -DIF). A -DIF regime suppresses growth of stems in many plant species, including Arabidopsis (Myster and Moe, 1995; Moe and Heins, 2000; Bours et al., 2013). However, economic realization of -DIF in greenhouses is only possibly when outside temperatures are relatively low, and therefore additional or alternative means of plant growth control are needed. These may be found in wavelength-specific additional light treatments using Light Emitting Diodes (LEDs).

Cell elongation in Arabidopsis hypocotyls under warm day/cold night (+DIF) conditions depends on auxin and ethylene biosynthesis and signaling, and expression of the transcription factors (TFs) PHYTOCHROME INTERACTING FACTOR 3, 4 and 5 (PIF3, 4, 5) (Bours et al., 2015). PIF4 and PIF5 act upstream, while PIF3 acts downstream in the signaling cascade regulating hypocotyl elongation (Bours et al., 2015). Indeed, PIFs are major regulators of plant cell elongation in response to light and temperature signals (Leivar and Quail, 2011; Leivar and Monte, 2014; Quint et al., 2016; Legris et al., 2017; Pham et al., 2018). PIFs stimulate cell elongation by transcriptional activation of genes involved in processes related to cell elongation such as cell wall modification and auxin biosynthesis, signaling and transport, while PIFs are also involved in repression of genes involved in photomorphogenesis (Leivar and Quail, 2011; Leivar and Monte, 2014; Quint et al., 2016; Legris et al., 2017; Pham et al., 2018). The PIFs interact with phytochromes, and interaction with the active Pfr form of phytochrome photoreceptors regulates PIF protein stability as function of the Red:Far Red (R:FR) light ratio (Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018). In addition, PIF activity is also regulated at post-transcriptional level through interactions with the blue light photoreceptors cryptochromes CRY1 and CRY2 (Bordage et al., 2016; Pedmale et al., 2016).

The bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5) often acts antagonistically to PIFs (Kami et al., 2010; Lau and Deng, 2010; Leivar and Quail, 2011; Gangappa and Botto, 2016). While PIFs act as positive regulators of skotomorphogenesis, HY5 promotes photomorphogenesis by stimulating expression of genes regulating processes such as photosynthetic machinery assembly, pigment production and chloroplast development (Lee et al., 2007; Zhang et al., 2013). Activities of HY5 and PIF4 are interdependent, as HY5 and PIF4 can bind to the same E/G-box promoter elements of PIF4 target genes. Indeed, several studies have shown that HY5 and PIF4 compete for the same promoter site of genes involved in Reactive Oxygen

Species (ROS) responses (Chen et al., 2013), photosynthesis (Toledo-Ortiz et al., 2014) and cell elongation (e.g. the auxin biosynthesis gene *YUCCA8* and α-expansin encoding gene *EXPANSIN A8*) (Gangappa and Kumar, 2017). HY5 has also been shown to positively regulate its own transcription in the light through direct binding of its promoter (Abbas et al., 2014; Binkert et al., 2014) and negatively regulates transcription of *PIF4* (Lee et al., 2007; Delker et al., 2014). Like the PIFs, HY5 is also regulated by light: blue light positively regulates *HY5* transcription (de Wit et al., 2016) and HY5 protein stability through CRY1-medaited destabilization of the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)-SUPPRESSOR OF PHYA1 (SPA1) complex, which targets HY5 for degradation in the dark (Osterlund et al., 2000; Saijo et al., 2003; Zhu et al., 2008; Lian et al., 2011; Liu et al., 2011).

Since light quality affects transcription and protein activity of both PIFs and HY5, wavelength-specific light pulses may potentially be used to control PIF and/or HY5 activity and therefore plant growth in greenhouses. Such light pulses may be used as alternative or in addition to a -DIF treatment. The energy efficiency of LEDs makes it a realistic option to use LEDs as additional light sources to manipulate both light intensity and quality in greenhouses to realize light conditions for desired plant growth (Singh et al., 2015; Bantis et al., 2018). However, even with our current insights, predicting the optimal timing and long-term effect of a short LED light pulse on overall activity of PIFs and HY5, and thereby on growth, is challenging for several reasons. First of all, most fundamental insights on light regulation of plant cell elongation are based on studies of Arabidopsis seedling hypocotyls and may not always directly apply to more mature stages of development. Second, much of the research on light signaling in plants is done under constant artificial light. This is in contrast to the natural changing broad-spectrum sunlight conditions experienced by plants in greenhouses, as function of sun position, weather and seasonal photoperiod. Finally, it has been shown that light signaling responses in plants are gated by the circadian clock (Salter et al., 2003; Feike et al., 2016). Therefore, the same added LED light pulse given at different times of the day may elicit different responses, or may change the phase of clock-regulated gene expression, so that effects may be outside the window of added light treatment.

It takes several days, if not weeks, to accurately determine the growth effect of a given light treatment. Indeed, it will be very time-consuming and labor intensive to screen for the endless variations and combinations that can be used in added light color, intensity, duration and timing on growth of plants to find optimal conditions. Transcription of key growth regulators such as PIFs and HY5 and their target genes can be monitored directly and semi-continuously using ff-luciferase (ffLUC) reporter plants in a custom build growth cabinet, in which ffLUC activity can be imaged, and light and temperature conditions can be controlled (LUMINATOR; Chapter 2 of this thesis). Transcriptional responses of selected ffLUC reporter genes to selected subtle light treatments can be observed for one or two days. This may be used to identify light treatments which induce changes in transcriptional activity, which over prolonged time may affect growth in a desired way. However, light conditions under LUMINATOR are crude compared to full sunlight spectrum, as only red (R), blue (B) and far red (FR) LED lights are used (Chapter 2 of this thesis). Therefore, light pulse treatments that give desired transcriptional responses

for growth manipulation in LUMINATOR need to be re-evaluated under more realistic light and temperature conditions. For this we used custom-built growth cabinets equipped with LED modules mimicking full sunlight spectral composition (SOLINATOR). Finally, light treatments were also tested in commercial greenhouses with added LED light under natural sunlight conditions. Here we evaluated whether a two-day transcriptional response to added light pulse treatments, aimed at limiting growth of the model plant Arabidopsis, and as monitored under the artificial light conditions of LUMINATOR, is sufficient to predict the long-term (2 weeks) growth responses of Arabidopsis and tomato plants in SOLLINATOR and real greenhouses (Figure 1).

As mentioned above, PIF proteins are unstable in the light, while HY5 protein is unstable in the dark (Osterlund et al., 2000; Saijo et al., 2003; Zhu et al., 2008). Therefore, transcriptional activity of *PIF*s in the light may be less relevant for stimulation of elongation, while transcriptional activity of *HY5* in the dark may be less relevant for suppression of elongation. Therefore, in order to evaluate the potential effect of light pulses on growth regulation by PIFs and HY5, the short term direct transcriptional response to an added light pulse of the *PIF::LUC* and *HY5::LUC* reporters was monitored as well as the integrated activity over the day or night period.

We tested the effect of additional R or B LED light during the first and last hour of the photoperiod on ffLUC reporter activity driven by promoters of *PIF3*, *PIF4*, *PIF5* (implicated in elongation responses) or *HY5* (implicated in suppression of elongation responses). Because regulation of PIFs by light is mainly at post-transcriptional level (Leivar and Quail, 2011; Leivar and Monte, 2014; Ma et al., 2016; Pedmale et al., 2016; Pham et al., 2018), *PIF* transcription may not directly be indicative of PIF protein activity. A reporter line for the promoter activity of the PIF4/5-target gene *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) (Kunihiro et al., 2011; Sun et al., 2013; Leivar and Monte, 2014) was included as indicator of effective PIF4/5 protein activity. Key responses of ffLUC reporters were validated by qRT-PCR.

We found some discrepancies between ffLUC reporter activity and responses of the endogenous gene. However, overall results indicate that the added R light treatment strongly reduces integral *IAA29* activity during the night. Transcriptional responses to added B light were observed for *HY5* and *PIF4*: integrated *PIF4* activity was reduced during the day, while integrated *HY5* activity was increased during the day. The transcriptional responses predict reduced growth under both of the added light treatments. To test these predictions, the effect of the added light treatments was tested on growth of tomato plants for two weeks in SOLINATOR and greenhouse. The prediction that the added R light treatment suppresses growth was confirmed by growth experiments in SOLINATOR, but in greenhouses added red light did not always lead to reduced growth. The added B light treatment did not lead to reduced growth in SOLINATOR, but did suppress growth in the greenhouse.

Overall, results presented here show that subtle effects of light pulse treatments can be monitored using ffLUC reporter plants and LUMINATOR, and that short-term light treatments can have effects on transcriptional activity outside the time window during which the light treatment is given. Furthermore, depending on the growth conditions,

translation of the insights into transcriptional regulation of key genes involved in elongation responses in Arabidopsis may have predictive value for estimating effects on plant growth in tomato.

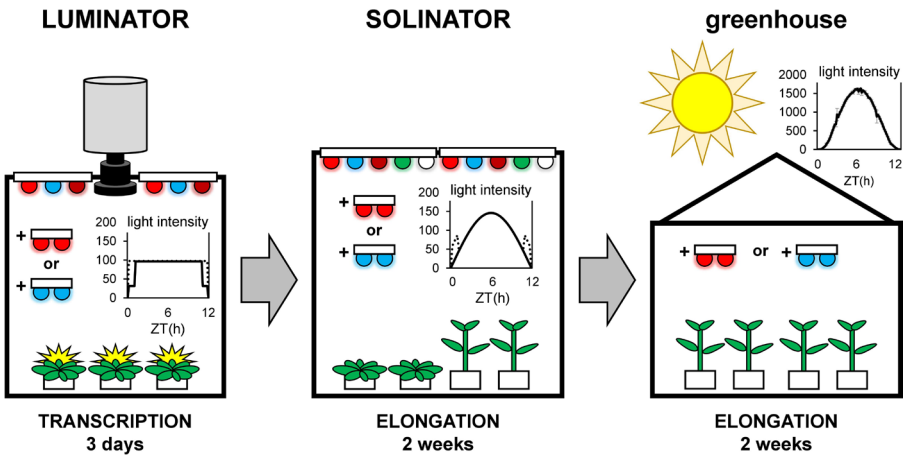


Figure 1: From lab to greenhouse. Schematic outline of research strategy. In LUMINATOR, the effect of one-hour additional blue or red LED light at the start and end of the photoperiod on expression of key growth-regulating genes is tested during three days using Arabidopsis firefly-luciferase (ffLUC) reporter plants. In SOLINATOR, the effect of the additional blue or red LED light treatments on growth of tomato is tested over a period of two weeks. In SOLINATOR light and temperature conditions are closer to natural conditions than in LUMINATOR. Finally, the effect of the effect of the additional blue or red LED light treatments on growth of tomato is tested in a greenhouse.

Material and Methods

Plant material

Arabidopsis Col-0 (N1092) seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). *PIF4::LUC* seeds were provided by Salomé Prat (CNB-CSIC, Madrid, Spain). *PIF3::LUC, PIF5::LUC, IAA29::LUC and HY5::LYC* seeds were generated as described below. All reporter lines are in Col-0 background. Tomato seeds were of the line *Solanum lycopersicum* cv. 'Money maker'.

LUMINATOR experiments

Growth conditions: Seeds of Arabidopsis Col-0 WT and ffLUC reporter lines were stratified in the dark for three days at 5°C and sown on 4x4x4cm rockwool blocks (Grodan, Roermond, The Netherlands) soaked in Hyponex nutrient solution (Unifarm, Wageningen, The Netherlands). Plants were pre-grown in a climate chamber (12hL/12hD; 150 µmole m^{-2} s^{-1} light provided by fluorescent tubes; 22°C; relative humidity (RH) at 65%). Plants were watered with Hyponex nutrient solution twice a week. After 26 days, ffLUC reporter plants for obtaining diurnal ffLUC activity profiles or Col-0 plants for qRT-PCR validation experiments were transferred to a custom-made climate cabinet

fitted with a CCD camera for semi-continuous imaging of ffLUC reporter plants under controlled light and temperature conditions (LUMINATOR; Chapter 2 of this thesis). Directly before transfer to LUMINATOR reporter plants were watered by soaking the rockwool blocks in Hyponex solution, which allows for growth for 4 days without additional watering. In LUMINATOR, plants were grown under 12hL/12hD cycles at constant temperature (T=22°C) and relative humidity (RH=70%).

Light treatments: A schematic representation of light treatments in LUMINATOR is shown in Figure 2. In LUMINATOR, control light conditions included a 1-hour step illumination gradient (light ramping) at start-day and end-day, to mimic altered light intensity and quality in morning and evening under natural light conditions. During this ramp total light intensity is 30 µmole m^{-2} s^{-1} and during the remaining hours of the photoperiod 100 µmole m^{-2} s^{-1} (Supplemental Figure S1A) The ratio blue:red:far red light during ramping is 1:2:1 and during the remaining hours of the photoperiod 3:6:1. As a result, the phytochrome photostationary state (PSS) during light ramping (0.75) was lower than during the remainder of the photoperiod (0.82) (Supplemental Figure S1C). For the additional red (R) light treatment, light intensity of R light during ramping was increased by 60 µmole m^{-2} s^{-1}. This leads to an increase in light intensity and PSS (Supplemental Figure S1A+C). For the additional blue (B) light treatment, light intensity of B light during ramping was increased by 55 µmole m^{-2} s^{-1} (Supplemental Figure S1B). Light spectra of all light settings are shown in Supplemental Figure S2.

SOLINATOR experiments

Growth conditions: Arabidopsis Col-0 seeds were stratified and pre-grown the same way as those used for LUMINATOR experiments. After three weeks the plants were transferred to either of two custom-made climate cabinets (SOLINATOR), in which light was provided by LED artificial sunlight research modules generation 1 (Specialty Lighting Holland B. V., Breda, The Netherlands). Tomato seeds were sown on 4x4x4cm rockwool blocks, which were soaked in 0.5x tomato nutrient solution (Yara Benelux BV, Vlaardingen, The Netherlands) before sowing, and stratified in the dark for 24h at 5°C. Seeds were then transferred to a climate chamber (12hL/12hD; 150 µmole m^{-2} s^{-1} light provided by fluorescent tubes; 25°C; RH75%), and after 8 days plants were transferred to four SOLINATOR cabinets in which different light treatments were provided (described below). Arabidopsis and tomato plants in SOLINATOR were grown under 12hL 25°C / 12hD 15°C cycles (+DIF conditions). The RH was constant 65%. Plants were watered three times a week with 0.5x nutrient solution. Arabidopsis plants were harvested after 10 days and tomato after 14 days for growth measurements.

Light treatments: Under control light conditions in SOLINATOR both light intensity and quality were variable during each 12h photoperiod, mimicking naturally occurring variations in light intensity (depending on wavelength) and resulting PSS over the day more closely than in LUMINATOR (Supplemental Figure S1). Maximum total light intensity reached at mid-day was 146 µmole m^{-2} s^{-1}, PSS varied between 0.65 and 0.72. For the additional R or B light treatments, 50 µmole m^{-2} s^{-1} of additional R or B light was

provided during the first and/or last hour of the photoperiod, which was provided by GreenPower Deep Red or Blue LED production modules, 2nd generation (Philips Lighting, Eindhoven, The Netherlands) with max. spectral output at 659 nm (± 10) and 453 nm (± 10), respectively. A schematic representation of the effect of the additional light treatments on light intensity and PSS are shown in Supplemental Figure S1.

Greenhouse experiments

Growth conditions: Tomato seeds were sown in potting medium ('Zaai/Stek Medium', Horticoop, Slingerland Potgrond, Katwijk, the Netherlands). Seeds were transferred to a greenhouse compartment for germination (natural photoperiod, 21-22°C; RH60%). Seven days after sowing, tomato seedlings were transferred into single pots (volume: 1.25 L), using substrate LP4 (Horticoop). After 14 days the tomato plants were transferred to either of two greenhouse departments, in which +DIF conditions (with natural photoperiod and light intensity) were realized. Temperature and RH in these compartments are shown in Supplemental Figure S3. Each greenhouse compartment was divided into 6 plots that each contained a custom-made table on which the plants were grown. Plants were watered regularly using tab water; all nutrients necessary for plant growth were available in the substrate. After two weeks growing under +DIF, plants were harvested for growth measurements. Greenhouse compartments were located in Wageningen, The Netherlands, at 40°N, 90°W, at 180 m elevation above sea level. Experiments were replicated in time: Experiment 1 ran from March 1 until March 14, 2018, Experiment 2 ran from March 20 until April 2, 2018.

Light treatments: Tomato plants in greenhouse compartments were grown under natural photoperiods. Under control conditions, no additional lighting was provided in the greenhouse compartments. Since these naturally change over time, day-length varied between 10h54m and 13h04m during the experiments. Average Daily Light Integral (DLI) during the experiments is shown in Supplemental Figure S3C. At night, black-out screens were used to prevent light contamination. For the additional R or B light treatments, 61 μmole m^{-2} s^{-1} (Experiment 1) or 37 μmole m^{-2} s^{-1} (Experiment 2) of additional red or blue light was provided during the first and last hour of the photoperiod. The additional light was provided by GreenPower Deep Red or Blue LED production modules, 2nd generation (Philips Lighting, Eindhoven, The Netherlands) with max. spectral output at 659 nm (± 10) and 453 nm (± 10), respectively. These were mounted on a frame, hanging at least 34cm above the top of the plants. Each light treatment (control and additional R or B) was provided to two plots per greenhouse compartment. To avoid light contamination, black-out screens were placed around each plot (white-colored side facing the plants).

Generation of ffLUC reporter lines

Part (2925bp) of the intergenic region upstream of the *PIF3* start codon, the full (2442bp) intergenic region upstream of the *PIF5* start codon, part (2500bp) of the of the intergenic

region upstream of the *IAA29* start codon and the full 756 bp intergenic region upstream of the *HY5* (AT5G11260) start codon were PCR amplified (primers listed in Supplemental Table S1) using Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). Subsequently, PCR products were cloned by TOPO® Cloning reaction (Invitrogen, Carlsbad, CA, USA) into the pENTR™ TOPO® entry vector. To generate *promoter::LUC* expression constructs the entry vectors containing the different promoter sequences were recombined into pGREEN-GW-Luc68 destination vectors by LR recombination using Gateway® LR Clonase® II enzyme mix (Invitrogen, Carlsbad, CA, USA). The *promoter::LUC* expression constructs were transformed to electrocompetent *Agrobacterium tumefaciens* (AGL0) and Arabidopsis Col-0 plants were transformed by floral dip transformation (Clough and Bent, 1998). Positive transformants were selected on 1xMS agar plates supplemented with 10mg/L BASTA and transferred to soil for propagation until homozygous lines were obtained.

Analyzing ffLUC activity in Arabidopsis rosettes

After they were transferred to LUMINATOR, Arabidopsis ffLUC reporter plants were allowed to acclimate at least one full day before imaging started. First full day of imaging was always under control conditions. Additional R or B light during ramping hours was (if provided) on day 2 and day 3. Luciferin was applied by spraying plants twice a day with 1mM D-luciferin (Promega, Fitchburg, WI, USA) in water, right after imaging at ZT(h)=2 and ZT(h)=8.5. Imaging and quantification of ffLUC activity was done as described before (Chapter 2 of this thesis).

RNA extraction and qRT-PCR for gene expression analysis

For gene expression analysis shoots of 4-week-old Col-0 plants were harvested separately and immediately frozen in liquid nitrogen. The frozen material was homogenized using a Mixer Mill MM 400 (Retsch, Haan, Germany) and total RNA was isolated using the InviTrap® Spin Plant RNA Mini kit (Stratec, Birkenfeld, Germany) according to manufacturer's instructions. Subsequently genomic DNA was removed with the TURBO DNA-*free*™ kit (Invitrogen, Carlsbad, CA, USA). Quality of the RNA was checked by running 2µl on agarose gel and concentration and purity was determined by NanonDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA). For each sample 1µg RNA was used to synthesize cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR reactions were performed on the CFX Connect™ Real-Time PCR Detection System using SYBR® Green qPCR mix and CFX Maestro™ software was used to analyse the data (all Bio-Rad, Hercules, CA, USA). Expression levels were calculated using the ΔΔCt method (Livak & Schmittgen, 2001), relative to expression levels of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780). Primers used are listed in Supplemental Table S2.

Auxin measurements

Shoots of 4-week-old Col-0 plants were harvested separately and immediately frozen in liquid nitrogen and the frozen material was homogenized using a Mixer Mill MM 400 (Retsch, Haan, Germany). Next the samples were freeze-dried and 2-4mg per sample was used to extract and determine levels of indole-3-acetic acid (IAA) in ng per mg (dry weight), as described in (Kolachevskaya et al., 2017).

Plant growth measurements

Arabidopsis were harvested after 10 days of treatments and tomato plants after 14 days of treatment by cutting them at the level of the soil/rockwool surface. Arabidopsis rosettes were photographed from the top and ImageJ software was used to determine total rosette leaf area (cm^2), diameter (cm) and circumference (cm). Plant stem length of tomato (hypocotyl+epicotyl+internodes) in cm was measured by ruler. Total leaf area (cm^3) was determined using a leaf area meter (Li-3000-100, Li-Cor Biosciences, NE, USA). The harvested Arabidopsis and tomato plants were dried in a forced air oven at 70°C for 48 hours and weighed to determine dry weight (g) of the above-ground tissue of each plant separately.

Statistical analysis

Statistical significance of differences in ffLUC activity, endogenous mRNA transcript levels, IAA levels and plant growth parameters were determined by Student's *t*-test ($p<0.05$).

Results

Diurnal activity of key ffLUC reporters for plant growth

In order to test whether a diurnal transcriptional response of key genes involved in growth regulation (e.g. *PIFs* and *HY5*) can be used to assess the long-term effect of short-term light treatments on growth, different Arabidopsis luciferase reporter lines were used. The integral activity of PIF4 and PIF5, including post-transcriptional regulation, may be monitored in the transcriptional response of PIF4/5 target genes such as *IAA29*, which is involved in auxin signaling (Kunihiro et al., 2011; Sun et al., 2013; Leivar and Monte, 2014). As key regulator of the suppression of elongation the activity of transcription factor HY5 was selected (Kami et al., 2010; Lau and Deng, 2010; Leivar and Quail, 2011; Gangappa and Botto, 2016). To monitor the transcriptional regulation of *PIF*s, *HY5* and *IAA29* we obtained *PIF4::LUC* reporter lines (López Salmerón, 2013) and constructed our own *PIF3::LUC*, *PIF5::LUC*, *IAA29::LUC* and *HY5::LUC* reporter lines using approximately 1-3 kb promoter fragments upstream from the transcription start site (detailed description in Material and Methods). For each construct several independent transformed Arabidopsis lines were obtained, and one representative line was chosen to

make homozygous for the ffLUC reporter to be used for further research. For each reporter line the diurnal ffLUC reporter activity was measured in 4-week-old Arabidopsis rosette plants. Plants were pre-grown in growth cabinets with fluorescent lights under a diurnal 12hL/12hD cycle and subsequently transferred to LUMINATOR for ffLUC activity measurements. LUMINATOR is a custom-build growth cabinet with LED light controls and a sensitive camera for ffLUC activity imaging (Chapter 2 of this thesis). Plants in LUMINATOR are grown under 12hL/12hD cycles and illumination was provided by red (R), blue (B) and far red (FR) LEDs. To crudely mimic natural changes in light intensity and quality at dawn and dusk (Supplemental Figure S1, left panels), a light ramping interval is used with reduced light intensities of blue and red during the first and last hour of each photoperiod (Supplemental Figure S1 right panels). The spectra of light conditions in LUMINATOR are shown in Supplemental Figure S2. The effect of light ramping on total light intensity and phytochrome photostationary state (PSS) is given in Supplemental Figure S1C. A schematic representation of the default light conditions used in LUMINATOR are shown in Figure 2 (top panel). During ramping PSS=0.75 compared to PSS=0.82 during the remaining hours of the photoperiod (Supplemental Figure S1C). Plants were acclimatized to growth in LUMINATOR for one day before start of ffLUC image acquisition every 30 minutes (7 minutes acquisition time). To evaluate the reproducibility of the reporter gene activities over the day plants were imaged for three subsequent days under 12hL/12hD cycles at constant 22°C. Representative ffLUC activity images of the different ffLUC reporter plants at ZT=0 to ZT=14 are shown in Supplemental Figure S4. The ffLUC activity in each reporter plant was quantified, corrected for background activity, and the average diurnal ffLUC activity of each set of six identical reporter plants is plotted in Figure 3A.

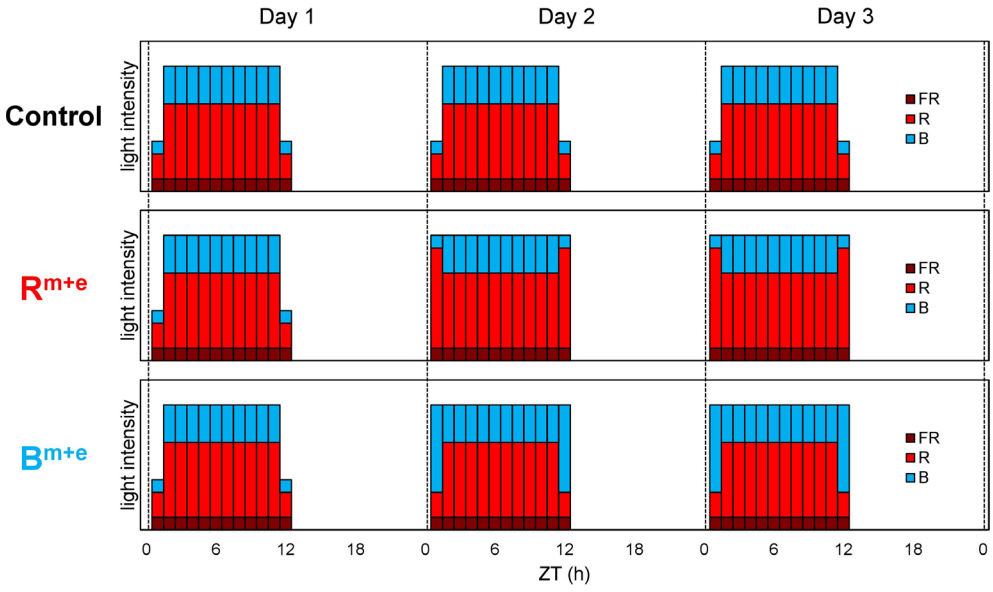


Figure 2: Schematic representation of light treatments in LUMINATOR. Upper panel: Light conditions control experiment (every day 1h light ramp during first and last hour of photoperiod). Middle panel: light conditions additional red light (R^{m+e}) experiment. Lower panel: light conditions additional blue light (B^{m+e}) experiment. Dark red bars represent light intensity far red (FR) LEDs, red bars light intensity red (R) LEDs and blue bars light intensity blue (B) LEDs. Max. light intensity is 100 µmole m^{-2} s^{-1}.

Diurnal ffLUC reporter activity

The activity of *PIF3::LUC* and *PIF4::LUC* and *PIF5::LUC* reporters show regular patterns over the three days (Figure 3A). The phase of *PIF4::LUC* and *PIF5::LUC* expression is at the end of the photoperiod, while the phase of *PIF3::LUC* expression is at the beginning of the night. This pattern is similar to endogenous *PIF3-5* expression in Arabidopsis Col seedlings grown under 12hL/12hD cycles (Mockler et al., 2007). In contrast, the diurnal activity of *IAA29::LUC* and *HY5::LUC* are much more irregular, with two transient peaks in ffLUC activity during the day (at ZT(h)=2.5 and 8) (Figure 3A). These transient peaks during the day relate to the two timepoints at which plants are sprayed with the substrate luciferin (see Material and Methods and Chapter 2 of this thesis) and suggest a sensitivity of especially the *IAA29* promoter activity to application of luciferin. Although *IAA29* is a target of PIF4 and PIF5, the expression profile of *IAA29::LUC* does not correlate well with that of *PIF4::LUC* and *PIF5::LUC*. During the day *PIF4*/*5* expression may be limited by degradation of the PIF proteins through interaction with PHY Pfr. The induction at start of the night may be explained by a rapid increase in PIF4/5 protein stability in the dark. In addition, other factors may be involved in the transcriptional regulation of *IAA29* (Figure 3A).

Integral ffLUC reporter activity during day and night

The overall elongation response as function of *PIF* transcription may be limited during the day as PIFs are destabilized through interaction with red light-activated PHYB (Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018). Similarly, transcription of *HY5* during the night may be less relevant as HY5 protein is degraded in the absence of light through interaction with the COP1-SPA complex (Osterlund et al., 2000; Saijo et al., 2003; Zhu et al., 2008). Therefore, total ffLUC reporter activity was quantified separately for each day and night period, and normalized to the integral ffLUC activity for the day period of the first day (Figure 3B). These analyses show a strong diurnal regulation of *PIF4::LUC* activity, with high transcriptional activity integrated over the day, and low activity integrated over the night. In contrast, activities of *PIF5::LUC* and *PIF3::LUC* are much more equal over the day and night period. The integral activity of *HY5::LUC* during the day is slightly higher than during the night (Figure 3B), consistent with a positive feedback of stable HY5 protein during the day on *HY5* transcription (Abbas et al., 2014; Binkert et al., 2014).

Response to light ramping interval at start and end day

The ffLUC activity of the different reporters during the light-dark and dark-light transitions was analyzed in more detail. For this, the activities during the first two hours of the day or the night (around the light ramping) were normalized to ZT(h)=0 and ZT(h)=11, respectively, and are plotted in detail in Figure 3C. For comparison, the activity during the first day ramping intervals were compared to the activity during ramping intervals of the third day. Results show that the expression profile of the different reporters is more or less the same for the first and third day, except for *PIF4::LUC* activity, which is slightly stronger induced at day 3 (Figure 3C). The *PIF4::LUC* activity is strongly induced during light ramping after dark, but not during ramping at the end of the photoperiod or during the light-dark transition. In contrast to *PIF4::LUC*, the *PIF5::LUC* is initially downregulated during light ramping at dawn, and *PIF5::LUC* does not show a strong response to the light ramping at end of the day. However, *PIF5::LUC* is strongly induced, like *PIF4::LUC*, under the full light conditions of the day (Figure 3A+C), suggesting that induction of *PIF5::LUC* may be more susceptible to PSS. Activity of *PIF3::LUC* is also initially downregulated during light ramping at dawn, but does not appear sensitive to the changes in light conditions from ramping to full light (Figure 3C). Activity of *IAA29::LUC* shows no response to the morning or evening light ramping conditions, but *IAA29::LUC* shows a strong increase in expression after the light-dark transition. Thus, also this fine analysis of *IAA29::LUC* does not show strong correlation with the activity of the genes that encode its putative direct regulators: PIF4 and PIF5. The *HY5::LUC* reporter shows an initial small downregulation in activity during dawn ramping, and is subsequently strongly induced under full light during the day, while *HY5::LUC* does not respond during light ramping at the end of the day (Figure 3C).

Results show that our system allows for the dissection of short-term fine details in transcriptional response to changing light conditions (ramping) during the day and for the quantification of the long-term integral day/night activity of the different key genes. Already under these default conditions the different key genes linked to plant growth show different light regulation. Therefore, we next tested how these different key genes respond to an additional R light pulse given during the ramping light period at dawn and dusk.

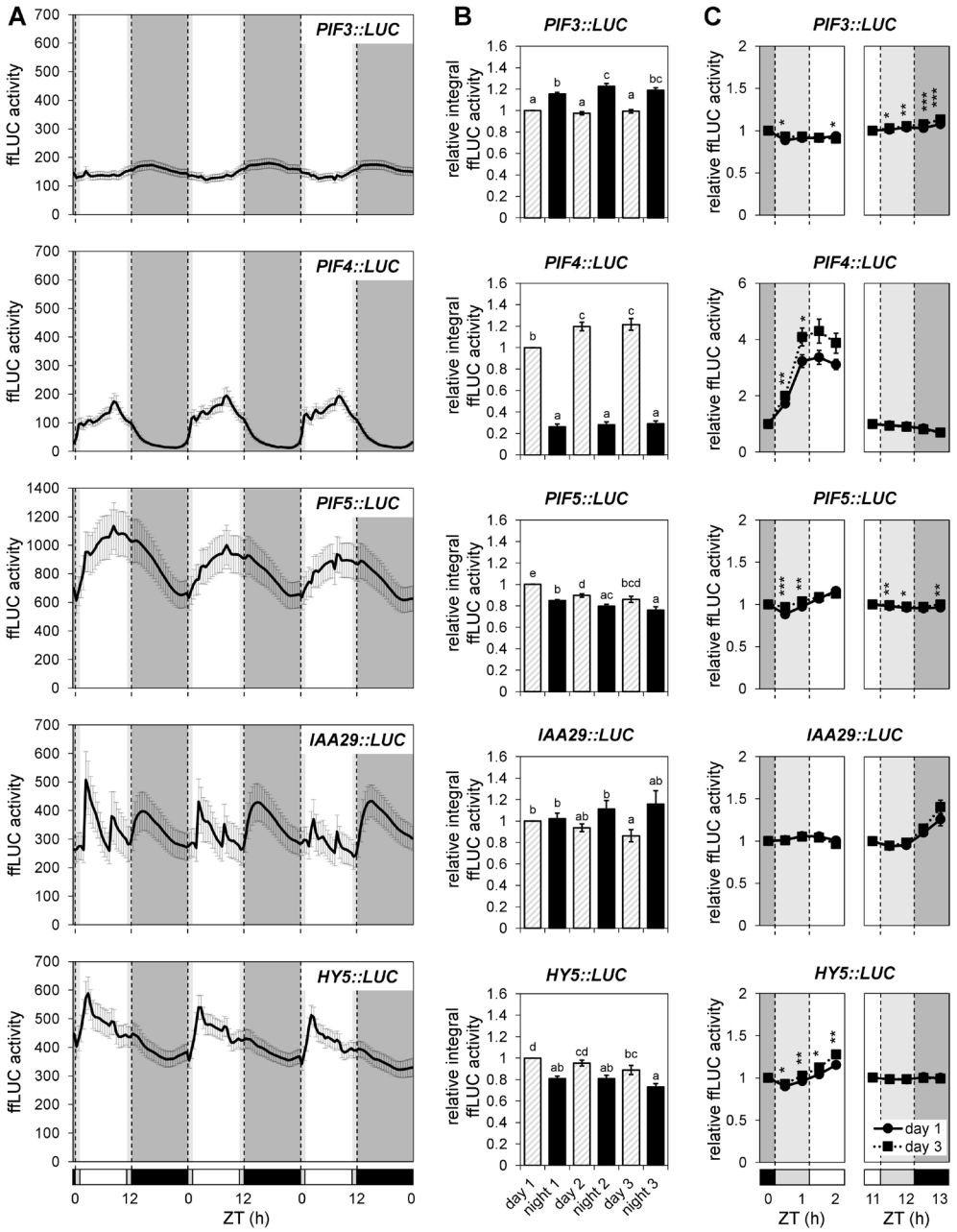


Figure 3: Activity of ffLUC reporters for key regulators of plant growth. A: Diurnal ffLUC activity profile of 4-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Data are mean luminescence ± SE (n=6), measured every 30 min. **B**: Graphs show ffLUC activity of reporters in A, integrated over day (white/grey bars) or night period (black bars), relative to integrated ffLUC activity over day period of the first day. Data are mean relative integral luminescence ± SE (n=6). **C**: Graphs show ffLUC activity of reporters in A during start day (left panels) and end day (right panels) of

day 1 and 3, relative to ffLUC activity at ZT(h)=0 or ZT(h)=11. Data are mean relative luminescence ± SE (n=6). Data Figure B and C were calculated using data day 1 and 3 shown in Figure A. In Figure A and C, dark grey graph areas and black bars underneath graphs represent dark period, light grey areas and bars represent light ramping (30 µmole m^{-2} s^{-1}; R:FR=2) and white areas and bars represent day light (100 µmole m^{-2} s^{-1}; R:FR=6). Different letters in B indicate significant differences in integral ffLUC activity ($p<0.05$, Student's t-test). Asterisks in A indicate significantly different from relative integral ffLUC activity on day 1 (*$p<0.05$, **$p<0.01$, ***$p<0.001$, Student's t-test).

Effect of added R light pulse at dawn and dusk on ffLUC reporter activity

Additional R light results in an increased R:FR light ratio and PSS, which leads to increased levels of PHY in the active Pfr form. Therefore, additional R light, may be effective in further suppression of PIF protein activity at the onset of the day or, when given at end-of-day, may be effective in suppression of PIF protein activity during the night, both of which may be reflected in expression of the PIF4/5 target *IAA29*. To test the effect of an additional R light treatment on transcriptional activity of the different reporters, 4-week old plants were adapted for one day to default light conditions in LUMINATOR. Subsequently, the ffLUC activity was imaged for one day under default light conditions including ramping (control light treatment), followed by two days under default light condition plus additional one-hour R light pulse of 60 µmole m^{-2} s^{-1} during the morning and evening (R^{m+e} treatment). A schematic representation of the light conditions during the R^{m+e} experiment in LUMINATOR is shown in Figure 2 (middle panel). The effect of added R light during ramping on total light intensity and PSS is given in Supplemental Figure S1. The direct transcriptional response during the added R light pulse treatment (normalized to expression before R light pulse) is shown in Figures 4A+C and the average integral ffLUC activity for the day and night period (normalized to expression without R light pulse) is shown in Figure 5. The full ffLUC activity profiles over the three days can be found in Supplemental Figure S5A.

Direct transcriptional responses to R^{m+e} treatment

To compare the short-term transcriptional response of the different reporters to additional R light, the relative ffLUC activity at the start (normalized to ZT(h)=0; Figure 4A) and end (normalized to ZT(h)=11; Figure 4C) of the day under R^{m+e} treatment conditions was compared with the relative ffLUC activity under control conditions. Results show that activity of the *PIF::LUC* and *HY5::LUC* reporters is not strongly responsive to the altered R:FR ratio during the R^{m+e} treatment, both at dawn and at dusk. The *IAA29::LUC* activity shows the strongest response to the R light pulse given at dusk. While expression of *IAA29::LUC* is not affected during the R light pulse, expression is no longer upregulated at the beginning of the dark period (Figure 4C). This suggests increased levels of PHY Pfr at the onset of the night, resulting in higher turnover of PIF4/5 protein and lower expression of their target gene *IAA29*.

To study whether changes in promoter activity, as shown by ffLUC reporter activity, correspond to changes in endogenous gene expression, the transcript levels of the corresponding endogenous genes in Arabidopsis Col-0 WT plants were measured by

qRT-PCR at selected time points (Figure 4B+D). Results show that for both control and R^{m+e} light treatment, endogenous gene expression shows stronger up- or downregulation during ramping than the corresponding ffLUC reporters. However, consistent with the response of the ffLUC reporters, the mRNA transcript levels of endogenous *PIF3*, *PIF4*, *PIF5* and *HY5* in morning and evening were not strongly affected by the R^{m+e} light treatment. The endogenous *IAA29* gene shows upregulation at dawn without R light pulse, but downregulation at dawn with R light pulse (Figure 4B), which is not reflected in the *IAA29::LUC* activity. At dusk, endogenous *IAA29* expression shows strong upregulation during ramping, which is absent when a R light pulse is given at dusk (Figure 4D). These discrepancies between ffLUC reporters and corresponding endogenous gene mRNA transcript levels have been shown before and are related to a general delay of about 1.5 hours between the phase of endogenous gene activity and the phase in ffLUC reporter activity (Chapter 2 of this thesis).

Integrated ffLUC reporter activity in response to R^{m+e} treatment

The shift in phase of expression between ffLUC reporter and endogenous gene may be less relevant when activity is integrated over the full day or full night. To determine the long-term effect of the added R light pulses, the integral relative ffLUC activity for the third day- and night-period under R^{m+e} (Figure 5; red bars) was compared to the integral relative ffLUC activity for the same periods under control conditions (Figure 5; black bars). This comparison shows that overall activity of *PIF4::LUC* is not significantly affected by the R^{m+e} treatment, activity of *PIF5::LUC* is increased both during the day and night, while *PIF3::LUC* activity is only slightly decreased during the night under the R^{m+e} treatment (Figure 5). The strongest response to additional R light was observed for the *IAA29::LUC* reporter. In line with the strong direct response to the added R light in the evening, integral activity of *IAA29::LUC* during the night was strongly reduced by the R^{m+e} treatment, while activity during the day was not significantly affected (Figure 5). This is consistent with PIF protein activity being reduced by the added R light during the end of day and PIF4/5 protein targeting the *IAA29* promoter. The decrease in night-time integral *IAA29::LUC* activity is caused by the absence of the transient peak in *IAA29::LUC* activity during the night that is observed under control conditions (Figure 3A), but not when additional R light is provided (Supplemental Figure S5A). These results suggest that the level of PIF proteins during the night is determined by the (relative) amount of R light provided at the end of the day. The integral *HY5::LUC* activity is slightly reduced during the day period, but not affected for the night period (Figure 5). Overall, the integral transcriptional response of the growth-related transcription factor genes show conflicting results for the prediction of long term growth responses to the R^{m+e} treatment: decreased *IAA29* and *PIF3* activity during the night could potentially reduce elongation at night, while decreased *HY5* activity during the day could potentially result in increased elongation during the day and increased *PIF3* activity during the night could potentially increase elongation at night.

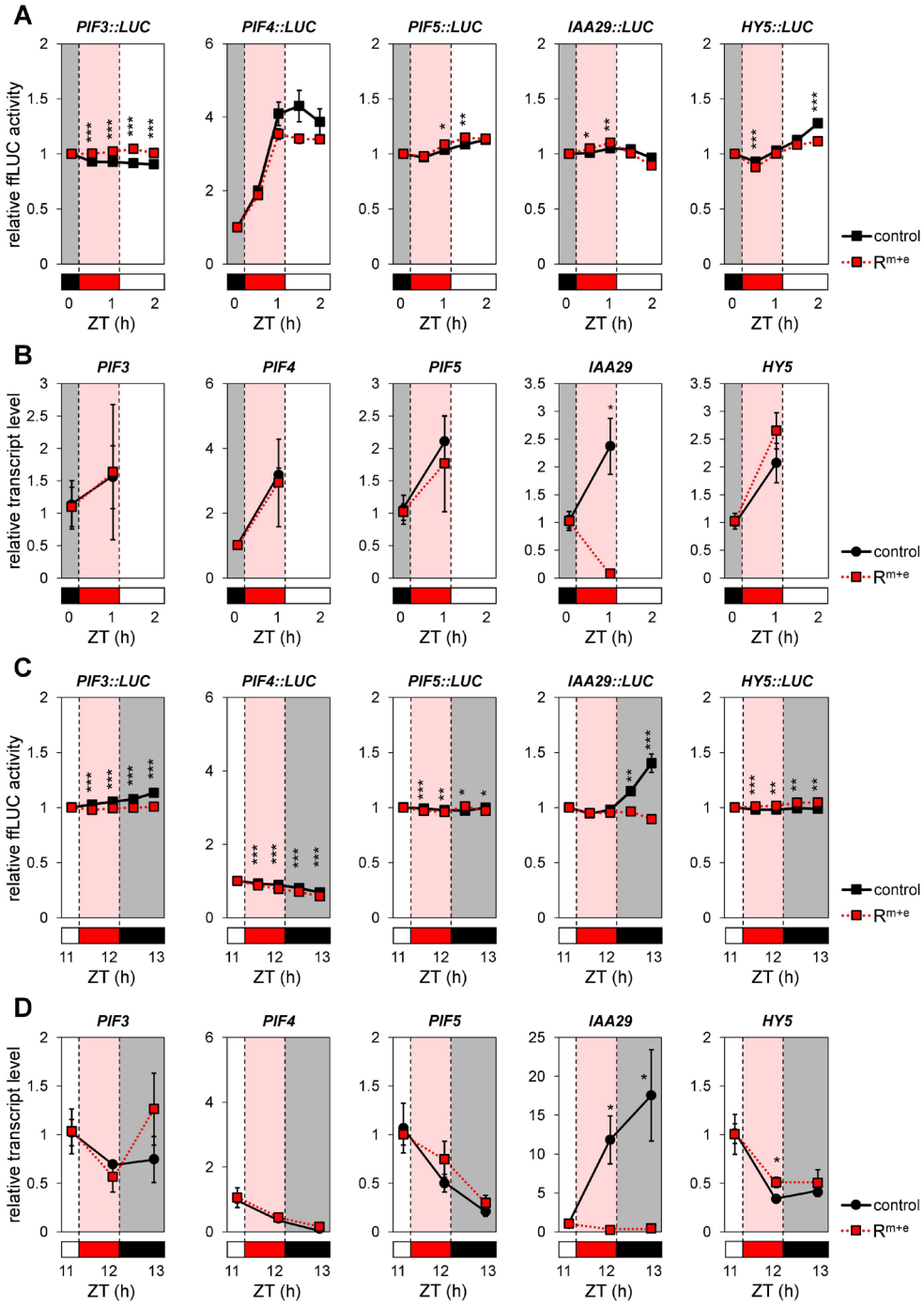


Figure 4: Direct transcriptional response to additional red light at dawn and dusk. Four-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control

treatment). Alternatively, additional red light (60 µmole m^{-2} s^{-1}) was provided during the ramping hours on the second and third day (R^{m+e} treatment). **A+C**: Graphs show activity of ffLUC reporters at start (**A**) and end (**C**) of the photoperiod on the third day, under control conditions (black squares, solid line) or R^{m+e} conditions (red squares, dashed line), relative to ffLUC activity at ZT(h)=0 or ZT(h)=11. Data are mean relative luminescence ± SE (n=6) calculated from data Figure 3A and Supplemental Figure S5A. **B+D**: Relative *PIF3*, *PIF4*, *PIF5*, *IAA29* and *HY5* mRNA transcript levels at start (**B**) and end (**D**) of the photoperiod on the third day in Col-0 WT plants under control conditions (black squared, solid lines) or R^{m+e} conditions (red squares, dashed lines). Data are mean relative transcript levels ±SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780) and normalized to ZT(h)=0 or ZT(h)=11. Dark grey graph areas and black bars underneath graphs represent dark period, red areas and bars represent ramping with additional red light (100 µmole m^{-2} s^{-1}; R:FR=8) and white areas and bars represent day light (100 µmole m^{-2} s^{-1}; R:FR=6). *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative luminescence or transcript levels during control conditions.

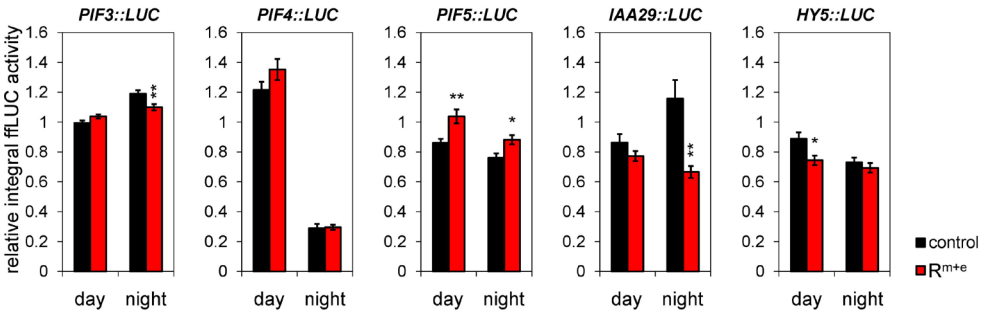


Figure 5: Effect of additional red light treatment on integral ffLUC reporter activity. Four-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control treatment). Alternatively, additional red light (60 µmole m^{-2} s^{-1}) was provided during the ramping hours on the second and third day (R^{m+e} treatment). Graphs show ffLUC activity of reporters under control conditions (black bars) or R^{m+e} conditions (red bars), integrated over day or night period of the third day, relative to integrated ffLUC activity over day period of the first day. Data are mean relative integral luminescence ± SE (n=6) calculated from data Figure 3A and Supplemental Figure S5A. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative integral ffLUC activity under control conditions.

Effect of added B light pulse at dawn and dusk on ffLUC reporter activity

The potential effect of added R light on elongation is through phytochromes, which (once activated) negatively regulate PIF protein stability. However, B light can also affect PIF protein activity, as B light-activated CRY1 blocks transcriptional activity of PIF4 (Ma et al., 2016), while CRY2 is also able to interact with PIFs (Pedmale et al., 2016). In addition, B light may indirectly affect transcription of *PIF*s and PIF target genes by increased HY5 protein accumulation and binding of HY5 at *PIF* and PIF target promoters (Lian et al., 2011; Liu et al., 2011; de Wit et al., 2016; Lian et al., 2018). At these promoters, HY5 competes for promoter binding sites with PIFs, potentially blocking transcription of *PIF*s and PIF target genes (Lee et al., 2007; Delker et al., 2014; Gangappa and Kumar, 2017).

An additional B light pulse may therefore be a good way to enhance suppression of plant elongation in greenhouses. The effect of added B light at dawn and dusk on transcriptional regulation of *PIFs, IAA29* and *HY5* was studied by providing additional 55 µmole m^{-2} s^{-1} of B light for one hour during morning and evening (B^{m+e}). A schematic representation of the light conditions during the B^{m+e} experiment in LUMINATOR is shown in Figure 2 (lower panel). The effect of added B light during light ramping on total light intensity and PSS is given in Supplemental Figure S1. The B^{m+e} treatment does not affect the R:FR ratio, but it does result in a slight decrease in PSS and increase in total light intensity during the ramping light period (Supplemental Figure S1). The response of ffLUC reporter activity and corresponding endogenous gene expression to the B^{m+e} light treatment was analyzed as described for the R^{m+e} light treatment. The diurnal ffLUC profiles of the different reporters under one day of control conditions followed by two days of B^{m+e} treatment is shown in Supplemental Figure S5B.

Direct transcriptional responses to B^{m+e} treatment

The direct transcriptional response of the different reporters to the B^{m+e} treatment was also analyzed in more detail and compared to the transcriptional response of the corresponding endogenous gene (Figure 6). Results show that, in response to the B^{m+e} light treatment, the endogenous gene expression is often stronger up- or downregulated at the start or end of the photoperiod than the corresponding ffLUC reporters, due to a delay in changes in ffLUC activity compared to changes in endogenous gene activity. This is similar to results of the R^{m+e} light treatment experiment (Figure 4). The *PIF::LUC* reporters show little response during the period of added B light in the morning, but a decrease in activity immediately following added B light, especially *PIF4::LUC* (Figure 6A). Analysis of the corresponding endogenous *PIF* gene expression indicates that expression of all *PIF*s is directly downregulated in response to added B light (Figure 6B). The *IAA29::LUC* reporter shows little response to added B light in the morning, but the endogenous *IAA29* gene is downregulated in response to added B light (Figure 6A+B). This is in agreement with *IAA29* being target of PIF activity. The *HY5::LUC* activity is increased after added B light in the morning, while expression of the endogenous *HY5* gene shows strong direct response to added B light at dawn, again indicating the delay between ffLUC reporter and corresponding endogenous gene activity (Figure 6A+B). Most reporters do not show a strong response in ffLUC activity to added B light at the end of the day (Figure 6C). In agreement with this, expression of endogenous *PIF*s and *IAA29* was not affected by added B light at the end of the day (Figure 6D). Only *HY5* expression shows an induction in response to added B light, which is abolished in the dark. This transient activity of *HY5* is not observed in the *HY5::LUC* activity (Figure 6C+D).

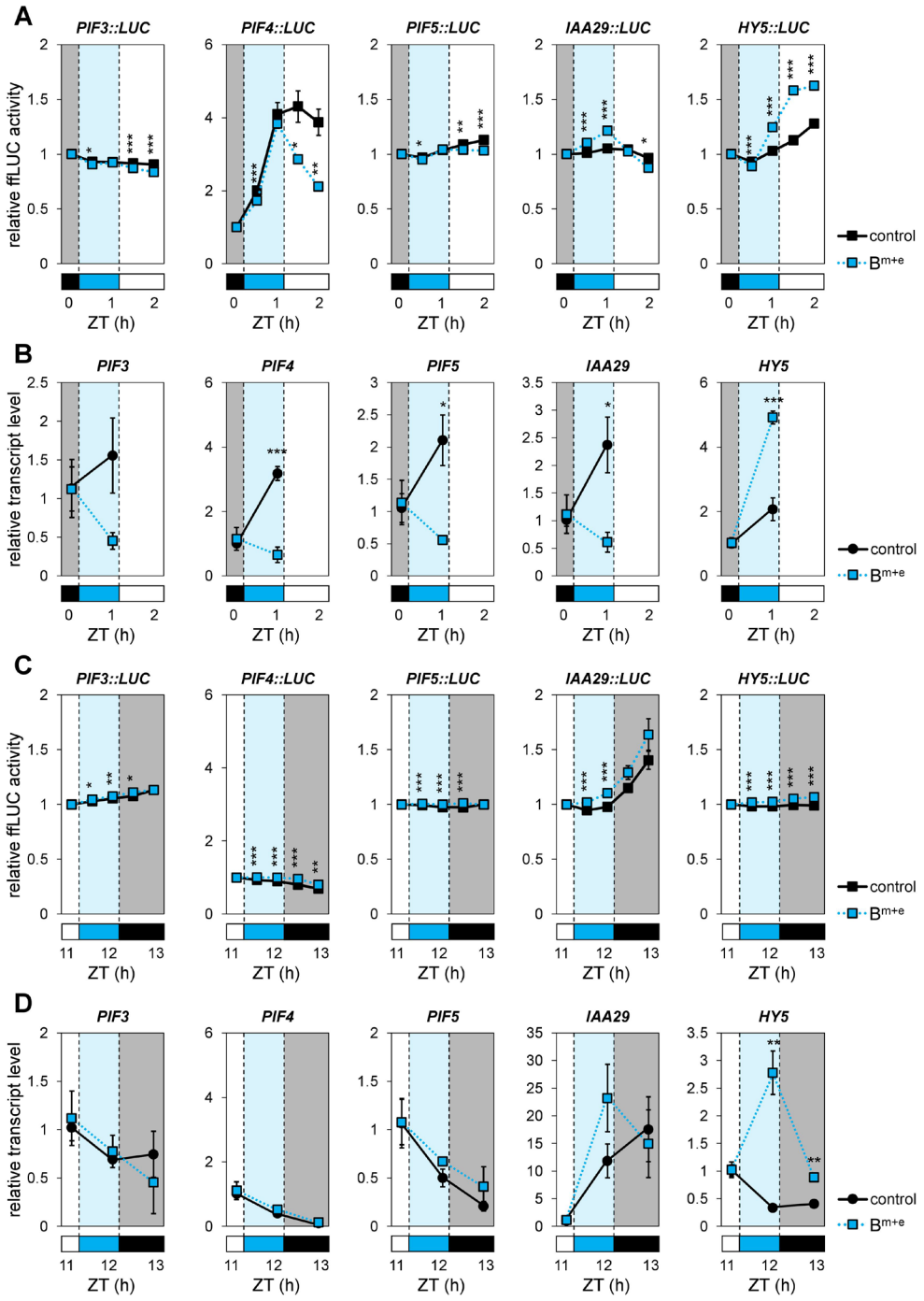


Figure 6: Direct transcriptional response to additional blue light at dawn and dusk. Four-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control

treatment). Alternatively, additional blue light (55 µmole m^{-2} s^{-1}) was provided during the ramping hours on the second and third day (B^{m+e} treatment). **A+C**: Graphs show activity of ffLUC reporters at start (**A**) and end (**C**) of the photoperiod on the third day, under control conditions (black squares, solid line) or B^{m+e} conditions (blue squares, dashed line), relative to ffLUC activity at ZT(h)=0 or ZT(h)=11. Data are mean relative luminescence ± SE (n=6) calculated from data Figure 3A and Supplemental Figure S5B. **B+D**: Relative *PIF3*, *PIF4*, *PIF5*, *IAA29* and *HY5* mRNA transcript levels at start (**B**) and end (**D**) of the photoperiod on the third day in Col-0 WT plants under control conditions (black squared, solid lines) or B^{m+e} conditions (blue squares, dashed lines). Data are mean relative transcript levels ±SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780) and normalized to ZT(h)=0 or ZT(h)=11. Dark grey graph areas and black bars underneath graphs represent dark period, blue areas and bars represent ramping with additional blue light (100 µmole m^{-2} s^{-1}; R:FR=8) and white areas and bars represent day light (100 µmole m^{-2} s^{-1}; R:FR=6). *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative luminescence or transcript levels during control conditions.

Integrated ffLUC reporter activity in response to B^{m+e} treatment

Figure 7 shows the average relative integral ffLUC reporter activity during day and during night for plants that received the B^{m+e} treatment (blue bars), compared to relative integrated ffLUC activity in plants grown under control conditions (black bars). Results show that the integral ffLUC activities for the *PIF3*, *PIF5* and *IAA29* reporters are not affected by the B^{m+e} treatment. The integral activity of *PIF4::LUC* is slightly decreased during the day in response to the B^{m+e} treatment (Figure 7). However, since PIF proteins may not be stable in the light, these changes in *PIF4* transcription during the day may not contribute strongly to elongation responses. In contrast, the integral *HY5::LUC* activity was significantly increased by the B^{m+e} treatment (Figure 7), both in dark and in light. Changes in *HY5* transcription at night may be less relevant due to COP1 targeting HY5 for degradation in the dark, but the increased *HY5* transcription could indicate a suppression of elongation under the B^{m+e} treatment.

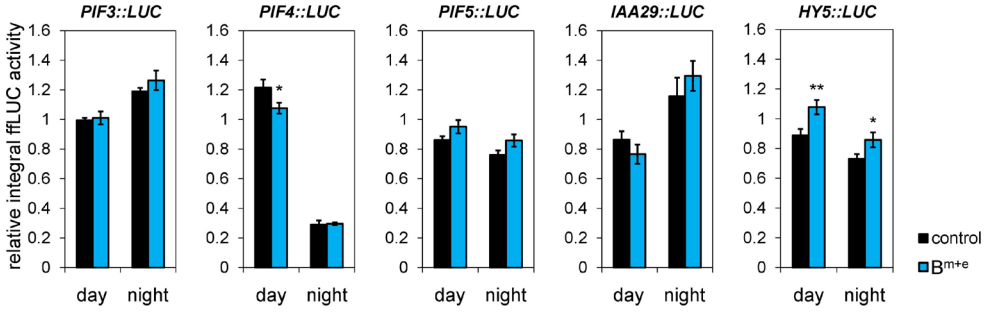


Figure 7: Effect of additional blue light treatment on integral ffLUC reporter activity. Four-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control treatment). Alternatively, additional blue light (55 µmole m^{-2} s^{-1}) was provided during the ramping hours on the second and third day (B^{m+e} treatment). Graphs show ffLUC activity of reporters under control conditions (black bars) or B^{m+e} conditions (blue bars), integrated over day or night period of the third day,

relative to integrated ffLUC activity over day period of the first day. Data are mean relative integral luminescence ± SE (n=6) calculated from data Figure 3A and Supplemental Figure S5B. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative integral ffLUC activity under control conditions

Plant growth responses to added the R or added B treatments

The effect of the R^{m+e} treatment on *PIF* and *HY5* activity is relatively small, while the effect on integral *IAA29* activity during the night is substantial. Therefore, overall prediction could be that elongation is reduced under prolonged R^{m+e} treatment. The B^{m+e} treatment results in a significant increase in integral *HY5* activity during the day and this could be predictive for a suppression of elongation in plants under B^{m+e} treatment. These predictions from transcriptional responses of the model plant Arabidopsis under the very basic artificial light conditions used in LUMINATOR were subsequently tested for crop plants grown in SOLINATOR. Tomato has the full complement of genes involved in elongation that have been characterized in Arabidopsis (*PIF*s, *HY5*, *IAA29*) and we assume that tomato PIFs and HY5 play a similar role in elongation in tomato as they do in Arabidopsis (Rosado et al., 2016; Batista-Silva et al., 2019; Hwang et al., 2019). The effect of added R or B light pulses on growth was tested for tomato (*Solanum lycopersicum* cv. 'Money maker') seedlings in SOLINATOR, where plants are grown under a LED array which mimics natural sunlight in combination with diurnal temperature cycles (12hL;25°C/12hD;15°C). Plants were grown for two weeks without added R or B light, or with added one-hour R or B light at start- and end-day (R^{m+e} and B^{m+e} treatments, respectively; SOLINATOR light conditions in Supplemental Figure S1). At the end of the two weeks hypocotyl length and leaf area were measured. Results show that tomato seedlings subjected to the R^{m+e} treatment are shorter than seedlings grown under control conditions (Figure 8A), while leaf area is not significantly affected (Figure 8C). This is consistent with the prediction based on the transcriptional analysis of key growth genes in Arabidopsis. In contrast, the B^{m+e} light treatment had no significant effect on stem elongation or on leaf area (Figure 8B+D), indicating that the higher expression of *HY5* during the day in Arabidopsis does not translate to reduced elongation in tomato.

Ultimately, the effect of added R or B light in morning and evening on growth of tomato plants was also tested in a greenhouse setting. For the greenhouse experiments 14-day-old tomato plants were used. Plants were grown for two weeks under natural photoperiod (10h54m-13h04m) or under natural photoperiod supplemented for one hour with additional R or B light at the start and end of each photoperiod (R^{m+e} and B^{m+e} treatments, respectively). Average temperatures were around 25°C during the day and around 17-18°C during the night (Supplemental Figure S3). This experiment was repeated once at a different time of the year. In the first experiment the light intensity of added R or B light was at 61 µmole m^{-2} s^{-1} (experiment 1), while in the second experiment the added R or B light had an intensity of 37 µmole m^{-2} s^{-1} (experiment 2). Because of the naturally changing photoperiods and daily variations in light intensity, the average Daily Light Integral (DLI) during the first experiment was lower than during the second experiment (Supplemental Figure S3C and Supplemental Table S3). The R^{m+e} light treatment in the

greenhouse results in an increase in plant length and leaf area for tomato plants in experiment 1 (average DLI= 3.30 mole m^{-2} day^{-1}, added R= 61 µmole m^{-2} s^{-1}), but results in reduced plant length and no change in leaf area in experiment 2 (average DLI= 4.67 mole m^{-2} day^{-1} with added R= 37 µmole m^{-2} s^{-1}) (Figure 8A+C). When additional B light is given in morning and evening this resulted in decreased plant length, both under additional B light with an intensity of 61 or 37 µmole m^{-2} s^{-1} (Figure 9B). The results for growth of tomato under B^{m+e} treatment conditions are consistent with the prediction based on transcriptional response of HY5 to the B^{m+e} treatment in Arabidopsis. The leaf area was only increased in response to added B light of higher intensity (Figure 9D). Effects on tomato shoot DW for the R^{m+e} and B^{m+e} treatments are shown in Supplemental Figure S6.

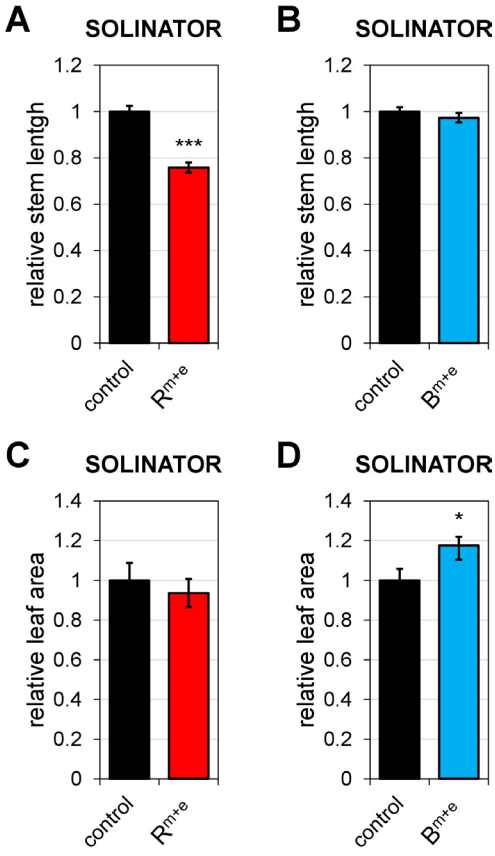


Figure 8: Effect of additional red/blue light treatment on growth of tomato in SOLINATOR. Relative stem length (**A**+**B**) and leaf area (**C**+**D**) of 22-day-old tomato (*Solanum lycopersicum* cv. 'Money maker') plants that were grown for two weeks in SOLINATOR cabinets under 12hL 25°C / 12hD 15°C cycles. Light in SOLINATOR was provided by LED artificial sunlight research modules, mimicking naturally occurring daily variations in sunlight intensity and quality. Daily Light Integral was 4.32 mole m^{-2} d^{-1}. Plants received one hour of additional red or blue light (50 µmole m^{-2} s^{-1}) at both the start and end of the photoperiod (R^{m+e} and B^{m+e} treatment, respectively) or they received no additional light (control treatment). Data are mean stem length/leaf area, normalized to stem length/leaf area of plants grown under control conditions (n=15) ± SE. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from plants grown under control conditions.

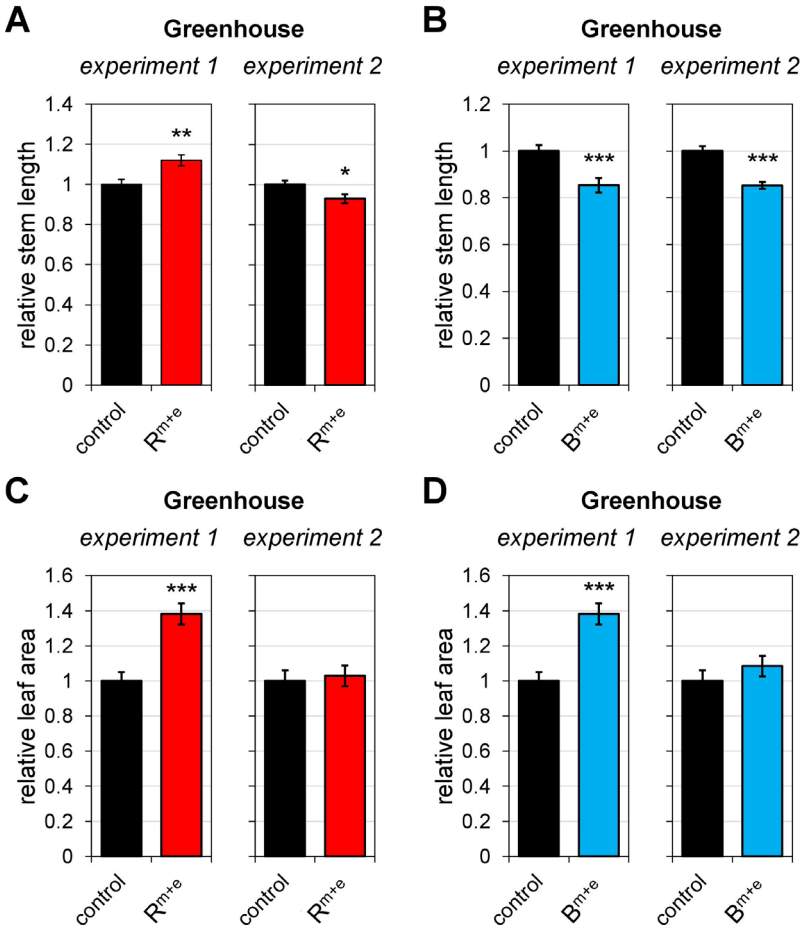


Figure 9: Effect of additional red/blue light treatment on growth of tomato in greenhouse. Relative stem length (**A**+**B**) and leaf area (**C**+**D**) of 28-day-old tomato (*Solanum lycopersicum* cv. 'Money maker') grown for two weeks in greenhouse compartments under natural photoperiods with day temperatures around 25°C and night temperatures around 17°C (see Supplemental Figure S3). Plants received one hour of additional red or blue light at the start and end of each photoperiod (R^{m+e} and B^{m+e} treatment, respectively) or they did not receive any additional light (control treatment). The experiment was repeated in time. Experiment 1 ran from March 1 until March 14, 2018. Experiment 2 ran from March 20 until April 2, 2018. Intensity of additional red/blue light was 61 µmole m^{-2} s^{-1} during experiment 1 and 37 µmole m^{-2} s^{-1} during experiment 2. Average Daily Light Integral (DLI) was 2.75-3.43 mole m^{-2} d^{-1} during experiment 1 and 4.26-4.66 mole m^{-2} d^{-1} during experiment 2. Data are mean stem length/leaf area, normalized to stem length/leaf area of plants grown under control conditions (n=24) ± SE. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from plants grown under control conditions.

Discussion

Discrepancies in *PIF4*/*5* expression and PIF4/5 target (*IAA29*) expression and auxin signaling activity (*IAA29*) and *PIF3* expression

Here we used Arabidopsis ffLUC reporter plants to assess the impact of short-term light treatments on transcriptional activity of some key genes in plant elongation responses. Results indicate that LUMINATOR can be used to detect the impact of R or B light pulses in morning and evening on both short term and integrated transcriptional response over full day or night period. For this we monitored the activity of *PIF4::LUC* and *PIF5::LUC*, and of a ffLUC reporter for the PIF4/5 target gene *IAA29* (Kunihiro et al., 2011; Sun et al., 2012). Since *IAA29* encodes an auxin signaling component and its expression is stimulated by high auxin levels (Reed, 2001; Lavy and Estelle, 2016; Pucciariello et al., 2018), the *IAA29::LUC* activity is both an indicator for PIF4/PIF5 protein activity, as well as a representative of auxin signaling activity. Using a *PIF3::LUC* reporter we also monitored activity of *PIF3*, which acts downstream of auxin signaling (Bours et al., 2015).

Results indicate that different *PIF* genes show different responses to the light treatments. For instance, integral *PIF5::LUC* activity was most responsive to added R light pulses (Figure 5), while integral *PIF4::LUC* activity was most responsive to added B light pulses (Figure 7). Genetic analysis places *PIF3* downstream of auxin signaling in the signal transduction pathway towards growth (Bours et al., 2015). However, the integral *PIF3::LUC* activity is only slightly reduced during the night in response to added R light pulses, while *IAA29::LUC* activity (representative of auxin signaling) is strongly reduced during the night in response to added R light pulses. Moreover, activity of the *PIF4::LUC* and *PIF5::LUC* reporters is not directly reflected in activity of the PIF4/5 target *IAA29*. This may be explained by extensive post-transcriptional regulation of PIF4/5 protein activity, which limits translation of *PIF* gene transcription into PIF target gene transcription.

PIF transcription directly responsive to B at dawn but not to R:FR

In contrast to PIF protein levels (Leivar and Quail, 2011; Leivar and Monte, 2014; Pham et al., 2018), our results show *PIF* transcription is not directly responsive to changes in R:FR ratio resulting from added R light (Figure 4). In contrast, *PIF* expression is directly responsive to added B light as *PIF3*/*4*/*5* mRNA levels are all downregulated by additional B light at dawn (Figure 6B). However, this is not strongly reflected by the *PIF::LUC* reporters, except for *PIF4::LUC* (Figure 6A). It could be that the promoter fragment used in the *PIF::LUC* reporters lack regulatory elements that are present in the full endogenous *PIF* genes (e.g. in 5' leader sequence or in introns). There is almost no transcriptional response of *PIF3*/*4*/*5* to added B light given at dusk (Figure 6C+D). This suggests that downregulation of *PIF* transcription at dusk may be fully controlled by interaction of the Evening Complex with *PIF* promoters (Nusinow et al., 2011).

IAA29 transcription directly responsive to R:FR at dawn and dusk and to B light at dawn

The absence of a direct transcriptional response of *PIF*s to added R light at dawn or dusk suggests that, at these time points, regulation of *PIF* transcription is not strongly controlled by phytochromes (Figure 4). This is in contrast to the PIF target gene *IAA29* (Figure 4), suggesting that effects of changes in R:FR on *IAA29* transcription is by post-transcriptional regulation of PIFs by phytochromes. Indeed, the *IAA29::LUC* activity profile under control conditions shows an increase at dawn, which is consistent with published results on endogenous *IAA29* expression in Arabidopsis, and can be explained by the increased *PIF4*/*5* transcription near the end of the night, confirming that *IAA29* is target of PIF4/5 (Figure 4A) (Mockler et al., 2007; Kunihiro et al., 2011; Sun et al., 2013; Seaton et al., 2015; Zhao et al., 2018). There is also a transient peak in *IAA29::LUC* activity at the beginning of the night under control conditions, suggesting a peak in PIF protein activity at the beginning of the night (Figure 3A). If the reduced R:FR during light ramping at dawn under control conditions (leading to reduced levels of PHY Pfr) is causal for the transient peak in *IAA29::LUC* activity (caused be increased PIF protein stability), then added R light is expected to increase PHY Pfr and reduce PIF protein activity at start of the night, resulting in reduced *IAA29* transcription. Indeed, added R light at dusk reduces the peak in *IAA29::LUC* activity at night (Supplemental Figure S5A). Regulation by R:FR near the end of the day is also observed in seedlings for the PIF target genes *IAA29* and *YUC8* (Mizuno et al., 2015). Recently it was shown that transcriptional activity of PIF proteins is suppressed towards the end of the day through the PSEUDO-RESPONSE REGULATORs 1 (TOC1), 5, 7 and 9 (PRR5, 7, 9) (Martin et al., 2018). The results of Mizuno *et al*. (2015) and results presented here suggest that this suppression is relieved by reduced R:FR at dusk. The level of B light, on the other hand, probably does not affect the regulation of PIF activity by TOC1 and PRR5/7/9, as additional B light did not lead to reduced *IAA29* transcription at dusk (Figure 6C+D). However, additional B light at dawn did lead to reduced *IAA29* transcription (Figure 6A+B). This may be the result of increased levels of B light-activated CRY1 negatively regulating transcriptional activity of PIF proteins (Ma et al., 2016).

Different response PIF4 targets *IAA29* and *YUC8*

In these experiments we used *IAA29::LUC* reporter plants as indicator of PIF protein activity. Another well-known PIF target is *YUC8*, encoding an enzyme for auxin biosynthesis (Sun et al., 2012). Although we constructed *YUC8::LUC* reporter plants, the ffLUC activity in these lines was too low to use in LUMINATOR. However, in the validation experiments, where we checked direct transcriptional responses of endogenous genes to the added R or added B light, we also analyzed *YUC8* transcription. Supplemental Figure S7 shows the results for the two PIF-induced targets *IAA29* and *YUC8*, which indicate that both genes are upregulated during the light ramping at dawn, and that this upregulation is more strongly suppressed by the added R light for *IAA29* than for *YUC8* (Supplemental Figure S7A). Moreover, while *IAA29* expression is upregulated during ramping at dusk and continues upregulation at onset of the night, expression of *YUC8* is

upregulated during ramping at dusk, but strongly downregulated at the onset of the night (Supplemental Figure S7C+D). For both genes the upregulation during ramping is abolished by the added R light at dusk (Supplemental Figure S7C). Added B light at dawn results in similar downregulation of expression of *IAA29* and *YUC8* (Supplemental Figure S7B), while added B light at dusk results in a more transient peak in *IAA29* activity, similar to the expression of *YUC8* (Supplemental Figure S7D). The difference in fine structure of the *IAA29* and *YUC8* transcription profile indicates that PIF protein activity is integrated differently for different PIF targets, suggesting there may be better PIF target reporters that link to elongation responses.

HY5 transcription direct responsive to B light at dawn but not to R light

Under control conditions, activity of *HY5::LUC* starts to increase towards the end of the night and peaks in the morning (Figure 3A). In response to additional B, but not R light, *HY5::LUC* activity is stimulated, leading to increased integral *HY5::LUC* activity during both day and night (Figure 6 and Figure 7). The direct transcriptional response of *HY5* to added B light is observed both at dawn and dusk, and therefore this response does not seem to be gated by the clock (Figure 6). Increased *HY5* transcription in response to higher levels of B light is in agreement with lower *HY5* transcription in response to lowered B light (de Wit et al., 2016). In contrast, *HY5* transcription is not directly responsive to added R light (Figure 4). Promoter binding activity of HY5, as well as HY5 protein stability were shown to be positively affected by B light (Osterlund et al., 2000; Saijo et al., 2003; Zhu et al., 2008; Lian et al., 2011; Liu et al., 2011; Lian et al., 2018). Since HY5 stimulates its own transcription (Abbas et al., 2014; Binkert et al., 2014), the increase in *HY5* transcription in response to added B light may be explained by increased HY5 protein and/or activity or reduced competition with transcriptional repressors at the *HY5* promoter.

Added R and added B light pulse treatments both reduce growth in tomato

The integral responses to the added R or added B light treatments were used to determine whether results obtained under the very basic artificial light conditions of LUMINATOR can be used to predict the long-term growth responses of plants to added light-pulse treatments, when plants are grown under a more realistic light spectrum as in SOLINATOR or greenhouses. The prolonged exposure to added R light pulses at dawn and dusk in SOLINATOR lead to a reduction in stem elongation of tomato seedlings (Figure 8A), while exposure to B light pulses at dawn and dusk did not affect tomato seedling hypocotyl length in SOLINATOR (Figure 8B). The strong reduction in integral *IAA29::LUC* activity at night in response to added R light pulses fits best with the reduced elongation under this treatment (Figure 5), confirming that *IAA29* is a better reporter for elongation activity than the individual *PIF*s. The small decrease in integral *PIF4::LUC* activity and increase in integral *HY5::LUC* activity during the day in response to added B light pulses (Figure 7) suggest an overall increased suppression of elongation. Although elongation of tomato seedlings was not affected by the added B light treatment in

SOLINATOR, elongation in tomato seedlings was suppressed by the added B light treatment in greenhouses in two independent experiments (Figure 9B).

Translating LUMINATOR results to growth responses to added R or B light pulses in greenhouse

Experimental data on the growth response of Arabidopsis to additional LED light treatments in SOLINATOR and greenhouses could unfortunately not be provided. However, additional experiments in LUMINATOR indicate that added R light pulses or added B light pulses given for three days, both result in reduced auxin levels in 4-week old Arabidopsis rosette plants (Supplemental Figure S8). As auxin is associated with elongation, this suggests that Arabidopsis plants exposed to added R or added B light could show reduced elongation compared to control conditions.

There are several obvious limitations in translating the results obtained under LUMINATOR to growth responses in SOLINATOR or in greenhouses. First of all, in LUMINATOR experiments are done at constant temperature due to the strong effect of temperature on LUC enzyme activity (Chapter 2 of this thesis). Both in SOLINATOR and greenhouse experiments a more realistic +DIF temperature regime was used (SOLINATOR: 25°C day, 15°C night; Greenhouse: ~25°C day, ~18°C night). As low temperature reduces the dark reversion of active PHY Pfr into inactive PHY Pr (Jung et al., 2016; Legris et al., 2016), this may amplify the effect of added R light on PIF protein activity at dusk compared to conditions in LUMINATOR. This temperature sensitivity of the added R light effect may be the cause of the different relative growth responses of tomato to control and added R light treatments during SOLINATOR (reduced plant growth) and greenhouse experiments (increased and reduced plant growth). Second, translating results from gene regulation in Arabidopsis to growth regulation in crop species such as tomato is only valid if regulation of elongation in the species is similar to that in Arabidopsis. Tomato has homologs of PIFs, IAA, YUC and HY5, but the role of these gene homologs in regulation of elongation responses in tomato is not well explored (Rosado et al., 2016; Batista-Silva et al., 2019; Wang et al., 2019). Growth responses of tomato in response to the light treatments tested here are not always consistent with predictions derived from LUMINATOR results on gene regulation. This suggests that tomato homologs of key growth-regulating components in Arabidopsis may not play exactly the same role in the regulation of elongation. Finally, the variable results obtained for tomato in response to added R light pulses in greenhouse in two experiments where temperature control was similar, indicates that a reduction of growth by added R light may only be effective when the intensity of the additional R light (relative to the DLI) is lower (Figure 9A; Supplemental Table S3). Thus, the effect of additional LED light treatments in greenhouses may depend on the overall light conditions in the greenhouse.

Supplemental Data

Supplemental Table S1: Primers used for cloning.

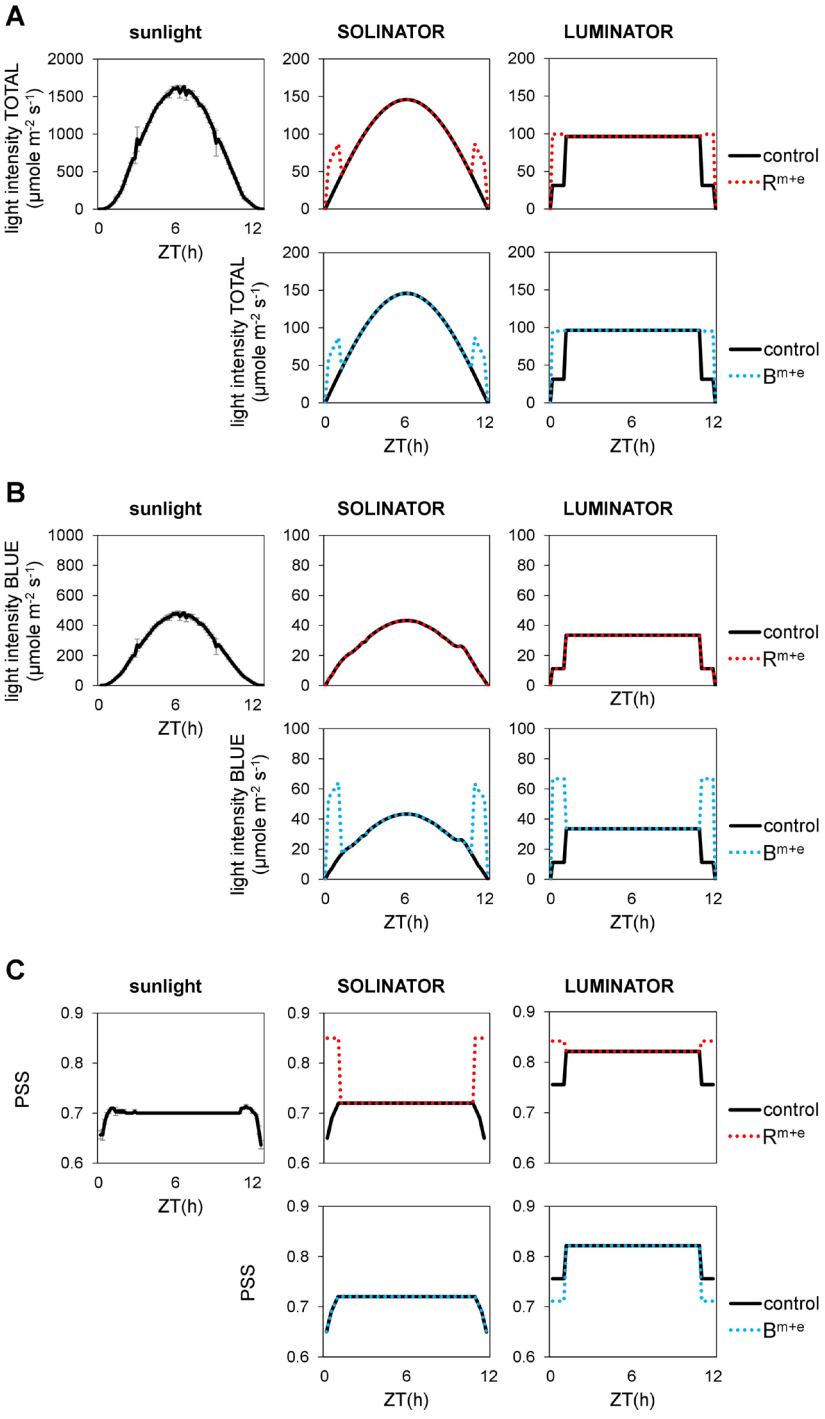
primer name	*sequence*
HY5 promoter fw	CACCTCTAATGTTAACGTTGAGATGGC
HY5 promoter rev	TTTTCTTACTCTTTGAAGATCGATCA
PIF3 promoter fw	CACCTCTTAAATCTACTTTTGACCCGT
PIF3 promoter rev	GGTGTTGCGTTTTACAGAAA
PIF5 promoter fw	CCACTTAGGGTTTTGACGTTTTGTTC
PIF5 promoter rev	GTCAGATCTGTAAAGACACTTAAATAAAG
IAA29 promoter fw	CACCAAATTGATCTGAAAATGTTGATGGG
IAA29 promoter rev	TTCTAAGGCAGCTTCGTCTTT

Supplemental Table S2: Primers used for qRT-PCR.

primer name	*TAIR id*	*sequence*
YLS8 fw	AT5G08290 (reference)	TTACTGTTTCGGTTGTTCTCCATTT
YLS8 rev	AT5G08290 (reference)	CACTGAATCATGTTCGAAGCAAGT
IPP2 fw	AT3G02780 (reference)	CATGCGACACACCAACACCA
IPP2 rev	AT3G02780 (reference)	TGAGGCGAATCAATGGGAGA
PIF3 fw	AT1G09530	CTGAAAGGAGACGGCGTGATAG
PIF3 rev	AT1G09530	CAGATAGTAACCAGACGCCATTGAC
PIF4 fw	AT2G43010	ACCTCAGCAGTTCATACGTCAG
PIF4 rev	AT2G43010	TGTACCGGGTTTTGGCAAAC
PIF5 fw	AT3G59060	GCTCCAAGCACAGAACCAAATC
PIF5 rev	AT3G59060	AACATGTCCGCTGGTTGTTG
IAA29 fw	AT4G32280	ATCACCATCATTGCCCGTAT
IAA29 rev	AT4G32280	ATTGCCACACCATCCATCTT
YUC8 fw	AT4G28720	TGTATGCGGTTGGGTTTACGAGGA
YUC8 rev	AT4G28720	CCTTGAGCGTTTCGTGGGTTGTTT
HY5 fw	AT5G11260	GTCGGAGAAAGTCAAAGGAAGC
HY5 rev	AT5G11260	TTCCTCTCTCTTGCTTGCTGAG

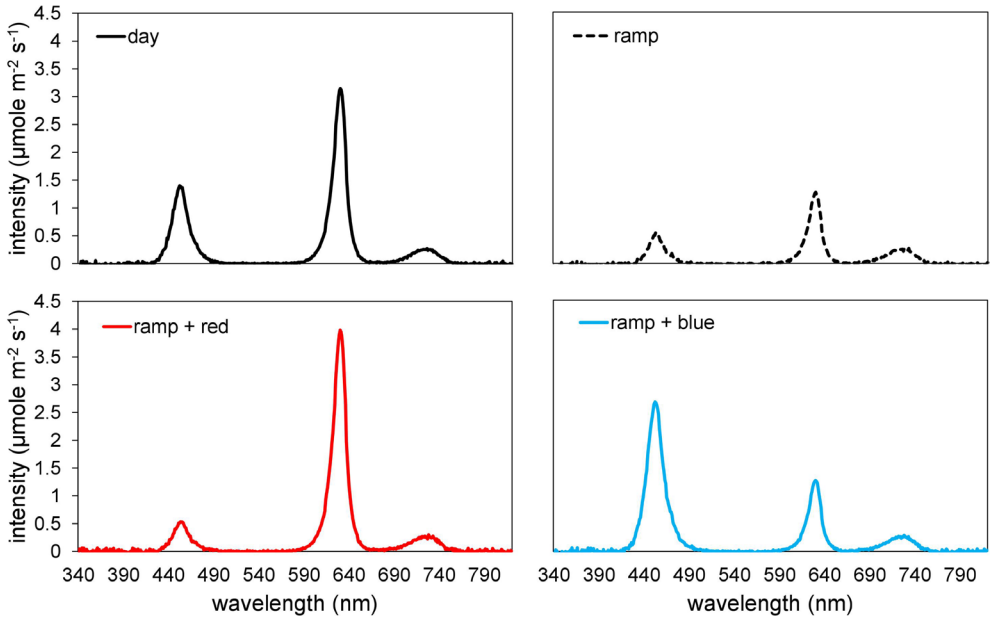
Supplemental Table S3: Daily Light Integral during different light treatments in LUMINATOR, SOLINATOR and greenhouse. Average Daily Light Integrals (DLI) during control light treatment (control), additional red light at dawn and dusk treatment (R^{m+e}), or additional blue light at dawn and dusk treatment (B^{m+e}) in LUMINATOR, SOLINATOR and greenhouses during both experiments. The DLIs are in mole photons m^{-2} d^{-1}. The DLI of greenhouse experiments is average DLI over 14 days. The DLI of the additional red (+R) or blue (+B) light alone is in % of DLI under R^{m+e} or B^{m+e} light conditions, respectively.

	LUMINATOR	*SOLINATOR*	*greenhouse (exp. 1)*	*greenhouse (exp. 2)*
	constant T	*+DIF*	*+DIF*	*+DIF*
control	3.81	4.32	2.75	4.26
R^{m+e}	4.24	4.68	3.30	4.67
+R (% of DLI)	11.32 %	8.33 %	13.31 %	5.70 %
B^{m+e}	4.21	4.68	3.43	4.66
+B (% of DLI)	10.37 %	8.33 %	12.80 %	5.72 %

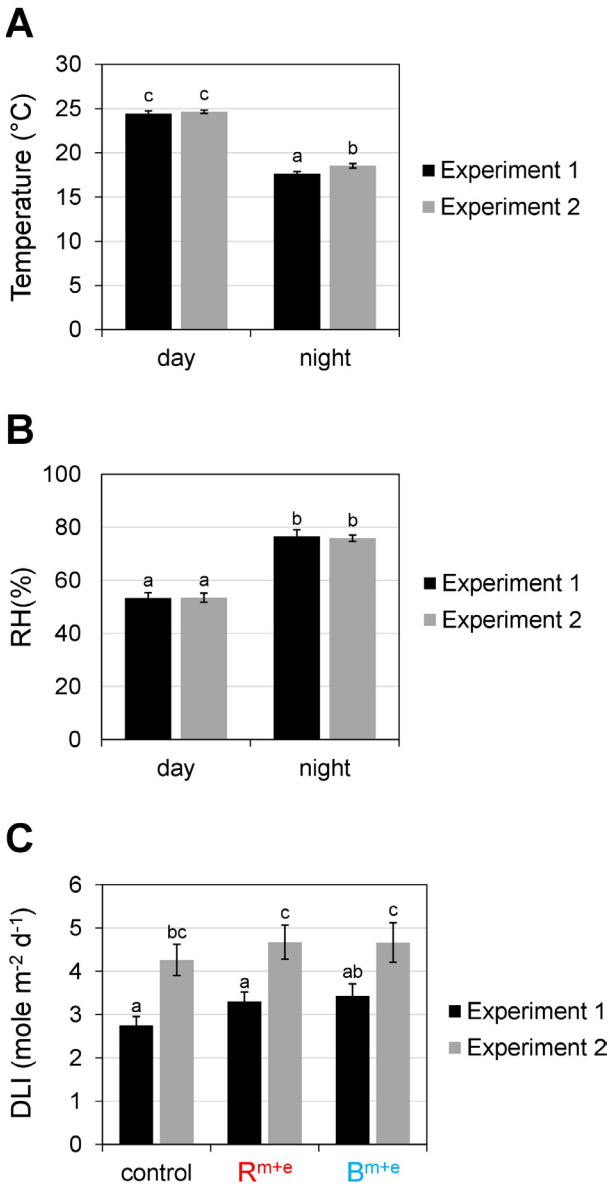


Supplemental Figure S1: light conditions SOLINATOR and LUMINATOR compared to natural sunlight. Daily variations in total light intensity (**A**), blue light intensity (**B**) and photostationary state (PSS; **C**) under natural sunlight (left panels), in SOLINATOR (middle panels) and in LUMINATOR (right panels).

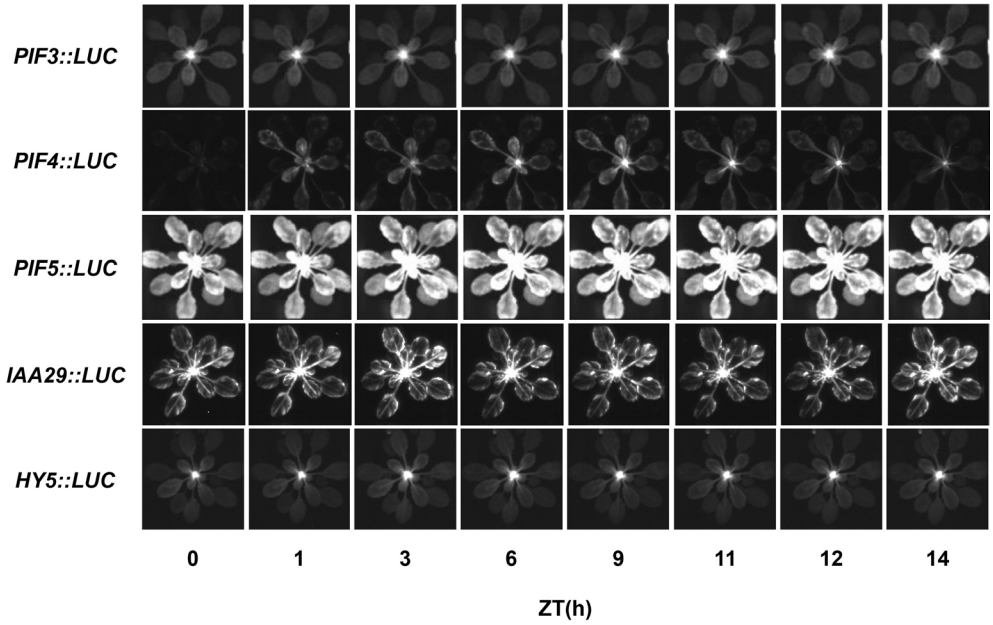
Natural sunlight was measured outside at Haarweg, Wageningen, The Netherlands (N 51° 58.2', E 5° 40.0') from the 28th until the 30th of September, 2011 (cloudless days) and data are average light intensities and PSS ±SE (3 days). Variations in light intensities and PSS under control conditions (solid black lines) in SOLINATOR and LUMINATOR were based on natural sunlight measurements, but not identical due to technical limitations of the systems. Graphs of SOLINATOR and LUMINATOR show light settings, and not actual measurements. The effect on the additional red light (R^{m+e}) treatment in SOLINATOR and LUMINATOR are shown by the red dashed lines, the effect of additional blue light (B^{m+e}) treatments by blue dashed lines.



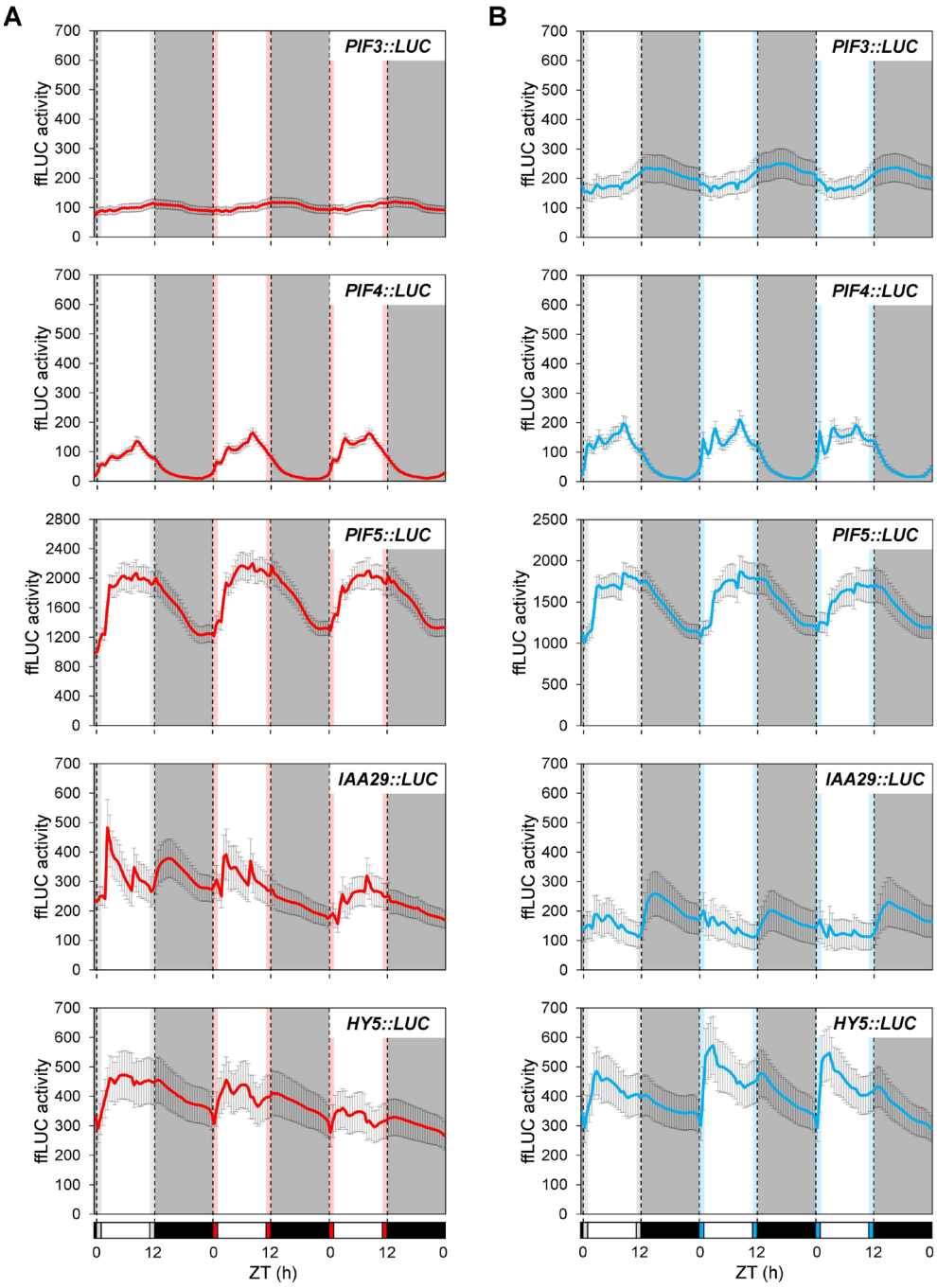
Supplemental Figure S2: Light spectra LUMINATOR settings. Light spectra of day (solid black line), ramp (dashed black line), ramp+ additional red light (solid red line) and ramp+ additional blue light (solid blue line) in LUMINATOR. Spectra were measured using a Flame-T spectroradiometer (Ocean Optics, Duiven, The Netherlands). For measurements sensor was placed at plant height in the center of the grid holding ffLUC reporter plants.



Supplemental Figure S3: Conditions in greenhouse compartments during experiments. A+B: Average temperature (**A**) and relative humidity (**B**) during the day or night in the greenhouse compartment. Data are mean average temperature in °C or mean average relative humidity (RH) in % during day or night ±SE (14 days). Temperature and RH were logged by one sensor (Hoogendoorn Growth Management, Vlaardingen, the Netherlands) placed in the center of the compartment. **C**: Average Daily light integral (DLI) in the greenhouse compartment for the different light treatments: control light treatment or everyday one-hour additional red (R^{m+e}) or blue (B^{m+e}) light at dawn and dusk. Data are DLI in mol photons m^{-2} d^{-1} ±SE (14 days). The intensity of visible light (400-700 nm) was logged by two sensors per growth table (LI-190R; LI-COR Biosciences, Lincoln, Nebraska, USA) that were placed in between the plants at canopy level. Black bars show data Experiment 1, grey bars show data Experiment 2. Different letters indicate significant differences in temperature, RH or DLI ($p<0.05$, Student's t-test).

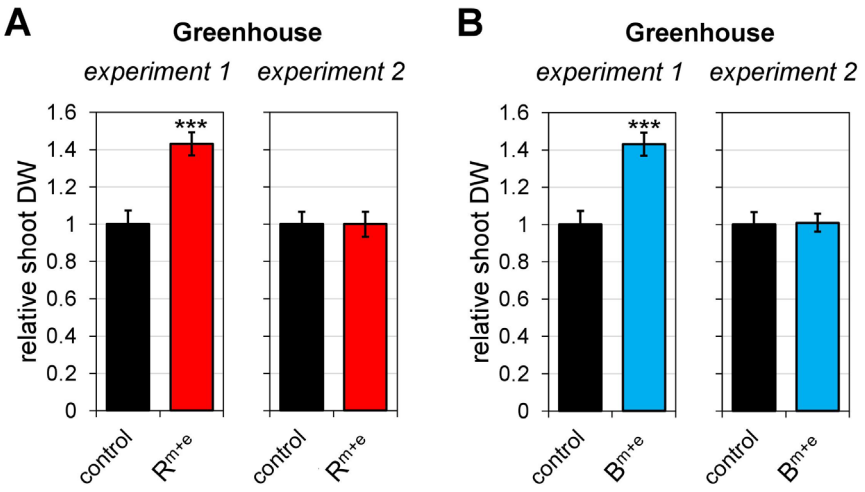


Supplemental Figure S4: Images of ffLUC activity in Arabidopsis reporter plants for key regulators of plant growth. Representative images of 30-day-old Arabidopsis *PIF3::LUC*, *PIF4::LUC*, *PIF5::LUC*, *IAA29::LUC* and *HY5::LUC* reporter plants grown under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Pictures were taken with LUMINATOR at different time points. Onset of day is after picture was taken at ZT(h)=0, night started after picture was taken at ZT(h)=12. Lights were always turned off during imaging and images were processed with ImageJ software.

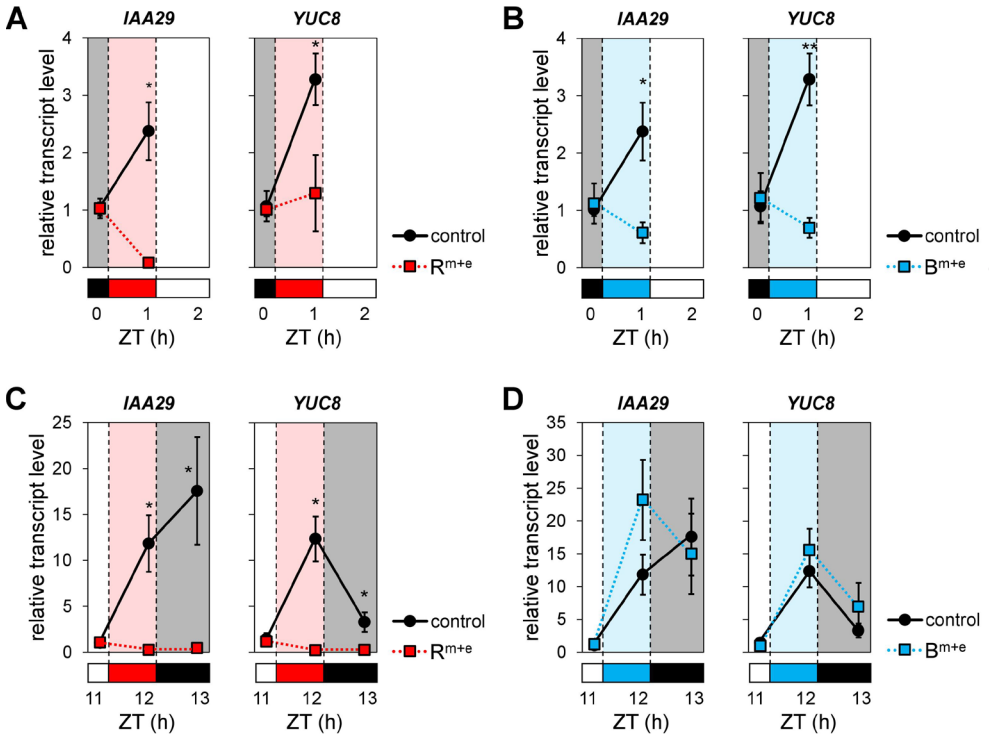


Supplemental Figure S5: Effect additional red or blue light treatments on diurnal activity of ffLUC reporters. Diurnal ffLUC activity profile of 4-week-old *PIF3::LUC, PIF4::LUC, PIF5::LUC, IAA29::LUC and HY5::LUC* Arabidopsis reporter plants grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). On the first day, light conditions included a one-hour light ramp at the start and end of the photoperiod (control conditions). On the second and third day additional red light (60

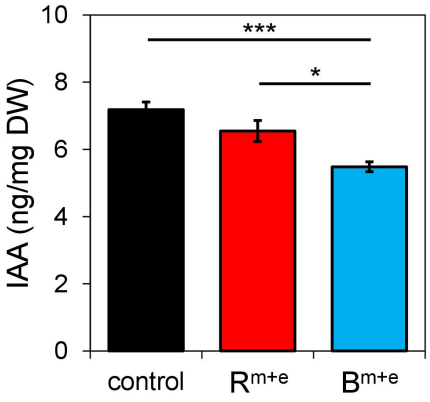
µmole m^{-2} s^{-1}; R^{m+e} treatment) or blue light (55 µmole m^{-2} s^{-1}; B^{m+e} treatment) was provided during the first and last hour of the photoperiod. Data are mean luminescence ± SE (n=6), measured every 30 min. Dark grey graph areas and black bars underneath graphs represent dark period, light grey areas and bars represent light ramping (30 µmole m^{-2} s^{-1}; R:FR=2), red areas and bars represent ramping with additional red light (100 µmole m^{-2} s^{-1}; R:FR=8), blue areas and bars represent ramping with additional blue light (100 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light (100 µmole m^{-2} s^{-1}; R:FR=6).



Supplemental Figure S6: Effect of additional red or blue light treatment on shoot biomass of tomato in greenhouse. Relative shoot dry weight (DW) of 28-day-old tomato (*Solanum lycopersicum* cv. 'Money maker') grown for two weeks in greenhouse compartments under natural photoperiods with day temperatures around 25°C and night temperatures around 17°C (see Supplemental Figure S3). Plants received one hour of additional red or blue light at the start and end of each photoperiod (R^{m+e} and B^{m+e} treatment, respectively) or they did not receive any additional light (control treatment). The experiment was repeated in time. Experiment 1 ran from March 1 until March 14, 2018. Experiment 2 ran from March 20 until April 2, 2018. Intensity of additional red/blue light was 61 µmole m^{-2} s^{-1} during experiment 1 and 37 µmole m^{-2} s^{-1} during experiment 2. Average Daily Light Integral (DLI) was 2.75-3.43 mole m^{-2} d^{-1} during experiment 1 and 4.26-4.66 mole m^{-2} d^{-1} during experiment 2. Data are mean shoot DW, normalized to shoot DW of plants grown under control conditions (n=24) ± SE. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from plants grown under control conditions.



Supplemental Figure S7: Effect of additional red or blue light treatment on endogenous expression of PIF-target genes *IAA29* and *YUC8*. Four-week-old Arabidopsis Col-0 WT plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control treatment). Alternatively, additional red light (60 µmole m^{-2} s^{-1}; R^{m+e} treatment) or blue light (55 µmole m^{-2} s^{-1}; B^{m+e} treatment) was provided during the ramping hours on the second and third day. Graphs show relative *IAA29* and *YUC8* mRNA transcript levels at start (**A**+**B**) and end (**C**+**D**) of day 3 in Col-0 WT plants grown under control conditions (black squared, solid lines) R^{m+e} conditions (red squares, dashed lines) and B^{m+e} conditions (blue squares, dashed lines). Data are mean relative transcript levels ±SE (n=3-4 biological replicates). Transcript levels are relative to those of reference genes *YLS8* (At5g08290) and *IPP2* (At3g02780) and normalized to ZT(h)=0 or ZT(h)=11. Dark grey graph areas and black bars underneath graphs represent dark period, red areas and bars represent ramping with additional red light conditions (100 µmole m^{-2} s^{-1}; R:FR=8), blue areas and bars represent ramping with additional blue light conditions (100 µmole m^{-2} s^{-1}; R:FR=2) and white areas represent day light conditions (100 µmole m^{-2} s^{-1}; R:FR=6). *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from relative transcript levels under control conditions.



Supplemental Figure S8: Effect of additional red or blue light treatment on endogenous auxin levels. Four-week-old Arabidopsis Col-0 WT plants were grown three subsequent days under 12hL/12hD cycles at constant temperature (22°C) and RH (70%). Light conditions included a one-hour light ramp at the start and end of the photoperiod of every day (control treatment). Alternatively, additional red light (60 µmole m^{-2} s^{-1}; R^{m+e} treatment) or blue light (55 µmole m^{-2} s^{-1}; B^{m+e} treatment) was provided during the ramping hours on the second and third day. Graph shows endogenous levels of indole-3-acetic acid (IAA) at the beginning of the night (ZT(h)=13) on day 3 in Col-0 WT plants grown under control conditions (black bars) R^{m+e} conditions (red bars) and B^{m+e} conditions (blue bars). Data are mean IAA levels in ng per mg dried plant material ±SE (n=4 biological replicates). *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from IAA levels under control conditions.

Chapter 6

General discussion

The science of controlling plant growth in greenhouses

In order to control elongation of stems and grow compact shaped plants year-round, it is common practice in horticulture to use chemical plant growth retardants (PGRs). However, alternative methods for plant growth control are needed because the use of chemical PGRs is increasingly restricted by government regulations due to environmental and health safety concerns (Bergstrand, 2017). Realizing a -DIF temperature regime (cold days/warm nights) as opposed to a naturally occurring +DIF regime (warm days/cold nights) is commonly used in horticulture to control plant growth without the use of chemicals (Carvalho et al., 2008; Bergstrand, 2017). To apply –DIF more efficiently, or to find alternatives for –DIF, we need to understand the molecular processes controlling plant growth and how these may be steered using controllable environmental cues. Previous research showed that -DIF suppresses cell elongation in Arabidopsis during the cold photoperiod through limiting auxin biosynthesis and signaling, leading to reduced auxin-induced ethylene biosynthesis and signaling (Bours et al., 2013; Bours et al., 2015). It was found that PHYTOCHROME INTERACTING FACTORs 4 and 5 (PIF4 and PIF5) act upstream to regulate auxin biosynthesis and that PIF3 acts downstream of ethylene signaling to regulate cell elongation responses to diurnal light/temperature conditions (Bours et al., 2013; Bours et al., 2015). Preliminary results also indicated that -DIF may induce a carbohydrate starvation status (Bours, 2014). In this thesis project we aimed to get a better understanding of how these different processes interact and may be manipulated by light treatments to enhance suppression of plant growth in greenhouses. The insights into the molecular regulation of the growth response to -DIF may lead to either a fine-tuning of the –DIF treatment or to alternative treatments to obtain the same objective. In this final discussion chapter, I give an overview of the most important results, how these relate to the new literature on plant growth regulation and address remaining questions. Finally, I will give suggestions to plant growers on how to obtain compact-shaped plants in greenhouses without the use of chemicals.

Growth regulation in seedlings versus adult rosette plants

Most studies that aim to dissect the molecular mechanisms of plant elongation responses are performed with Arabidopsis seedlings and focus on elongation of the hypocotyl. Studying growth responses in hypocotyls has the advantage that elongation responses are fast, are easy to score and require little space. Moreover, most of the hypocotyl elongation responses rely on cell elongation rather than on cell proliferation, making the hypocotyl a good model for studying the mechanisms underlying cell elongation. However, during development the architectural structure and age composition of the plant gets more complex due to the sequential formation and outgrowth of true leaves, internodes and axillary buds. Within leaves, different structures (e.g. leaf blade and leaf petiole) are formed with different developmental time windows, which may show different growth responses at any given moment. Depending on the developmental age of the leaf and its structure, growth may rely more on cell expansion of blade and petioles (older leaves) or cell proliferation (younger leaves). While growth of old leaves through cell

expansion relies mainly on the ability to take up water to increase turgor pressure for cell expansion, growth of young leaves through cell proliferation relies mainly on carbohydrate (CH) availability (Pantin et al., 2012). Due to the different requirements for growth of different leaves/structures, growth responses in more mature plants may not only depend on local developmental age and structure of the leaf, but also on transport of metabolites between different structures. This is nicely illustrated by our own results that show stronger growth reduction in response to -DIF in petioles and young developing leaves compared to the leaf blades of older leaves (Chapter 3). The challenge in this research was to determine the relevance of the fundamental insights into molecular control of hypocotyl cell elongation for the situation of more mature plants. The different response-capacity of different parts of the leaf makes that local growth responses in adult plants are more diverse and graded. Although the fundamental molecular mechanisms controlling local growth may be the same as in hypocotyls of seedlings, local conditions in adult plants may be much stronger influenced by plant architecture (e.g. self-shading) and internal transport capacity (e.g. sugar transport between source and sink leaves). Both of these features are a function of light and temperature conditions as well. That means that all studies on growth responses driven by the endogenous circadian clock, influenced by local CH metabolism and regulated by key growth-regulating transcription factors (PIFs/ELONGATED HYPOCOTYL 5 (HY5)) preferably need to be studied separately in source and sink leaves. For this reason, we chose to study the effect of -DIF in combination with different light treatments on regulation of growth of mature Arabidopsis rosette plants, rather than using hypocotyl elongation assays. Previous research had already identified key transcription factors involved in regulation of elongation (PIFs and HY5; see Introduction Figure 2). To study the role of these transcription factors in growth we focused on the transcriptional regulation of these key transcription factors in the shoot under different light treatments (Chapter 2 and Chapter 5). These studies did not reveal major differences in the profile of transcriptional responses of the firefly luciferase (ffLUC) reporters to the different light regimes in source and sink leaves.

A good example of how responses in mature rosette plants may be different from that in seedlings is given in Chapter 2 where we show that in whole rosettes *PIF4* transcript levels are only slightly upregulated at the end of the night in response to high ambient temperatures, while it was previously shown that in seedlings *PIF4* transcript levels are significantly increased throughout the day and night (Zhu et al., 2016). This shows that transcriptional responses related to plant growth may indeed depend on the developmental stage of the plant.

RNAseq analysis gives an integral view of the transcriptional response to -DIF. However, the budget for RNAseq was limited and we had to choose between sampling at different time points and from different tissues. Since the growth response to -DIF is strongest in young leaves and petioles we chose to sample those two tissues for RNAseq analysis and for time points we chose end of the night and end of the day. Results of the RNAseq analysis are described in Chapter 3. In Chapter 4 we used the same tissues, but now sampled at multiple timepoints, to study the clock regulation of CH metabolism in more detail. The growth of young sink leaves mainly depends on CH and micronutrients

availability (Pantin et al., 2012). However, sink leaves do not produce sufficient CHs to support their growth potential and require import of sugars (in the form of sucrose) from older - source leaves (Turgeon, 1989). Source leaves produce an excess of sugars during the day, which is converted to starch. This starch can be mobilized during the night to sustain transport of carbohydrates to sink leaves (Turgeon, 1989). Therefore, metabolic activity and related local CH status differs between source and sink leaves, depending on local photosynthesis capacity and efficiency and timing of CH transport from source to sink (Turgeon, 1989; Kolling et al., 2015). The results of our separate analyses on sink material (sink leaves and petioles) and remaining source material of Arabidopsis rosettes grown under +DIF and -DIF confirms that starch metabolism is differentially regulated in source and sink, and that sucrose transport from source to sink may be limited in response to -DIF at the end of the night. This leads to a CH starvation status at end of night in sink leaves that inhibits growth during the subsequent day. This also stresses that the more complex structure of an adult plant is an essential part of understanding growth under different environmental conditions.

LUMINATOR: unique possibilities versus real challenges

In order to study the effect of -DIF on transcriptional responses related to growth and the clock, Arabidopsis ffLUC reporter plants were obtained or made. We used several clock reporter genes (*PSEUDO-RESPONSE REGULATOR 9* (*PRR9)*, *LATE ELONGATED HYPOCOTYL* (*LHY*), *GIGANTEA* (*GI)*, *TIMING OF CAB EXPRESSION 1* (*TOC1*)) and several reporters for key genes related to growth (*PIF3*/*4*/*5*, *HY5*, *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*)). Using ffLUC reporter plants allows for semi-continuous real-time monitoring of gene activity in planta. Although a reporter line only reveals the transcriptional activity of the selected promoter, the ffLUC reporter plants have the advantage that many different light conditions can efficiently be tested in a short time frame, and only potential interesting transcriptional responses may need to be validated by qPCR profiling.

In order to monitor ffLUC activity in reporter plants, two LUMINATOR cabinets were built, which allow for semi-continuous monitoring of ffLUC activity with a highly sensitive camera (Chapter 2). LUMINATOR consists of a custom-built box with LED light, temperature and humidity control, in which up to 36 adult Arabidopsis rosette plants can be grown and monitored for multiple days. The technical realization of LUMINATOR was mainly thanks to Maarten Wassenaar. Special wide view lenses with high light yield were used to reduce ffLUC activity capturing time, and special filters were used in front of the lenses to block chlorophyll fluorescence light. Changes in temperature have a big effect on humidity. For LUMINATOR, the relative humidity was kept around 70%-80% during experiments at 12°C-22°C or it was kept around 40% during experiments at >22°C. Later during this project, it became known that indeed humidity can have an effect on transcriptional responses (Georgii et al., 2017).

For correct interpretation of ffLUC activity measurements obtained with LUMINATOR we first performed some validation experiments, which are described in Chapter 2.

LUMINATOR was used to monitor ffLUC activity in a set of 4 four-week-old Arabidopsis reporter plants over several days. Those experiments demonstrated that substrate application is best by daily spraying. Temperature changes have a big effect on ffLUC activity, making interpretation of changes in ffLUC reporter activity under +DIF and -DIF very difficult, but not impossible. Therefore, to study the effect of light treatments, subsequent experiments were done at constant temperature. Under 12h light/ 12h dark cycles and at a constant temperature of 22°C, the diurnal ffLUC activity profiles of the reporters generally matched well with expression profiles of the corresponding endogenous genes (measured by qRT-PCR). Under these conditions LUMINATOR is very efficient to monitor responses of ffLUC reporters to one-hour additional LED light treatments (Chapter 2 and Chapter 5). Using LUMINATOR we could show that, besides immediate responses, added light pulses may also have effects outside the time-window during which the added light pulse is given. The high time resolution of ffLUC reporter activity also made it possible to determine integrated activity of ffLUC reporters, and thus of the related gene activity during the day or night interval. Such integrated activity profiles may be more relevant to determine growth responses than gene activity measurements at a single time point. Results in Chapter 5 show that the combination of LUMINATOR and ffLUC reporters provides a powerful and time-efficient tool to study transcriptional responses to different light treatments in more detail than would be possible with other methods (e.g. measuring gene expression by qRT-PCR).

Challenges in interpretation of in-planta ffLUC reporter activity

The validation experiments for results obtained in LUMINATOR identified several challenges in the interpretation of changes in ffLUC reporter activity. First of all, ffLUC reporters require the substrate luciferin to be able to produce the bioluminescent signal. In Chapter 2 we show that providing luciferin by spraying it two times a day on the plants is more effective than feeding luciferin to the roots. However, especially at high ffLUC reporter activity, the luciferin spray results in a small transient peak in ffLUC activity, indicating that at that timepoint luciferin was limiting the ffLUC activity and that such changes in ffLUC activity are not related to increased reporter activity. Second, it is possible that the selected upstream promoter fragment used in the ffLUC reporters may not include all regulatory elements of the related endogenous gene. Missing such elements may lead to differences between the ffLUC reporter expression profile and the expression profile of the related endogenous gene as determined by qPCR for selected timepoints. Difficulty in such comparisons is that the time-resolution of ffLUC reporter activity is usually higher than that obtained by qPCR profiling. Indeed, small but significant changes in ffLUC reporter activity in response to additional LED light do not always fully correlate to significant changes in mRNA transcript levels of the corresponding endogenous gene. A third challenge in the interpretation of ffLUC reporter activity is that we found that big diurnal peaks in ffLUC signals are delayed by up to two hours compared to the phase of diurnal fluctuations in mRNA transcript levels at 22°C, which is consistent with previous observations ((Millar et al., 1992); Chapter 2). Moreover, in Chapter 2 it is shown that this delay is increased to up to about six hours at 12°.

The activity of the ffLUC enzyme is directly affected by temperature, and temperature responses of ffLUC reporters are therefore not always dependent on changes in promoter activity (Koksharov and Ugarova, 2011). However, comparison of the different ffLUC reporter activities under regimes that include temperature cycles indicate that not all reporters show a similar response to the temperature change. When the effect of temperature is solely on ffLUC activity, one could expect a similar relative response to temperature change for all reporters. The fact that some reporters show different responses indicates that promoters in these reporters do contain regulatory elements that respond to temperature. For example, although both *PIF4::LUC* and *HY5::LUC* initially show an immediate drop in ffLUC activity in response to a temperature shift from constant 22°C to constant 12°C, *HY5::LUC* activity starts to increase after several hours, while *PIF4::LUC* activity further decreases (Chapter 2: Figure 6). This suggests that the *HY5* promoter is positively regulated at lower temperatures and the *PIF4* promoter is negatively regulated at lower temperatures, which is in agreement with generally increased *HY5* mRNA levels and decreased *PIF4* mRNA levels at 12°C compared to 22°C (Chapter 2: Supplemental Figure S13). Thus, interpreting the transcriptional regulation by use of ffLUC reporters under temperature cycles in LUMINATOR is complicated and needs careful validation by measuring mRNA transcript levels of the endogenous genes by qRT-PCR.

Transcriptional response to -DIF

To obtain an overview of processes affected by -DIF (compared to +DIF) RNAseq analysis was performed on mRNA and miRNA isolated from rosette centers (sink leaves and source leaf petioles) of Col-0 WT and *phyB-9* mutants (mutant line for the phytochrome B (PHYB) photoreceptor; Chapter 3). Due to budget limitations, sample timepoints were limited to end of the night (EN) and end of the day (ED). The RNAseq analysis for Col-0 WT identified several biological processes affected by -DIF, such as induction of cold acclimation. Of the 2706 differentially expressed genes (DEGs) under -DIF, 550 DEGs are detected at both ED and EN and this subset of genes is enriched for genes involved in cold acclimation. In addition, some DEGs related cold acclimation processes are only detected at ED, indicating that some aspects of cold acclimation are restricted to day-time. This -DIF-triggered cold-acclimation during the day is accompanied by the day-time accumulation of soluble sugars, such as sucrose and glucose (Chapter 4), which may help cold tolerance through membrane stabilization, ROS scavenging and photosystem protection (Tarkowski and Van den Ende, 2015).

Genes that are only upregulated at EN under -DIF are associated with processes linked to low energy status. In addition, many genes that are only downregulated at ED or EN, are associated with processes that link to the processes for promotion of cell growth, such as cell wall modification and water transport. This indicates that cell growth may be limited both at end day and at end night under -DIF. Furthermore, comparison of the set of genes that are differentially expressed under -DIF with the set of genes that are transcriptionally regulated by PIFs suggests that PIF protein accumulation/activity is downregulated under -DIF, especially at EN (Chapter 3: Figure 10). This is in agreement

with previous research showing that suppression of plant cell elongation under -DIF is linked to reduced activity of PIFs (Bours et al., 2015). Of the *PIF* genes, only *PIF4* expression was downregulated at ED, which may be indication that expression of *PIF4* is more sensitive to the combination of light and temperature than the expression of the other *PIF* genes. Indeed, PIF4 also plays a key role in elongation response to elevated temperature, as described in many papers on thermomorphogenesis, while this thermomorphogenic response of Arabidopsis seedlings was also found to be a function of light intensity (Qiu et al., 2019).

The RNA samples were also analyzed for differentially expressed miRNAs in response to -DIF. Unfortunately, the processing of these samples was not very good by the hotel service, so that no replicate values were obtained. Still, some miRNAs that potentially target growth related genes were identified that showed >2-fold differential counts in comparison of +DIF and -DIF samples, suggesting that at least part of the transcriptional response to -DIF may be through regulation of transcript stability by miRNAs (Chapter 3). The time within the project did not allow to further explore this type of regulation of growth in response to -DIF, for instance by validating the differential expression of the miRNA, or by studying the -DIF response of the related mutants and lines overexpressing such miRNAs.

Updated model for the regulation of PIF4

A search in Pubmed shows that since the start of this thesis work 114 new papers appeared on PIF4, confirming the key role this transcription factor plays in Arabidopsis plant growth and development. Indeed, our results in Chapter 3 suggest that under -DIF PIF protein activity and/or stability is suppressed and that specifically *PIF4* transcription is reduced. This raises the question which transcription factors are responsible for this light/temperature sensitive regulation of *PIF4* transcription and which proteins affect PIF protein activity or stability under -DIF. In the past years, several types of transcription factors were linked to *PIF4* transcription. These will be briefly discussed below. In addition, the past years several factors, besides PHYB, have been identified that affect PIF4 protein stability or activity, which will also be briefly summarized below. Figure 1 shows an updated version of the model presented in Chapter 1, based on our current insights into regulation of *PIF4* transcription and different novel components acting on PIF4 that are discussed below.

Transcriptional regulation of PIF4

Recently it was shown that upregulation of *PIF4* transcription under high ambient temperatures is mediated by the transcription factor BRASSINAZOLE RESISTANT 1 (BZR1), which promotes *PIF4* transcription through directly binding and activating the *PIF4* promoter (Ibanez et al., 2018). The increased activity of BZR1 is the result of increased levels of brassinosteroids (BRs), leading to inactivation of the kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2) (Bernardo-Garcia et al., 2014; Wang et al.,

2014; Ibanez et al., 2018). The BIN2 kinase phosphorylates BZR1, resulting in its degradation. Inactivation of BIN2 results in stabilized dephosphorylated BZR1 and accumulation of BZR1 in the nucleus, where it activates its target gene promoters (e.g. *PIF4*) (Wang et al., 2012; Bernardo-Garcia et al., 2014; Wang et al., 2014). Interestingly, while *PIF4* expression is directly positively regulated by BZR1, PIF4 also indirectly positively regulates *BZR1* expression in a positive feedback loop which sustains expression of *PIF4* and *BZR1* at high temperatures (Ibanez et al., 2018).

Members of the TEOSINTEBRANCHED1/CYCLOIDEA/PCF (TCP) family of transcription factors, TCP5, 13 and 17, are also positive regulators of *PIF4* transcription, and these factors also play a role in thermomorphogenesis (Han et al., 2019). By studying TCP5 in more detail, the authors show that this transcription factor stimulates *PIF4* transcription through direct binding to the *PIF4* promoter.

There are also several transcription factors that negatively regulate transcription of *PIF4*, including LUX, HY5 and PIF4 itself. The LUX protein component of the Evening Complex (EC), targets the EC to the promoters of *PIF4* and *PIF5* in vivo and suppresses *PIF4* (and *PIF5*) transcription (Nusinow et al., 2011). The growth-suppressing transcription factor HY5 works in part antagonistically to PIF4 by suppressing *PIF4* transcription through interaction with BZR1 or by binding to the G-box in the *PIF4* promoter (Lee et al., 2007; Delker et al., 2014; Li and He, 2016). In our research we also added a new component to transcriptional regulation of PIF4 as we identified PIF4 as a negative regulator of its own gene transcription (Shapulatov, 2019).

Regulation of PIF4 protein stability

It has been known for a long time that the activate Pfr form of the PHYB photoreceptor targets PIF4 (and other PIFs) for degradation. In addition to this negative role of PHYB Pfr on PIF4 protein stability, the DELLA proteins were also found to trigger degradation of PIF4 (and PIF1, 3 and 5) (Li et al., 2016). In turn, the pool of DELLA proteins is regulated by Gibberellins (GA), as DELLA proteins are targeted for degradation upon GA signaling (discussed in Chapter 1). Recently, the key energy sensor complexes SUCROSE NON-FERMENTATION1-RELATED KINASE 1 (SnRK1) and TARGET OF RAPAMYCIN COMPLEX 1 (TORC1), which are activated in response to CH shortage or excess respectively, were also found to affect PIF4 activity. Activated SnRK1 results in PIF4 phosphorylation and subsequent degradation of PIF4 protein (Hwang et al., 2019). In addition, inactivation of TORC1 and activation of SnRK1 results in degradation of BZR1, the positive transcriptional regulator of *PIF4* transcription, through autophagy (Zhang et al., 2016).

Blocking PIF4 protein transcriptional activity

PIF4 stimulates cell elongation by binding specific E- or G-box promoter elements and activating expression of target genes that promote cell elongation (Leivar and Monte, 2014). During the course of this project, an increasing number of proteins was found to regulate the transcriptional activity of PIF4 (and other PIFs) through direct interaction with PIF4 protein and/or its target promoters. For example, besides promoting *PIF4* transcription, BZR1 also positively regulates transcriptional activity of PIF4 at high ambient temperatures through direct interaction with PIF4 proteins and binding of PIF4 target promoters (Ibanez et al., 2018). In fact, BZR1 and PIF4 heterodimerization seems to be required for PIF-mediated cell elongation (Oh et al., 2012; de Lucas and Prat, 2014). Like BZR1, TCP5 also enhances transcriptional activity of PIF4 proteins through direct interaction with PIF4 proteins and binding of PIF4 to PIF4 target promoters (Han et al., 2019). Since the *PIF4* promoter can also be a target of PIF4, these interactions may also influence the transcription of *PIF4* itself. Moreover, since the effect of these proteins on PIF4 were only investigated for a limited set of PIF4 target promoters, it is not yet clear whether the same PIF4-protein interaction at promoter binding sites results in the same transcriptional activity (activation or repression) at different target promoters. Remarkably, in response to cold, which leads to BZR1 dephosphorylation, BZR1 binds and activates the promoters of *CBF1* and *CBF2* (*C-REPEAT/DRE-BINDING FACTORs*). CBF1 and CBF2 activity suppresses genes encoding GA biosynthesis enzymes, and thus under cold there is less GA, leading to stabilization of DELLA proteins. Since DELLAs can block PIF4 protein activity, this provides a mechanism through which (during cold) BZR1 may actually suppress PIF protein activity (Li et al., 2017; Planas-Riverola et al., 2019).

It has been shown that at high temperature, photoactivated cryptochrome 1 (CRY1) interacts with PIF4 to repress its transcriptional activity (Ma et al., 2016). However, under low blue light (due to canopy shade) CRY1/2 interacts with PIF4 to enhance its transcriptional activity (Pedmale et al., 2016; Xu et al., 2016).

Besides CRYs, HY5 and DELLA proteins, several core clock components have recently been shown to negatively regulate PIF4 activity. These clock components include PRR5, 7 and 9, TOC1 and the Evening Complex (EC) component EARLY FLOWERING 3 (ELF3) (Nieto et al., 2015; Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). For instance, ELF3 suppresses PIF4 (and PIF5) transcriptional activity by directly binding to PIF4 protein and blocking its binding to target promoters (Nieto et al., 2015).

The overview given in Figure 1 shows that the different levels of direct and indirect regulation of *PIF4* transcription, PIF4 protein stability and PIF4 protein activity, which includes direct negative feedback and indirect positive feedback interactions, is making it extremely difficult to translate the *PIF4* gene activity profiles (e.g. as monitored in *PIF4::LUC* reporter plants) to PIF4-target gene activity and to related growth responses under different light treatments. Here it seems that the more we know about the regulation, the more difficult it becomes to give it actual meaning for plant growth at normal or special (e.g. -DIF) diurnal conditions.

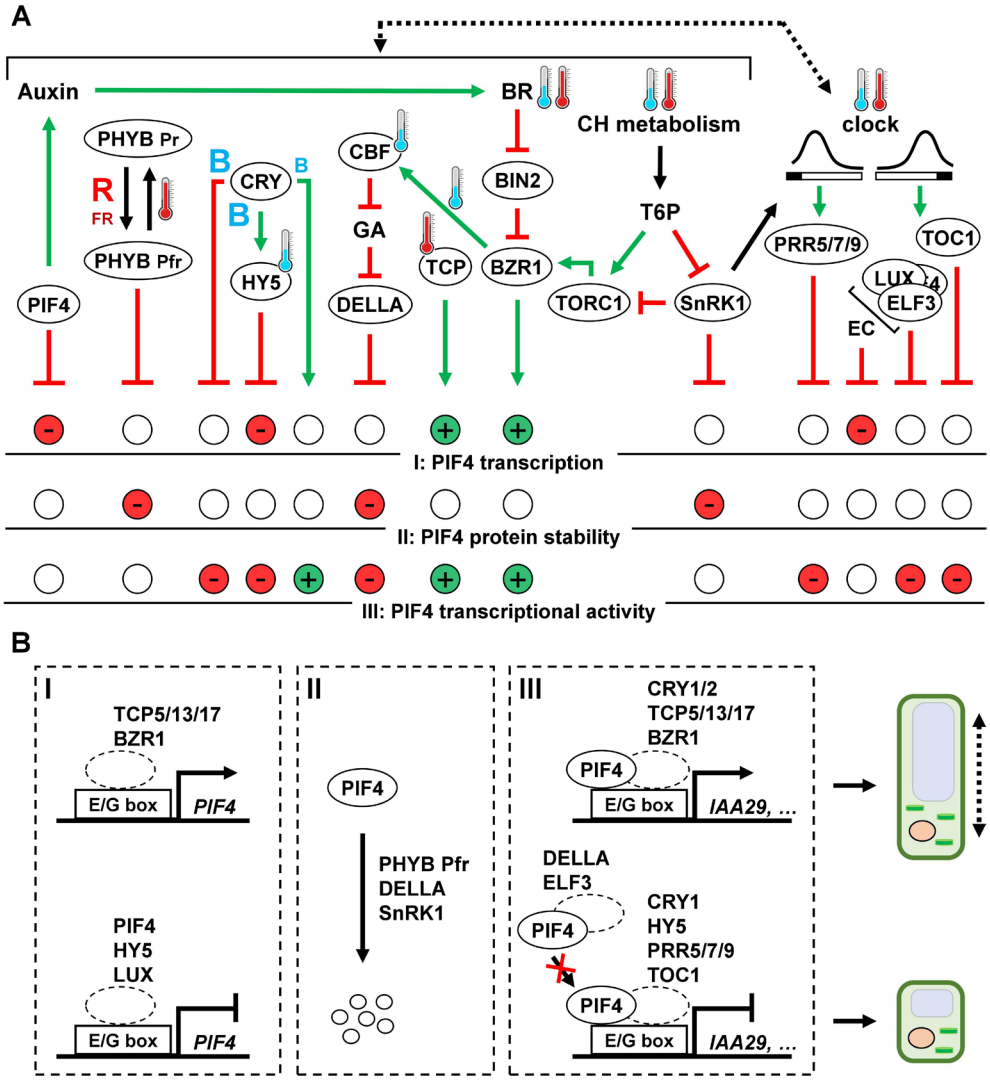


Figure 1: Updated model for the regulation of PIF4 in plant elongation responses. A: Model for regulation of molecular components that mediate light/temperature control of PIF4 on three different levels: *PIF4* transcription (I), PIF4 protein stability (II) and transcriptional activity of PIF4 at PIF4 target genes (III). **B**: Model showing control of plant cell elongation through regulation of *PIF4* transcription (I), PIF protein stability (II) and PIF4 transcriptional activity (III) by regulatory components shown in A. Models are updated versions of those in Figure 2 of Chapter 1, based on studies on regulation of PIF4 that were published during this project and discussed in the main text. Possible feedback interactions of PIF4 on upstream components in model are not shown. Ovals represent proteins or protein complexes. Black arrows indicate regulation; dashed arrow indicates regulation is not fully understood; green arrows indicate positive regulation; red bars indicate negative regulation. Color and sign in circles in A indicate whether regulatory component enhances (green; +), suppresses (red; -) or does not influence (white) activity of PIF4 on different levels. Thermometers in A indicate whether regulation/component is stimulated at high (red thermometer) or low (blue thermometer) temperature. Colored letters in A indicate regulation is stimulated by high blue light (large B), low blue light (small B) or by a high red/far-red light ratio (large R, small FR).

A role for PHYB in the transcriptional response to -DIF

Previously PHYB was identified as factor in the -DIF elongation response and recently it was shown that PHYB Pfr is involved in temperature sensing (Thingnaes et al., 2008; Bours et al., 2013; Jung et al., 2016; Legris et al., 2016; Qiu et al., 2019). In chapter 3 I describe the RNAseq analysis for both Col-0 WT and the *phyB-9* mutant. Comparison of the transcriptional response in these two genotypes helped identify which genes/processes affected by -DIF are under control of PHYB. Most of the DEGs involved in cell growth that are downregulated at EN in WT (including PIF-target genes) did not show up in the list of DEGs at EN in the *phyB-9* mutant (for example, see Chapter 3: Supplemental Table S5 and Supplemental Figure S4). These results indicate that PHYB is key to the differential regulation of growth-regulating genes in response to -DIF. Moreover, these genes may be causal for growth suppression under -DIF, as their suppression is reduced in the *phyB-9* mutant.

The role of PHYB in transcriptional regulation is through regulation of PIF protein stability. Upon illumination by red light, PHYB is converted from its inactive Pr form into its active Pfr form, which is able to enter the nucleus where it targets PIFs for degradation (Leivar and Monte, 2014). In time, PHYB Pfr is spontaneously converted back into its inactive Pr form. This process is called dark reversion because it is not dependent on light. During this thesis research two papers were published that showed that the rate of Pfr dark reversion to Pr is temperature sensitive (Jung et al., 2016; Legris et al., 2016). At high temperatures, the dark reversion rate is increased, leading to a smaller pool of active PHYB Pfr. Because of the warm night under -DIF, the dark reversion rate of the photoactivated PHYB at night is higher than under +DIF. Therefore, it is to be expected that suppression of PIF protein stability by PHYB Pfr is shorter lived under -DIF at night. Thus, from this role of PHYB Pfr in temperature sensing one would predict a higher PIF protein stability and activity under -DIF, and therefore increased growth. This is not observed under -DIF, indicating that other factors overrule this effect of temperature on PIF protein activity. The downregulation of genes involved in growth and the putative reduced PIF activity at EN thus indicates that under -DIF the downregulation of *PIF4* during the cold day is dominant over the potential increase in PIF4 protein activity during the warm night. This is in agreement with *PIF4* not being downregulated at the end of the day under -DIF in the *phyB-9* mutant (Chapter 3: Supplemental Table S3). Reduced *PIF4* transcription at end-day under -DIF limits PIF4 protein abundance at night. However, turnover of PIF4 protein at night may be reduced due to increased dark reversion of PHYB Pfr during the warm night under -DIF. The fact that *PIF4* transcription is not significantly downregulated in response to the cold day under -DIF in the *phyB-9* mutant suggests that PHYB is involved in the low temperature response of *PIF4* transcription. It is possible that this is through limited positive feedback on *PIF4* transcription via BR biosynthesis and the transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1) (Ibanez et al., 2018). Alternatively, this could be through enhanced PIF4 protein activity in suppression of its own transcription (Shapulatov, 2019).

Deeper analysis of the transcriptional regulation of PIF4 under -DIF

Our RNAseq analyses data provides a general overview of the processes affected by -DIF at two key time points (Chapter 3). However, including two timepoints limits our insight into the transcriptional response to -DIF over the full 24 hours of a day. In selecting the timepoints we reasoned that potentially the night-day and day-night transitions during -DIF may show the most changes in gene activity compared to +DIF. For some selected key genes, we measured mRNA transcript levels in source and sink material of adult Arabidopsis plants grown under +DIF or -DIF conditions every two hours during the day. The results are presented in Chapter 3, but here I discuss in more detail transcription factors PIF3/4/5 and HY5 (Figure 2). As expected from the RNAseq data, the qPCR confirmed that *PIF4* expression is most strongly downregulated under -DIF, while for *PIF3* and *PIF5* the expression is not significantly lower under -DIF. Moreover, the strongest downregulation of *PIF4* transcription under -DIF is at mid-day. During the course of this thesis work both transcription factors BZR1 and TCP5, 13 and 17 were identified as positive transcriptional regulators of *PIF4* during thermomorphogenesis (Ibanez et al., 2018; Han et al., 2019). It is possible that downregulation of *PIF4* transcription is caused by reduced expression of these transcriptional regulators of the *PIF4* gene. However, in the RNAseq data there is no indication of lower *BZR1* or *TCP* mRNA transcript levels under -DIF at ED or EN. Since the biggest difference in *PIF4* expression is at mid-day, in future experiments the expression level of *BZR1* and *TCP*s should also be quantified for this time-point.

Alternatively, the protein activity of BZR1 and/or TCPs could be reduced at low temperature, to explain the lower transcription of PIF4 during the day under -DIF. To determine whether protein activity of BZR1 is affected under -DIF I looked at expression of BZR1 and TCP target genes and compared genes that are differentially expressed under -DIF at ED and/or EN with genes found to be BZR1-regulated in seedlings (Oh et al., 2012). In addition, to determine whether protein activity of TCPs is affected under -DIF I looked at expression of TCP target genes and compared genes that are differentially expressed under -DIF at ED and/or EN with genes found to be regulated by TCP5, 13 and/or 17 in leaf petioles of 3-week-old plants (Han et al., 2019). Of the 2151 BZR1-regulated genes, only 217 were differentially expressed under -DIF and of the 491 TCP-regulated genes, only 78 were differentially expressed under -DIF. These results suggest that -DIF does not result in a strong reduction in activity of BZR1 and TCPs at ED or EN, and that reduced activity of these transcriptional regulators is most likely not the cause for the reduced *PIF4* expression.

Downregulation of *PIF4* transcription under -DIF may also be explained by increased transcription or protein activity of HY5, which is thought to suppress *PIF4* transcription. Expression of *HY5* is minimally affected under -DIF: downregulation of *HY5* at the start of the day is delayed by about two hours compared to under +DIF, both in source and sink leaves (Figure 2). At EN and ED the expression of *HY5* is not much different under +DIF and -DIF. This confirms that limited sampling for RNAseq may result in missing relevant differentially expressed genes at other timepoints. Although mRNA levels suggest only increased *HY5* transcription for a short time, integral *HY5::LUC* activity of

reporter plants grown under -DIF is significantly increased over the entire day and night period (Figure 3). Combined, these results indicate that under -DIF *HY5* transcription and possibly HY5 protein activity is increased (HY5 promotes its own transcription), while this may block *PIF4* transcription (Abbas et al., 2014; Binkert et al., 2014; Delker et al., 2014). This needs further confirmation by future research.

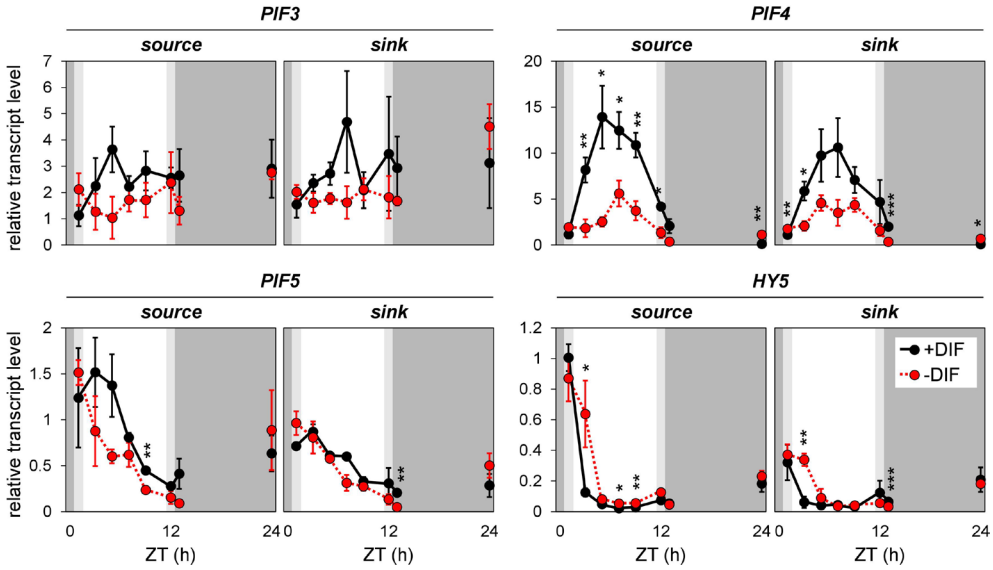


Figure 2: mRNA transcript levels of key growth-regulating genes in source or sink leaves under +DIF and -DIF. mRNA transcript levels of *PIF3*, *PIF4*, *PIF5* and *HY5* at ZT(h)=1, 3, 5, 7, 9, 12 and 24 in source or sink leaves of 32-day-old Arabidopsis Col-0 WT plants grown under +DIF (12hL 22°C/12hD 12°C; black dots, solid line) or -DIF conditions (12hL 12°C/12hD 22°C; red dots, dashed line). Dark grey graph areas represent dark period, white areas represent daylight (180 µmole m^{-2} s^{-1}), light grey areas represent dawn/dusk. Data are mean mRNA transcript levels ± SE (n=2-3 biological replicates of 6 pooled plants). Transcript levels are relative to those of reference genes *YLS8* (AT5G08290) and *IPP2* (AT3G02780) and normalized to mRNA transcript levels in source leaves under +DIF at ZT(h)=1. *p<0.05, **p<0.01 ***p<0.001 (Student's t-test) significantly different from levels under +DIF. Methods are described in more detail in Chapter 4 of this thesis.

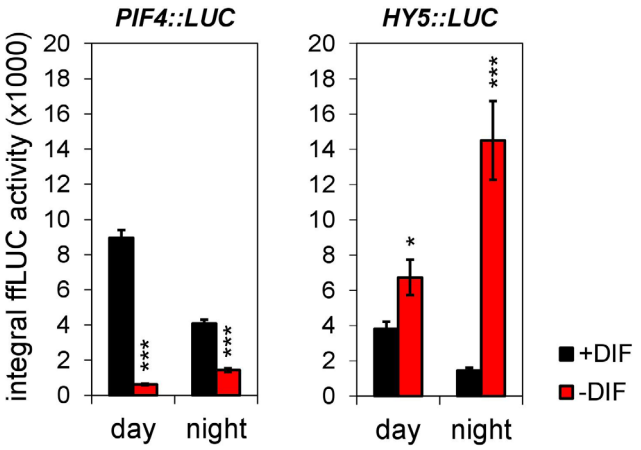


Figure 3: Effect -DIF on integral *PIF4::LUC* and *HY5::LUC* activity. Four-week-old *PIF4::LUC* and *HY5::LUC* Arabidopsis reporter plants were grown for two subsequent days under +DIF conditions (12hL 22°C/12hD 12°C) or -DIF conditions (12hL 12°C/12hD 22°C) in LUMINATOR. Light conditions under both conditions were identical as under control conditions described in Chapter 5. Graphs show ffLUC activity of reporters under +DIF (black bars) or -DIF (red bars), integrated over the two day- or night-periods. Data are mean integral luminescence ± SE (n=6). *p<0.05, **p<0.01, ***p<0.001 (Student's t-test) significantly different from integral ffLUC activity under +DIF conditions.

-DIF disrupts clock-regulated starch metabolism and induces CH starvation

In Chapter 4 we show that -DIF results in a CH starvation status at EN, both in source and sink leaves. Strangely, while the CH starvation at EN under -DIF links to reduced sucrose levels in sink leaves, it does not link to reduced sucrose levels in source leaves. Sugar levels are actually increased in source leaves at EN, suggesting that sugars are mobilized from starch at night but remain at a subcellular location in source leaves where they cannot contribute to formation of the sugar signaling molecule Trehalose-6-Phosphate (T6P). Our interpretation of these results is that starch mobilization during the night is faster than can be sustained for the full night, leading to a CH shortage in the cytosol of cells in source leaves. Presumably, the sugars that are derived from starch in source leaves are translocated to the apoplast of mesophyll cells. In apoplastic loading, sucrose that is released from starch in mesophyll cells enters the cell wall space (apoplast) and is taken up in the minor vein phloem by membrane transporters (Ayre, 2011). This uptake requires energy and is driven by a proton gradient over the plasma-membrane of the phloem cell. Once sugars are in the phloem at the source, the sugar transport is gradient driven, which requires an activity at the site of sink leaves that depletes sugars from the phloem. Similar to under -DIF, a reduction in export of CHs from source to sink leaves of Arabidopsis plants is also observed in plants in which CH starvation is induced by an extended night (Kolling et al., 2015). This changes the question of why there is reduced growth of sink leaves under -DIF to why there is no sugar transport from source to sink under -DIF. Future studies could therefore investigate sugar loading and unloading capacity in phloem in source and sink leaves. Indication that these activities may indeed be affected under -DIF come from the RNAseq data which shows that *SWEET11*, which is involved in sucrose phloem loading (Chen et al., 2012), is upregulated at EN under -DIF (Chapter 3).

In Arabidopsis, up to 60% of the total carbohydrates produced through photosynthesis during the day is stored as starch, which is subsequently mobilized at night to provide

carbohydrates to sustain growth and metabolic maintenance at night (Graf and Smith, 2011; Streb and Zeeman, 2012). Starch is broken down at night in a near-linear manner at a rate that results in starch levels being almost, but not completely depleted at the end of the night. This way the plant makes optimal use of its starch reserves, without reaching a CH starvation status at the end of the night. Therefore, under normal conditions the plant can sustain growth, while CH starvation would lead to a period of growth suppression (Gibon et al., 2004; Graf and Smith, 2011; Streb and Zeeman, 2012). We note that most studies on diurnal regulation of starch breakdown are done at constant temperature, while in nature light/dark cycles are accompanied by alternating temperature cycles. Light is usually associated with high temperatures and dark with lower temperatures.

In our experiments we noted that starch is actually far from depleted near the end of the night under +DIF (Chapter 4), which is different from experimental results shown in literature. One reason for this difference may be that the level of EN starch may actually be a function of the PAR light intensity during the day, which would need further investigation. However, the starch remaining at EN under our +DIF conditions does suggest that sink activity may be limiting. In other words, under a +DIF regime, factors other than carbohydrate availability become limiting for growth at night. Since utilization of sugars for growth requires micronutrients, this could also indicate that water transport of micronutrients can be limiting at night. Alternatively, the relatively high levels of starch detected at EN under +DIF could be due to the low maintenance respiration under +DIF (Pilkington et al., 2015). In contrast, maintenance respiration is increased under -DIF (Chapter 4) which, combined with a mis-regulation of starch mobilization during the night, leads to the starvation status at EN under -DIF. The mis-regulation of starch mobilization is remarkable as it has been shown that this is a strict function of photoperiod and the clock: depending on the given photoperiod, the rate of starch mobilization is adjusted such that starch is just not fully depleted at the end of the night (Graf et al., 2010). This regulation is so strict that when unexpectedly the night is extended by a few hours, the plant depletes its available starch reserves to sustain growth and gets into an CH starvation mode. The -DIF regime is one of the first environmental conditions that can disrupt this strict photoperiod control of rate of starch mobilization. Indeed, this effect of -DIF most likely relates to the disruption of the clock by -DIF, as illustrated by -DIF-induced phase shifts in clock-controlled leaf movement (Bours et al., 2013), and the differential phase shifts and altered amplitude of different core clock components (Figure 4; Chapter 4). It is still not known how the activity of the clock (or output of specific clock genes) can adjust the rate of starch mobilization according to the photoperiod. Since clock genes are transcription factors, it could be that regulation of at least some of the components in starch mobilization are under direct transcriptional control of clock genes. Regulation could also be through indirect effects of the clock acting at the protein level.

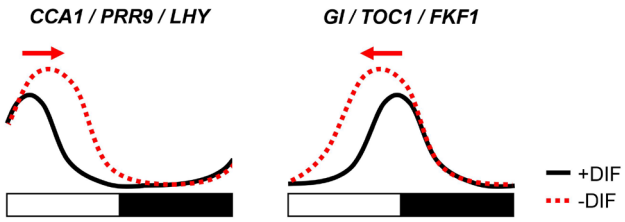


Figure 4: Effect -DIF on expression core clock genes. Schematic representation of diurnal expression of morning-phased clock genes (CCA1; PRR9; LHY) and evening-phased clock genes (GI; TOC1; FKF1) under +DIF (black solid line) and -DIF (red dashed line). Bars indicate photoperiod (white bars) and dark period (black bars). Figure is based on data presented in Figure 1 and Supplemental Figure S1 of Chapter 4.

One model suggests that the rate of starch degradation is set by integrating information about the starch levels at the start of the night (high after long day, lower after short day) and the remaining hours to the next (anticipated) dawn. This model for the regulation of starch breakdown is referred to as the arithmetic division (AD) model (Scialdone et al., 2013). While the AD model suggests convergence of parallel clock signals (measuring night period) and metabolic signals (starch levels at ED), an alternative model suggests that metabolic signals actually feedback to the clock. This model is named the retrograde signaling model. In the retrograde signaling model the metabolic signals result in a resetting of the phase in expression of some clock genes, while in turn this altered clock functioning somehow affects the rate of starch breakdown (Feugier and Satake, 2012; Seki et al., 2017). The retrograde signaling model is supported by studies showing that sugars can regulate circadian rhythms (Haydon et al., 2013; Shin et al., 2017; Frank et al., 2018). For example, low cellular sugar levels lead to a delay in the rhythmic expression of the clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Haydon et al., 2013; Frank et al., 2018). This is through negative regulation of *CCA1* expression by a signaling cascade that includes PRR7, bZIP63 and the KIN10 (SnRK1.1) subunit of the energy sensor SnRK1, which is activated under conditions of low sugar availability (CH starvation) (Frank et al., 2018). The activation of SnRK1 in response to CH starvation is the result of relieved suppression of SnRK1 activity by T6P, a marker for sugar availability (Zhang et al., 2009; Mair et al., 2015; Figueroa and Lunn, 2016). Consistent with this retrograde signaling model, we also showed that the delayed phase in rhythmic expression of morning clock gene *CCA1* under -DIF coincides with a CH starvation status, as indicated by induction of the CH starvation marker genes (e.g. *DARK-INDUCIBLE 6* (*DIN6*/*ASN1*)) at the end of the night (Chapter 3).

CH starvation links to clock-suppression of *PIF4* transcription and reduced PIF4 stability, thus explaining reduced growth under -DIF

As described above, the altered clock gene expression under -DIF links to the CH starvation status at the end of the night. The altered clock gene expression may provide another explanation for decreased PIF protein activity under -DIF, as this may lead to increased levels of the clock proteins PRR5/7/9 and TOC1. It has been shown that these clock components are able to interact with PIFs and possibly directly bind to promoter

regions of PIF-induced target genes, thereby repressing transcriptional activity of PIFs (Soy et al., 2016; Zhu et al., 2016; Martin et al., 2018). The repression of PIF transcriptional activity by the clock results in restriction of PIF-mediated growth responses to specific times of the day, at which these clock components are not expressed (Soy et al., 2016; Zhu et al., 2016). The increased amplitude and delayed phase of *PRR9* expression and the increased amplitude and advanced phase of *TOC1* expression under -DIF potentially leads to PRR9 and TOC1 protein levels being higher and present during a longer period of the day. This would shorten the window for potential PIF transcriptional activity during the day and may explain the reduced PIF-mediated growth under -DIF. In addition to negatively regulating *PIF4*/*5* transcription through interaction with other members of the EC, the clock component ELF3 also interacts with PIF proteins in an EC-independent manner and prevents PIF4 from activating its target genes (Nieto et al., 2015). Since ELF3 plays such an important role in the regulation of PIFs on both transcriptional and protein levels, it would be interesting to see whether the expression of *ELF3* is also affected by -DIF. It is possible that, like the other evening-phased clock genes (e.g. *GI* and *TOC1*), *ELF3* and other members of the EC show an advanced phase in expression. An advanced phase in expression of EC components would also partially explain the reduced PIF4 transcription under -DIF (Figure 1; Chapter 3).

Recently it was found that CH starvation may also affect stability of the PIF4 protein itself. This was discovered in the context of thermomorphogenesis: while heat stimulates hypocotyl elongation, this elongation response is inhibited when sugar levels are low. When cellular sugar levels are low, levels of the sugar signaling molecule T6P are reduced, which allows for the KIN10 subunit of the SnRK1 complex to be activated by GEMINIVIRUS REP-INTERACTING KINASE 1 (GRIK1) (Hwang et al., 2019). The activated KIN10 then phosphorylates PIF4, which is subsequently targeted for degradation (Hwang et al., 2019). Assuming that this mechanism also operates at lower temperatures, this suggests that KIN10 is more active during -DIF-induced carbohydrate starvation at EN, resulting in enhanced degradation of PIF4. This model provides a mechanistic link between CH starvation and reduced elongation under -DIF. Suppression of PIF protein abundance in response to CH starvation is consistent with reduced expression of PIF4-target genes under -DIF at EN (Chapter 3).

While SnRK1 is involved in growth suppression under low energy conditions, the TORC1 complex is involved in the stimulation of growth under high energy conditions. TORC1 activity is stimulated by T6P and suppressed in response to CH starvation, probably through phosphorylation of the RAPTOR (regulatory-associated protein of TOR) subunit of TORC1 by SnRK1 (Lastdrager et al., 2014; Nukarinen et al., 2016; Shi et al., 2018). It was shown that TORC1 stimulates growth through preventing BZR1 degradation, which allows for BZR1-induced *PIF4* expression and BZR1 interacting with PIF4 proteins to positively regulate its transcriptional activity (Zhang et al., 2016). Therefore, the induced EN CH starvation under -DIF may also reduce *PIF* transcription and PIF protein activity indirectly through reduced TORC1 activity, leading to reduced BZR1 abundance.

Manipulating growth with timed light pulses

Besides a fundamental understanding of the light and temperature regulation of growth, the other, more applied goal of the project was to come up with new ways to control plant growth in greenhouses in order to avoid the use of chemical plant growth retardants (PGRs). The -DIF regime is commonly used in horticulture to control plant growth, but cannot be realized throughout the year. As previous research and results presented in this thesis show, the growth suppression in response to a -DIF regime is associated with reduced expression of *PIF* genes and PIF-induced target genes (e.g. *IAA29*) (Chapter 3; (Bours et al., 2015)). We studied whether added LED light of specific wavelength can be used to manipulate expression of *PIF*s and PIF target genes (Chapter 2 and 5). Since both *PIF* transcription and PIF protein activity is affected by its antagonist HY5, we also studied the response of the *HY5* gene to selected light treatments (Chapter 2 and 5). Pulses of LED light can trigger responses of PIFs and PIF target genes in less than 15 minutes (Salter et al., 2003; Soy et al., 2016), but we show that there are also long-term effect of added light treatments (Chapter 2 and 5). Such long-term effects on transcriptional activity need to be considered to fully appreciate the potential effect on elongation. However, our data presented in Chapter 5 also show that integrated *PIF4::LUC* transcriptional activity does not correlate with the integrated response of PIF4 targets (e.g. *IAA29::LUC* expression). This is explained by all the different post-transcriptional effects on PIF protein activity (See Figure 1). Such post-transcriptional effects may have been captured by a *PIF4::PIF4-LUC* reporter construct, which includes effects on stability of the *PIF4::LUC* fusion protein. However, activity of such a reporter was too low in rosette plants to be of use in LUMINATOR. The HY5 protein promotes its own expression and we can therefore interpret *HY5::LUC* expression as representative for HY5 protein activity. Because HY5 negatively regulates *PIF4* expression, the activity of the *PIF4::LUC* reporter also integrates HY5 protein accumulation/activity. This influence of HY5 on *PIF4* gene activity is confirmed by the opposite response of the *HY5::LUC* and *PIF4::LUC* reporters to added B light at start day and end day (Chapter 2 and 5).

Both light and temperature responses are gated by the clock, while light and temperature signals also both entrain the clock. Gating of light responses under +DIF may therefore by slightly different to gating of light responses under -DIF. Cold acclimation responses leading to growth suppression are gated towards beginning of the day, as *CBF* transcription is only increased during this period in response to cold (Fowler et al., 2005). Presumably this is the result of suppression of *CBF* expression in the evening by clock components PRR5, 7 and 9, TOC1 and the EC (Nakamichi et al., 2009; Keily et al., 2013), while the morning clock components LHY and CCA1 stimulate *CBF* transcription (Dong et al., 2011). Gating of cold responses towards the day may also be caused by the fact that HY5, which is degraded in the dark, plays an essential role in cold acclimation (Catala et al., 2011). The gating of growth responses to low temperature to the day period is consistent with the fact that a -DIF regime leads to suppression of growth, while a +DIF regime does not. The gating of growth responses to temperature may be a consequence of the gating of transcriptional responses to temperature cues. This is supported by our transcriptomics data in Chapter 3, which showed that many genes that facilitate cell

elongation (e.g. *PIF4*, *PIP*s, and *EXPA*s) are only differentially expressed at one timepoint under -DIF, indicating their response to temperature is time-of-day-dependent. Below I briefly discuss how the response to red (R), blue (B) or R+B light pulses is gated:

Gated response to added R+B (no light ramping): The experiments for testing the response of PIFs to added LED light are complicated by the gating of transcriptional responses to light signals by the endogenous clock (Salter et al., 2003; Soy et al., 2016). Instead of providing additional LED light throughout the entire day, we provided one-hour additional light pulses during the morning or evening to test the effect on expression of *PIF3*/*4*/*5*, *IAA29* and *HY5*. Under control conditions in LUMINATOR, the intensities of R and B light were reduced during the first and last hour of the photoperiod to roughly mimic natural changes in light intensity and quality at dawn and dusk. Conditions during these hours are referred to as light ramping conditions in this thesis. In Chapter 2 we show that adding both B and R light during the first hour and last hour of the photoperiod (no light ramping) leads to an immediate decrease in *PIF4::LUC* activity, while *HY5::LUC* was increased. Although the added R+B light in the morning triggered a strong short term expression response for *PIF4* and *HY5*, there was no significant effect on the integrated *PIF4::LUC* and *HY5::LUC* activity over the day. The direct response in *PIF4*/*HY5* promoter activity to the added light at the end of the day was very limited. However, analysis of the integral *PIF4::LUC* activity during the subsequent night suggests that long term transcription of *PIF4* during the night is reduced (Chapter 2). As PIF protein is more stable in the dark, this suggests a lower PIF-induced elongation activity during the night after the R+B evening pulse. The responses of *HY5* and *PIF4* activity to the added R+B light at dawn and dusk (no light ramping) lead to the prediction that such as light regime may be used to suppress plant growth. Indeed, young tomato plants grown under +DIF (L25°C/D15°C) conditions for two weeks under a light regime without light ramping in SOLINATOR climate cabinets developed shorter stems than plants grown under a ramping light regime mimicking natural changes in light intensity and quality during the day.

Gated response to added B: In Chapter 5 we show that added B light during the first hour of the day, but not during the last hour, results in immediate downregulation of *PIF4* transcription as *PIF4::LUC* activity and endogenous *PIF4* mRNA transcript levels were decreased. A similar response was observed for *PIF3*, *PIF5* and *IAA29*. Consistent with a reciprocal regulation between PIF4 and HY5, the *HY5::LUC* activity and *HY5* mRNA transcript levels were upregulated in response to added B light. Besides the immediate short-term response, added B light leads to a decrease in integral *PIF4::LUC* activity during the photoperiod and an increase in integral *HY5::LUC* activity during photoperiod and dark period. The gated response to added B light (response in morning, no response in evening) may be related to the effect of B light on HY5. B light activates CRY1 and activated CRY1 inhibits formation of the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)-SUPPRESSOR OF PHYA (SPA) complex, which targets HY5 for degradation (Lian et al., 2011; Liu et al., 2011). In addition, activated CRY1 positively regulates HY5 transcriptional activity by preventing the binding of AGB1 (β-subunit of G-protein complex), to HY5, which blocks HY5 transcriptional activity (Lian et al., 2018). Since *HY5* expression peaks in the morning, this would explain the strongest effect of B light on *HY5*

in the morning. The increased HY5 protein levels can subsequently reduce *PIF* transcript levels and PIF protein activity when provided during the first hour of the photoperiod.

The gating of the growth response to added B light pulses was tested in SOLINATOR on Arabidopsis and tomato plants grown under +DIF (L22°C/D12°C for Arabidopsis; L25°C/D15°C for tomato). Consistent with gating of the transcriptional response of *HY5*, added B light in the evening alone (B^e) had no effect on growth of Arabidopsis, while only providing added B light in the morning (B^m) was as efficient as providing added B light in the morning and evening (B^{m+e}) in reducing growth (Figure 5). In tomato B^m, but not B^{m+e} or B^e, leads to a significant reduction in stem length (Figure 6B). Although the B^e treatment did not lead to a reduction in stem length, leaf area was reduced, suggesting that these two growth parameters may be regulated, at least partially, through different signaling pathways. To see whether the effect of added R/B light pulse treatments on plant growth is dependent on temperature conditions, light treatments in SOLINATOR were also provided in combination with a -DIF regime (L12°C/D22°C for Arabidopsis; L15°C/D25°C for tomato). The results for Arabidopsis show that -DIF leads to reduced growth and that, like under +DIF conditions, the B^{m+e} and B^m treatments lead to a reduction in growth under -DIF (Figure 5). Thus, B^{m+e} and B^m appear to enhance the growth suppressing effect of -DIF. However, in tomato, none of the tested additional B light treatments lead to a reduction in plant height when the plants were grown under -DIF conditions (Figure 6B+D). In fact, B^{m+e} and B^m actually lead to an increase in tomato stem length under -DIF in SOLINATOR. Assuming that, like in Arabidopsis, suppression of growth by both -DIF and added light pulses in tomato is linked to downregulation of PIFs, it is possible that downregulation of PIF activity is already maximized under -DIF and therefore does not allow for additional regulation by light pulses. That only in tomato additional light is not able to enhance the effect of -DIF would be consistent with Arabidopsis being less sensitive to low temperatures than tomato (Weiss and Egea-Cortines, 2009).

Gated response to added R: one hour of added R light in morning or evening did not affect *PIF::LUC* or *HY5::LUC* activity, nor did it affect endogenous mRNA transcript levels of *PIF3*/*4*/*5* and *HY5*. Thus, the responses of *PIF4* and *HY5* to added R+B light in the morning (Chapter 2) are most likely caused by the fraction of B light. However, the *IAA29::LUC* reporter, as well as *IAA29* mRNA transcript levels, show a very strong downregulation to the added R light pulse in the evening, suggesting that added R light reduces PIF protein activity during the night. The added R light increases the R:FR ratio, leading to an increased pool of active PHYB Pfr which targets PIFs for degradation. Consequently, expression of PIF-induced target genes such as *IAA29* is reduced. Interestingly, the *IAA29::LUC* activity is reduced throughout the night in response to the added R light pulse in the evening, indicating the effectiveness of this short light treatment on long term PIF activity in the dark. This suggest that without added R light the PIF activity during the night is mainly from PIFs produced at the end of day (peak in *PIF4* expression), because *PIF4*/*5* transcription is strongly repressed during the night under a 12hL/12hD diurnal regime (Chapter 2; (Nusinow et al., 2011)). Added R light for suppression of PIF activity is therefore most effective shortly following the peak in *PIF* transcription. Previous studies with Arabidopsis also showed that the growth response of

seedlings to changes in the R:FR light ratio is strongest when applied at dusk (Salter et al., 2003; Mizuno et al., 2015; Soy et al., 2016). Like for the added B light treatments, gating of the growth response of tomato to added R light was tested under +DIF or -DIF conditions in SOLINATOR. Added R light in the morning (R^m) did not affect stem elongation in tomato, while both added R light in the evening alone (R^e) and added R light in the morning and evening (R^{m+e}) leads to a reduction in stem elongation (Figure 6A).

It was shown that increased intensities of R light enhanced the effect of -DIF on growth of *Campanula isophylla* (Moe et al., 1991). However, in tomato, none of the tested additional R light treatments lead to a reduction in plant height when the plants were grown under -DIF conditions in SOLINATOR (Figure 6A+C).

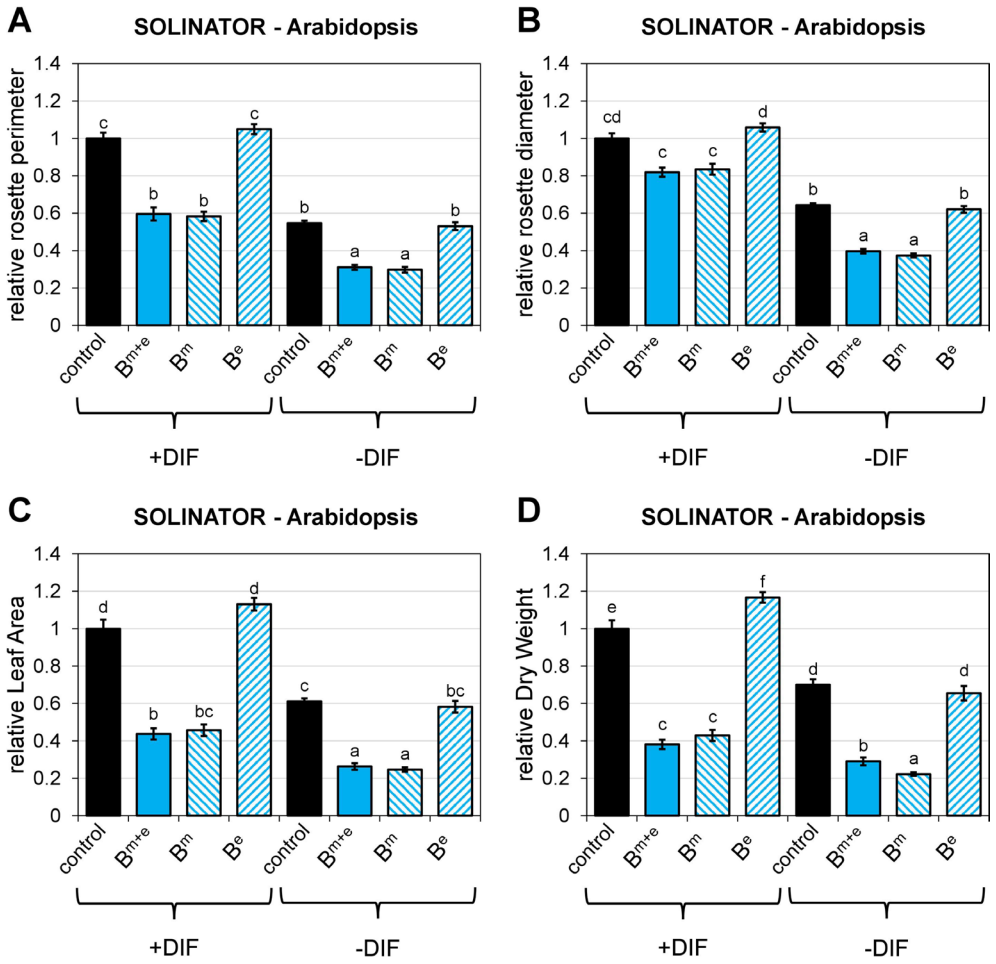


Figure 5: Effect added blue light pulses on growth of Arabidopsis in SOLINATOR. Relative rosette perimeter (**A**), diameter (**B**), leaf area (**C**) and shoot dry weight (**D**) of 31-day-old Arabidopsis Col-0 WT plants that were grown for 10 days in SOLINATOR climate cabinets under +DIF conditions (12hL 22°C / 12hD 12°C) or -DIF conditions (12hL 12°C / 12hD 22°C) and subjected to different additional blue light treatments. Plants received one hour of additional blue light (50 µmole m^{-2} s^{-1}) at both the start and end of the photoperiod (B^{m+e}), only at the start of the photoperiod (B^{m}), only at the end of the photoperiod (B^{e}), or they received no additional light (control). Data are mean values, normalized to values for plants grown under control conditions (n=15) ± SE. Different letters indicate significant differences between means across light and temperature treatments (Fisher's protected least significant difference test, p<0,05; Dunn's post hoc test, α=5%).

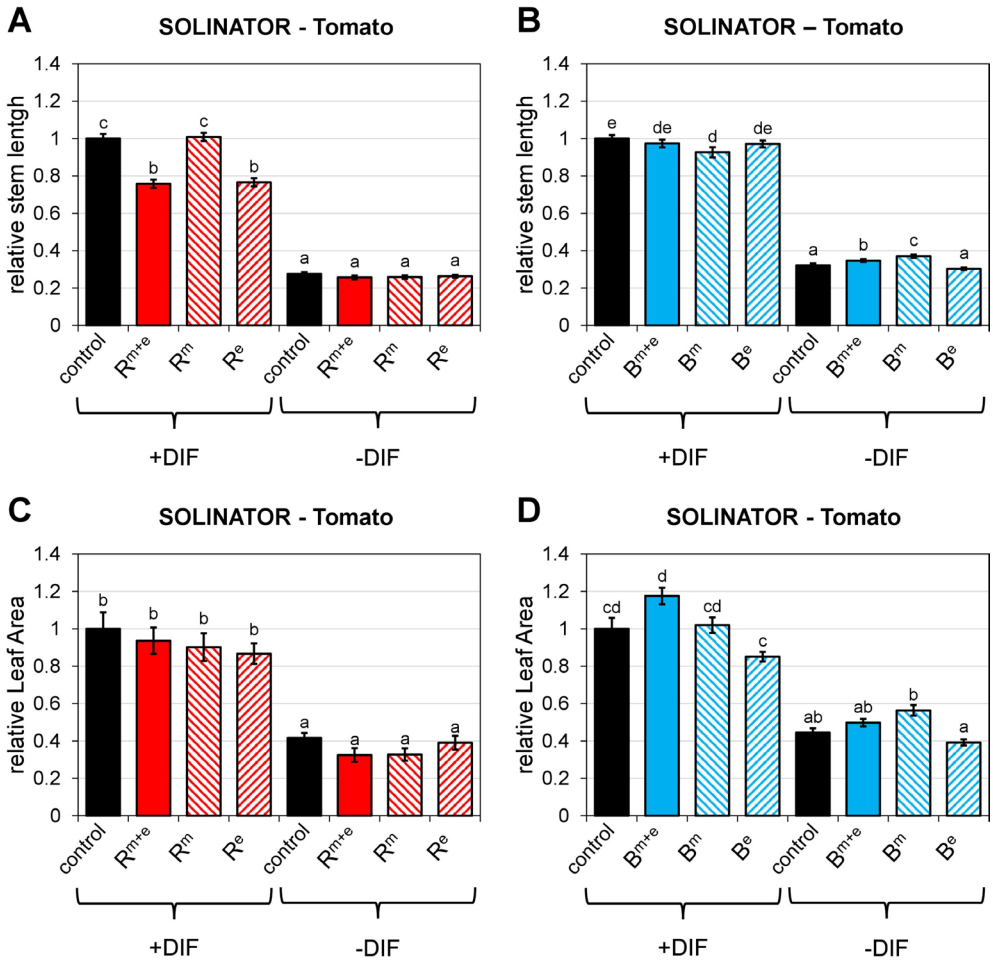


Figure 6: Effect added red or blue light pulses on growth of tomato in SOLINATOR. Relative stem length (**A**+**B**) and leaf area (**C**+**D**) of 22-day-old tomato plants (*Solanum lycopersicum* cv. 'Money maker') that were grown for two weeks in SOLINATOR cabinets under +DIF conditions (12hL 25°C / 12hD 15°C) or -DIF conditions (12hL 15°C / 12hD 25°C) and subjected to different additional light treatments. Plants received one hour of additional red or blue light (50 µmole m^{-2} s^{-1}) at both the start and end of the photoperiod (R^{m+e}/B^{m+e}), only at the start of the photoperiod (R^{m}/B^{m}), only at the end of the photoperiod (R^{e}/R^{e}), or they received no additional light (control). Data are mean values, normalized to values for plants grown under control conditions (n=15) ± SE. Different letters indicate significant differences between means across light and temperature treatments (Fisher's protected least significant difference test, p<0,05; Dunn's post hoc test, α=5%).

Translating laboratory results to practice: novel growth protocols

Our experiments have yielded several suggestions for manipulation of plant growth in greenhouses through added LED light treatments. The experiments that lead to these recommendations were mostly done at light intensities commonly present in greenhouses. However, we have also learned throughout this project that the

effectiveness of some treatments is also a function of light intensity and not only a function of light quality. This may be especially relevant for protocols that function by induction of a CH starvation status. When plants are grown under high light intensity, so that starch reserves at the end of the day are very high, it may take more extreme measures to push the plant into a CH starvation status at EN.

Timed added B and R light pulses to control plant growth

In Chapter 5 we show that growth of tomato plants in greenhouses can be reduced by using additional R or B LED light for only one hour at the start and end of the day. However, the intensity of the added LED light should not be too high (DLI added LED light <6% of the total DLI) and this light treatment does not work in combination with -DIF (low day temperature). Thus, at the end of this project we conclude that one of the initial aims: enhancing the effect of -DIF by light treatments, did not work in the greenhouse. However, the project did provide alternatives for -DIF that can be used under normal +DIF conditions and therefore may be of more practical use. The greenhouse experiments were conducted in early spring, at relatively low light intensities and with a photoperiod of 12-13 hours. Future experiments should be conducted to determine whether one hour of additional B or R light at the start and end of the day also suppresses plant growth under higher light intensities or longer days, when it may require higher intensities of additional LED light to change light composition and growth responses to light cues may be restricted to other moments within the photoperiod.

Controlling growth through induced CH starvation by intermittent extended night

We have shown in different ways that inducing a CH Starvation status inhibits growth (Chapter 4). This concept has been applied to control plant growth under +DIF conditions in greenhouses, by intermittently extending the night by 3 hours. Indeed, this does reduce leaf elongation in Arabidopsis (Chapter 4). Moreover, the intermitted extended night may be combined with the -DIF treatment to enhance the CH starvation status reached by -DIF, especially during periods of the year that -DIF regime cannot be fully realized. This is something that can be tested in commercial setting of greenhouses. However, it should be noted that whether plant gets into CH starvation due to higher night-temperature (like under -DIF) or an extended night depends on the amount of starch reserves at the onset of night. Since light intensity and daylength are important determinants of starch accumulation, the effectiveness of -DIF and intermittent extended night treatments may depend on the light intensity and/or day-lengths during the treatments.

Controlling growth through induced CH starvation by spraying with trehalose

Several studies show that increasing T6P levels by exogenous application of trehalose inhibits growth. This may be partially due to limiting the ability of plants to mobilize starch (Wingler et al., 2000; Schluepmann et al., 2004; Martins et al., 2013). We show that

spraying leaves with trehalose indeed increased starch accumulation and suppressed growth (Chapter 4), suggesting that this can be used to reduce plant growth in greenhouses. However, as T6P is a marker for sugar availability and positively regulates growth through suppressing SnRK1 and activating TORC1 (Lastdrager et al., 2014), plant growth may actually be increased in response to exogenous application of trehalose under conditions where sugar production from starch is not limiting for growth (e.g. under long days or higher light intensity).

Future perspectives

Combined treatments?

The results in Figure 6 show that, for reducing stem length in tomato, added B light is only effective in the morning and added R light is only effective in the evening. Therefore, a combination of additional B light in the morning and additional R light in the evening may be especially effective in suppressing plant growth. Additional light treatments above may also be used in combination with other treatments mentioned above. However, it is not sure whether combined treatments are actually more effective in controlling plant growth. For instance, changing the CH status of the plant may change time-window during which light cues can affect plant growth.

Better reporters?

A substantial part of the research described in this thesis focused on studying the regulation of key growth-regulating genes (*PIF3*/*4*/*5*, *IAA29*, *HY5*) by timed additional LED light pulses using ffLUC reporter plants in combination with LUMINATOR. We showed that using our ffLUC reporters can help identify light treatments that may be used to control plant growth in greenhouses within a couple of days. But we also learned that it may be more useful to use more reporters of PIF target genes, as activity of these integrate the transcriptional and post-transcriptional regulation of PIFs. During this project we also developed *YUC8::LUC* reporter lines, but these could not be used in our experiments due to its weak activity in adult plants. A reporter for the α-expansin gene *EXP8* would also potentially be a good marker for plant growth, providing that its expression is strong enough in adult plants.

A reporter for this gene would especially be informative since PIFs, BZR1 and HY5 are all able to bind the promoter of *EXP8* and while PIFs and BZR1 activate its expression, HY5 blocks its expression (Bours et al., 2015; Gangappa and Kumar, 2017). As a result, an *EXP8::LUC* reporter may provide integrated information on protein dynamics of PIFs, BZR1, and HY5.

UV-B for growth control?

Here we only tested whether expression of growth-regulating genes could be altered by additional R+B/R/B light pulses of one hour at the start and end of the day. However, in

the search for novel (light) treatments for growing compact plants, it would also be interesting to provide additional light at other timepoints, or provide light of different wavelengths, depending on the time of the day. As UV-B light has been shown to be involved in the suppression of cell elongation through HY5 stabilization (Yin and Ulm, 2017), LUMINATOR may also be used to study the genetic responses to additional UV-B light treatments in order to identify potentially effective UV-B light treatments for the control of plant growth.

Mineral transport at night?

Carbohydrates that are mobilized for growth are of no use when no micronutrients (e.g. Zn, Mg, Fe) are available to produce the building blocks like amino acids and lipids. It is possible that the controlled mobilization of starch during the night is actually somehow coupled to the local availability of micronutrients. In this view, transport of micronutrients may be more effective during the unnatural warm night of -DIF, causing an unnatural enhanced utilization of starch during the night which leads to a CH starvation mode. If the mobilization of starch at night is indeed coupled to local micronutrient availability, and such availability of micronutrients is indeed regulated by photoperiod, this suggests that the rate of starch mobilization may be manipulated through by-passing natural micronutrient transport. This may be tested by spraying leaves with micronutrients at the onset of the night. We have performed preliminary experiments using rosette plants expressing the CH starvation reporter grown under +DIF. When grown under +DIF the starvation reporter is hardly active at the EN under +DIF, confirming that photoperiod control of starch mobilization prevents reaching a CH starvation status. However, after a night at which leaves were sprayed with micronutrients the plants do show upregulation of this reporter at the end of the night, indicating that this treatment can induce a starvation status under +DIF. Whether this in the long run also inhibits growth still needs to be determined, since the extra nutrients on the leaf may actually stimulate growth more during the subsequent day.

Extended use of LUMINATOR

During this thesis project LUMINATOR was used for identification of light treatments that have a desired effect on growth-regulating genes. Using the proper reporters, the system may also be used to search for light treatments that potentially can be used to control other physiological/developmental processes such as flowering or plant resilience. This is now being realized in a follow up project at PPH named ‘LEDs make it resilient’, in which PhD candidate Mara Meisenburg is investigating how light treatments may be used to enhance plant defense responses against herbivores.

Concluding remarks

This thesis project aimed at increasing our understanding of the underlying molecular mechanisms through which plant growth is suppressed under -DIF, while another aim was to use acquired insights to develop new growth protocols for greenhouses. The collaboration with the Horticulture and Production Physiology group and the willingness of several companies to facilitate on-site trial experiments provided us with the unique possibility to test and validate some of these protocols. We showed that a temporary induction of CH starvation status is linked with a reduction in plant growth, regardless of how CH starvation is reached. This insight was key to several of novel protocols that were suggested to be used in greenhouses. The key role of CH status also explains how PAR light intensity may modulate and restrict the application of some of the growth protocols based on CH starvation status. The work presented in this thesis demonstrates the effectiveness of designing protocols based on fundamental insights compared to empiric testing of growth protocols.

References

Abbas N, Maurya JP, Senapati D, Gangappa SN, Chattopadhyay S (2014) Arabidopsis CAM7 and HY5 physically interact and directly bind to the HY5 promoter to regulate its expression and thereby promote photomorphogenesis. Plant Cell 26: 1036-1052

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 20: 2117-2129

Alonso-Peral MM, Li J, Li Y, Allen RS, Schnippenkoetter W, Ohms S, White RG, Millar AA (2010) The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in Arabidopsis. Plant Physiol 154: 757-771

Arnholdt-Schmitt B, Costa JH, de Melo DF (2006) AOX–a functional marker for efficient cell reprogramming under stress? Trends in plant science 11: 281-287

Atkin OK, Loveys BR, Atkinson LJ, Pons TL (2006) Phenotypic plasticity and growth temperature: understanding interspecific variability. J Exp Bot 57: 267-281

Ayre BG (2011) Membrane-transport systems for sucrose in relation to whole-plant carbon partitioning. Mol Plant 4: 377-394

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448: 938-942

Baena-Gonzalez E, Sheen J (2008) Convergent energy and stress signaling. Trends Plant Sci 13: 474-482

Bantis F, Smirnakou S, Ouzounis T, Koukounaras A, Ntagkas N, Radoglou K (2018) Current status and recent achievements in the field of horticulture with the use of light-emitting diodes (LEDs). Scientia Horticulturae 235: 437-451

Batista-Silva W, Medeiros DB, Rodrigues-Salvador A, Daloso DM, Omena-Garcia RP, Oliveira FS, Pino LE, Peres LEP, Nunes-Nesi A, Fernie AR, Zsogon A, Araujo WL (2019) Modulation of auxin signalling through DIAGETROPICA and ENTIRE differentially affects tomato plant growth via changes in photosynthetic and mitochondrial metabolism. Plant Cell Environ 42: 448-465

Bergstrand K-JI (2017) Methods for growth regulation of greenhouse produced ornamental pot- and bedding plants – a current review. Folia Horticulturae 29: 63-74

Bernardo-Garcia S, de Lucas M, Martinez C, Espinosa-Ruiz A, Daviere JM, Prat S (2014) BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth. Genes Dev 28: 1681-1694

Binkert M, Kozma-Bognar L, Terecskei K, De Veylder L, Nagy F, Ulm R (2014) UV-B-responsive association of the Arabidopsis bZIP transcription factor ELONGATED HYPOCOTYL5 with target genes, including its own promoter. Plant Cell 26: 4200-4213

Blasing OE, Gibon Y, Gunther M, Hohne M, Morcuende R, Osuna D, Thimm O, Usadel B, Scheible WR, Stitt M (2005) Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in Arabidopsis. Plant Cell 17: 3257-3281

Bordage S, Sullivan S, Laird J, Millar AJ, Nimmo HG (2016) Organ specificity in the plant circadian system is explained by different light inputs to the shoot and root clocks. New Phytol 212: 136-149

Bours R (2014) Antiphase light and temperature cycles disrupt rhythmic plant growth: the Arabidopsis jetlag.

Bours R, Kohlen W, Bouwmeester HJ, van der Krol A (2015) Thermoperiodic control of hypocotyl elongation depends on auxin-induced ethylene signaling that controls downstream PHYTOCHROME INTERACTING FACTOR3 activity. Plant Physiol 167: 517-530

Bours R, Muthuraman M, Bouwmeester H, Van der Krol A (2012) OSCILLATOR: A system for analysis of diurnal leaf growth using infrared photography combined with wavelet transformation. Plant Methods 8: 1-12

Bours R, van Zanten M, Pierik R, Bouwmeester H, van der Krol A (2013) Antiphase light and temperature cycles affect PHYTOCHROME B-controlled ethylene sensitivity and biosynthesis, limiting leaf movement and growth of Arabidopsis. Plant Physiol 163: 882-895

Braidwood L, Breuer C, Sugimoto K (2014) My body is a cage: mechanisms and modulation of plant cell growth. New Phytol 201: 388-402

Buwalda F, Kempkes FLK, Pronk AA, Dieleman JA, Janse J, Heij G (2007) Temperatuurverlaging in de ochtend. *In*. Wageningen UR Glastuinbouw, Wageningen

Cao S, Song Y, Su L (2007) Freezing sensitivity in the gigantea mutant of Arabidopsis is associated with sugar deficiency. Biologia plantarum 51: 359-362

Cao S, Ye M, Jiang S (2005) Involvement of GIGANTEA gene in the regulation of the cold stress response in Arabidopsis. Plant Cell Rep 24: 683-690

Carvalho S, van Noort F, Postma R, Heuvelink E (2008) Possibilities for producing compact floricultural crops. *In*. Wageningen UR Greenhouse Horticulture

Casal JJ, Balasubramanian S (2019) Thermomorphogenesis. Annual review of plant biology 70: 321-346

Caspar T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of Arabidopsis thaliana (L.) deficient in chloroplast phosphoglucomutase activity. Plant Physiology 79: 11-17

Caspar T, Lin T-P, Kakefuda G, Benbow L, Preiss J, Somerville C (1991) Mutants of Arabidopsis with altered regulation of starch degradation. Plant physiology 95: 1181-1188

Catala R, Medina J, Salinas J (2011) Integration of low temperature and light signaling during cold acclimation response in Arabidopsis. Proc Natl Acad Sci U S A 108: 16475-16480

Cha JY, Kim J, Kim TS, Zeng Q, Wang L, Lee SY, Kim WY, Somers DE (2017) GIGANTEA is a co-chaperone which facilitates maturation of ZEITLUPE in the Arabidopsis circadian clock. Nat Commun 8: 3

Chattopadhyay S, Ang L-H, Puente P, Deng X-W, Wei N (1998) Arabidopsis bZIP Protein HY5 Directly Interacts with Light-Responsive Promoters in Mediating Light Control of Gene Expression. The Plant Cell 10: 673-683

Chaumont F, Tyerman SD (2014) Aquaporins: highly regulated channels controlling plant water relations. Plant Physiol 164: 1600-1618

Chen D, Xu G, Tang W, Jing Y, Ji Q, Fei Z, Lin R (2013) Antagonistic basic helix-loop-helix/bZIP transcription factors form transcriptional modules that integrate light and reactive oxygen species signaling in Arabidopsis. Plant Cell 25: 1657-1673

Chen L-Q, Qu X-Q, Hou B-H, Sosso D, Osorio S, Fernie AR, Frommer WB (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science 335: 207-211

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12: 444-451

Cho H-T, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 97: 9783-9788

Cho H-T, Cosgrove DJ (2002) Regulation of root hair initiation and expansin gene expression in Arabidopsis. The Plant Cell 14: 3237-3253

Clack T, Mathews S, Sharrock RA (1994) The phytochrome apoprotein family inArabidopsis is encoded by five genes: the sequences and expression of PHYD and PHYE. Plant molecular biology 25: 413-427

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The plant journal 16: 735-743

Cosgrove DJ (2000) Loosening of plant cell walls by expansins. Nature 407: 321

Covington MF, Harmer SL (2007) The circadian clock regulates auxin signaling and responses in Arabidopsis. PLoS Biol 5: e222

Craig DS, Runkle ES (2016) An intermediate phytochrome photoequilibria from night-interruption lighting optimally promotes flowering of several long-day plants. Environmental and Experimental Botany 121: 132-138

Crawford AJ, McLachlan DH, Hetherington AM, Franklin KA (2012) High temperature exposure increases plant cooling capacity. Current Biology 22: R396-R397

Crevillen P, Ventriglia T, Pinto F, Orea A, Merida A, Romero JM (2005) Differential pattern of expression and sugar regulation of Arabidopsis thaliana ADP-glucose pyrophosphorylase-encoding genes. J Biol Chem 280: 8143-8149

Cuijpers LHM, Vogelezang JVM (1992) DIF and temperature drop for short-day pot plants. Acta Horticulturae 327: 25-32

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic acids research 39: W155-W159

de Lucas M, Daviere JM, Rodriguez-Falcon M, Pontin M, Iglesias-Pedraz JM, Lorrain S, Fankhauser C, Blazquez MA, Titarenko E, Prat S (2008) A molecular framework for light and gibberellin control of cell elongation. Nature 451: 480-484

de Lucas M, Prat S (2014) PIFs get BRright: PHYTOCHROME INTERACTING FACTORs as integrators of light and hormonal signals. New Phytol 202: 1126-1141

de Montaigu A, Toth R, Coupland G (2010) Plant development goes like clockwork. Trends Genet 26: 296-306

De Wet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli. Proceedings of the National Academy of Sciences 82: 7870-7873

de Wit M, Keuskamp DH, Bongers FJ, Hornitschek P, Gommers CMM, Reinen E, Martinez-Ceron C, Fankhauser C, Pierik R (2016) Integration of Phytochrome and Cryptochrome Signals Determines Plant Growth during Competition for Light. Curr Biol 26: 3320-3326

Delker C, Sonntag L, James GV, Janitza P, Ibanez C, Ziermann H, Peterson T, Denk K, Mull S, Ziegler J, Davis SJ, Schneeberger K, Quint M (2014) The DET1-COP1-HY5

pathway constitutes a multipurpose signaling module regulating plant photomorphogenesis and thermomorphogenesis. Cell Rep 9: 1983-1989
Di Ferdinando M, Brunetti C, Fini A, Tattini M (2012) Flavonoids as antioxidants in plants under abiotic stresses. *In* Abiotic stress responses in plants. Springer, pp 159-179
Dong MA, Farre EM, Thomashow MF (2011) Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis. Proc Natl Acad Sci U S A 108: 7241-7246
Dowson-Day MJ, Millar AJ (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis. The Plant Journal 17: 63-71
Edwards KD, Akman OE, Knox K, Lumsden PJ, Thomson AW, Brown PE, Pokhilko A, Kozma-Bognar L, Nagy F, Rand DA, Millar AJ (2010) Quantitative analysis of regulatory flexibility under changing environmental conditions. Mol Syst Biol 6: 424
Eimert K, Wang S-M, Lue W, Chen J (1995) Monogenic recessive mutations causing both late floral initiation and excess starch accumulation in Arabidopsis. The Plant Cell 7: 1703-1712
Erwin JE, Heins RD, Karlsson MG (1989) Thermomorphogenesis in Lilium longiflorum. American Journal of Botany 76: 47-52
Fankhauser C, Batschauer A (2016) Shadow on the Plant: A Strategy to Exit. Cell 164: 15-17
Feeney KA, Putker M, Brancaccio M, O'Neill JS (2016) In-depth Characterization of Firefly Luciferase as a Reporter of Circadian Gene Expression in Mammalian Cells. J Biol Rhythms 31: 540-550
Feike D, Seung D, Graf A, Bischof S, Ellick T, Coiro M, Soyk S, Eicke S, Mettler-Altmann T, Lu KJ, Trick M, Zeeman SC, Smith AM (2016) The Starch Granule-Associated Protein EARLY STARVATION1 Is Required for the Control of Starch Degradation in Arabidopsis thaliana Leaves. Plant Cell 28: 1472-1489
Feng S, Martinez C, Gusmaroli G, Wang Y, Zhou J, Wang F, Chen L, Yu L, Iglesias-Pedraz JM, Kircher S, Schafer E, Fu X, Fan LM, Deng XW (2008) Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. Nature 451: 475-479
Feugier FG, Satake A (2012) Dynamical feedback between circadian clock and sucrose availability explains adaptive response of starch metabolism to various photoperiods. Front Plant Sci 3: 305
Figueroa CM, Lunn JE (2016) A Tale of Two Sugars: Trehalose 6-Phosphate and Sucrose. Plant Physiol 172: 7-27
Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139: 1795-1805
Flis A, Sulpice R, Seaton DD, Ivakov AA, Liput M, Abel C, Millar AJ, Stitt M (2016) Photoperiod-dependent changes in the phase of core clock transcripts and global transcriptional outputs at dawn and dusk in Arabidopsis. Plant Cell Environ 39: 1955-1981
Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J (1999) GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. The EMBO Journal 18: 4679-4688
Fowler SG, Cook D, Thomashow MF (2005) Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol 137: 961-968

Frank A, Matiolli CC, Viana AJC, Hearn TJ, Kusakina J, Belbin FE, Wells Newman D, Yochikawa A, Cano-Ramirez DL, Chembath A, Cragg-Barber K, Haydon MJ, Hotta CT, Vincentz M, Webb AAR, Dodd AN (2018) Circadian Entrainment in Arabidopsis by the Sugar-Responsive Transcription Factor bZIP63. Curr Biol 28: 2597-2606 e2596

Franklin KA, Lee SH, Patel D, Kumar SV, Spartz AK, Gu C, Ye S, Yu P, Breen G, Cohen JD (2011) Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. Proceedings of the National Academy of Sciences 108: 20231-20235

Fung-Uceda J, Lee K, Seo PJ, Polyn S, De Veylder L, Mas P (2018) The Circadian Clock Sets the Time of DNA Replication Licensing to Regulate Growth in Arabidopsis. Dev Cell 45: 101-113 e104

Furuya M, Schäfer E (1996) Photoperception and signalling of induction reactions by different phytochromes. Trends in Plant Science 1: 301-307

Gangappa SN, Botto JF (2016) The Multifaceted Roles of HY5 in Plant Growth and Development. Mol Plant 9: 1353-1365

Gangappa SN, Kumar SV (2017) DET1 and HY5 Control PIF4-Mediated Thermosensory Elongation Growth through Distinct Mechanisms. Cell Rep 18: 344-351

Georgii E, Jin M, Zhao J, Kanawati B, Schmitt-Kopplin P, Albert A, Winkler JB, Schäffner AR (2017) Relationships between drought, heat and air humidity responses revealed by transcriptome-metabolome co-analysis. BMC plant biology 17: 120

Gibon Y, Blasing OE, Palacios-Rojas N, Pankovic D, Hendriks JH, Fisahn J, Hohne M, Gunther M, Stitt M (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant J 39: 847-862

Gil KE, Park CM (2019) Thermal adaptation and plasticity of the plant circadian clock. New Phytol 221: 1215-1229

Gilmour SJ, Fowler SG, Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant molecular biology 54: 767-781

Goh HH, Sloan J, Dorca-Fornell C, Fleming A (2012) Inducible repression of multiple expansin genes leads to growth suppression during leaf development. Plant Physiol 159: 1759-1770

Gómez LD, Baud S, Gilday A, Li Y, Graham IA (2006) Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. The Plant Journal 46: 69-84

Gonzali S, Pistelli L, De Bellis L, Alpi A (2001) Characterization of two Arabidopsis thaliana fructokinases. Plant Science 160: 1107-1114

Gould PD, Locke JC, Larue C, Southern MM, Davis SJ, Hanano S, Moyle R, Milich R, Putterill J, Millar AJ, Hall A (2006) The molecular basis of temperature compensation in the Arabidopsis circadian clock. Plant Cell 18: 1177-1187

Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. Proc Natl Acad Sci U S A 107: 9458-9463

Graf A, Smith AM (2011) Starch and the clock: the dark side of plant productivity. Trends in Plant Science 16: 169-175

Greenham K, McClung CR (2015) Integrating circadian dynamics with physiological processes in plants. Nat Rev Genet 16: 598-610

Grindal G, Moe R, Junttila O (1998) The role of gibberellin and phytochrome in DIF-mediated stem elongation. *In* XXV International Horticultural Congress, Part 4: Culture Techniques with Special Emphasis on Environmental Implications 514, pp 205-212

Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular cell 30: 214-226

Han X, Yu H, Yuan R, Yang Y, An F, Qin G (2019) Arabidopsis Transcription Factor TCP5 Controls Plant Thermomorphogenesis by Positively Regulating PIF4 Activity. iScience 15: 611-622

Hanano S, Domagalska MA, Nagy F, Davis SJ (2006) Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes to Cells 11: 1381-1392

Hannah MA, Heyer AG, Hincha DK (2005) A global survey of gene regulation during cold acclimation in Arabidopsis thaliana. PLoS Genet 1: e26

Hansen LL, van den Burg HA, van Ooijen G (2017) Sumoylation Contributes to Timekeeping and Temperature Compensation of the Plant Circadian Clock. J Biol Rhythms 32: 560-569

Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nature reviews Molecular cell biology 8: 774

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science 290: 2110-2113

Havaux M, Kloppstech K (2001) The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsisnpq and tt mutants. Planta 213: 953-966

Haydon MJ, Mielczarek O, Frank A, Roman A, Webb AAR (2017) Sucrose and Ethylene Signaling Interact to Modulate the Circadian Clock. Plant Physiol 175: 947-958

Haydon MJ, Mielczarek O, Robertson FC, Hubbard KE, Webb AA (2013) Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. Nature 502: 689-692

Henriques R, Papdi C, Ahmad Z, Bögre L (2018) Circadian Regulation of Plant Growth. *In* Annual Plant Reviews online, pp 1-29

Herrero E, Kolmos E, Bujdoso N, Yuan Y, Wang M, Berns MC, Uhlworm H, Coupland G, Saini R, Jaskolski M, Webb A, Goncalves J, Davis SJ (2012) EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. Plant Cell 24: 428-443

Hsu PY, Harmer SL (2014) Wheels within wheels: the plant circadian system. Trends Plant Sci 19: 240-249

Huq E, Quail PH (2002) PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. The EMBO Journal 21: 2441-2450

Huq E, Tepperman JM, Quail PH (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. Proc Natl Acad Sci U S A 97: 9789-9794

Hwang G, Kim S, Cho JY, Paik I, Kim JI, Oh E (2019) Trehalose-6-phosphate signaling regulates thermoresponsive hypocotyl growth in Arabidopsis thaliana. EMBO Rep: e47828

Ibanez C, Delker C, Martinez C, Burstenbinder K, Janitza P, Lippmann R, Ludwig W, Sun H, James GV, Klecker M, Grossjohann A, Schneeberger K, Prat S, Quint M (2018) Brassinosteroids Dominate Hormonal Regulation of Plant Thermomorphogenesis via BZR1. Curr Biol 28: 303-310 e303

Ibañez C, Poeschl Y, Peterson T, Bellstädt J, Denk K, Gogol-Döring A, Quint M, Delker C (2015) Developmental plasticity of Arabidopsis thaliana accessions across an ambient temperature range. bioRxiv. *In*. Cold Spring Harbor Labs Journals

Izumi M (2019) Roles of the Clock in Controlling Starch Metabolism. Plant Physiol 179: 1441-1443

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280: 104-106

Jang K, Gil Lee H, Jung S-J, Paek N-C, Joon Seo P (2015) The E3 Ubiquitin Ligase COP1 Regulates Thermosensory Flowering by Triggering GI Degradation in Arabidopsis. Scientific Reports 5: 12071

Johansson M, Koster T (2019) On the move through time - a historical review of plant clock research. Plant Biol (Stuttg) 21 Suppl 1: 13-20

Jung J-H, Domijan M, Klose C, Biswas S, Ezer D, Gao M, Khattak AK, Box MS, Charoensawan V, Cortijo S (2016) Phytochromes function as thermosensors in Arabidopsis. Science 354: 886-889

Kami C, Lorrain S, Hornitschek P, Fankhauser C (2010) Light-Regulated Plant Growth and Development. *In* Plant Development, pp 29-66

Kamioka M, Takao S, Suzuki T, Taki K, Higashiyama T, Kinoshita T, Nakamichi N (2016) Direct Repression of Evening Genes by CIRCADIAN CLOCK-ASSOCIATED1 in the Arabidopsis Circadian Clock. Plant Cell 28: 696-711

Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research 28: 27-30

Kaplan F, Guy CL (2004) β-Amylase induction and the protective role of maltose during temperature shock. Plant Physiology 135: 1674-1684

Kaplan F, Guy CL (2005) RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. The Plant Journal 44: 730-743

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature biotechnology 17: 287

Keily J, MacGregor DR, Smith RW, Millar AJ, Halliday KJ, Penfield S (2013) Model selection reveals control of cold signalling by evening-phased components of the plant circadian clock. Plant J 76: 247-257

Keuskamp DH, Sasidharan R, Vos I, Peeters AJ, Voesenek LA, Pierik R (2011) Blue-light-mediated shade avoidance requires combined auxin and brassinosteroid action in Arabidopsis seedlings. Plant J 67: 208-217

Kim H, Kim Y, Yeom M, Lim J, Nam HG (2016) Age-associated circadian period changes in Arabidopsis leaves. J Exp Bot 67: 2665-2673

Knight MR, Knight H (2012) Low-temperature perception leading to gene expression and cold tolerance in higher plants. New Phytol 195: 737-751

Koini MA, Alvey L, Allen T, Tilley CA, Harberd NP, Whitelam GC, Franklin KA (2009) High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. Current Biology 19: 408-413

Koksharov MI, Ugarova NN (2011) Thermostabilization of firefly luciferase by in vivo directed evolution. Protein Eng Des Sel 24: 835-844

Kolachevskaya OO, Sergeeva LI, Floková K, Getman IA, Lomin SN, Alekseeva VV, Rukavtsova EB, Buryanov YI, Romanov GA (2017) Auxin synthesis gene tms1 driven by tuber-specific promoter alters hormonal status of transgenic potato plants and their responses to exogenous phytohormones. Plant cell reports 36: 419-435

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proceedings of the National Academy of Sciences 102: 11118-11123

Kolling K, Thalmann M, Muller A, Jenny C, Zeeman SC (2015) Carbon partitioning in Arabidopsis thaliana is a dynamic process controlled by the plants metabolic status and its circadian clock. Plant Cell Environ 38: 1965-1979

Koornneef M, Rolff E, Spruit JP (1980) Genetic Control of Light-inhibited Hypocotyl Elongation in Arabidopsis thaliana (L.) Z. Pflanzenphysiol. 100: 147-160

Kotting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol 13: 321-329

Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 42: D68-73

Kumar SV, Wigge PA (2010) H2A.Z-containing nucleosomes mediate the thermosensory response in Arabidopsis. Cell 140: 136-147

Kunihiro A, Yamashino T, Nakamichi N, Niwa Y, Nakanishi H, Mizuno T (2011) Phytochrome-interacting factor 4 and 5 (PIF4 and PIF5) activate the homeobox ATHB2 and auxin-inducible IAA29 genes in the coincidence mechanism underlying photoperiodic control of plant growth of Arabidopsis thaliana. Plant and Cell Physiology 52: 1315-1329

Kusakina J, Gould PD, Hall A (2014) A fast circadian clock at high temperatures is a conserved feature across Arabidopsis accessions and likely to be important for vegetative yield. Plant Cell Environ 37: 327-340

Lalonde S, Tegeder M, Throne-Holst M, Frommer W, Patrick J (2003) Phloem loading and unloading of sugars and amino acids. Plant, Cell & Environment 26: 37-56

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology 10: R25

Lastdrager J, Hanson J, Smeekens S (2014) Sugar signals and the control of plant growth and development. J Exp Bot 65: 799-807

Lau OS, Deng XW (2010) Plant hormone signaling lightens up: integrators of light and hormones. Curr Opin Plant Biol 13: 571-577

Lavy M, Estelle M (2016) Mechanisms of auxin signaling. Development 143: 3226-3229

Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, Tongprasit W, Zhao H, Lee I, Deng XW (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell 19: 731-749

Legris M, Klose C, Burgie ES, Rojas CCR, Neme M, Hiltbrunner A, Wigge PA, Schäfer E, Vierstra RD, Casal JJ (2016) Phytochrome B integrates light and temperature signals in Arabidopsis. Science 354: 897-900

Legris M, Nieto C, Sellaro R, Prat S, Casal JJ (2017) Perception and signalling of light and temperature cues in plants. Plant J 90: 683-697

Leivar P, Monte E (2014) PIFs: systems integrators in plant development. Plant Cell 26: 56-78

Leivar P, Monte E, Oka Y, Liu T, Carle C, Castillon A, Huq E, Quail PH (2008) Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Curr Biol 18: 1815-1823

Leivar P, Quail PH (2011) PIFs: pivotal components in a cellular signaling hub. Trends Plant Sci 16: 19-28

Lepistö A, Kangasjärvi S, Luomala E-M, Brader G, Sipari N, Keränen M, Keinänen M, Rintamäki E (2009) Chloroplast NADPH-Thioredoxin Reductase Interacts with Photoperiodic Development in Arabidopsis. Plant Physiology 149: 1261-1276

Li H, Ye K, Shi Y, Cheng J, Zhang X, Yang S (2017) BZR1 Positively Regulates Freezing Tolerance via CBF-Dependent and CBF-Independent Pathways in Arabidopsis. Mol Plant 10: 545-559

Li K, Yu R, Fan LM, Wei N, Chen H, Deng XW (2016) DELLA-mediated PIF degradation contributes to coordination of light and gibberellin signalling in Arabidopsis. Nat Commun 7: 11868

Li L, Foster CM, Gan Q, Nettleton D, James MG, Myers AM, Wurtele ES (2009) Identification of the novel protein QQS as a component of the starch metabolic network in Arabidopsis leaves. Plant J 58: 485-498

Li QF, He JX (2016) BZR1 Interacts with HY5 to Mediate Brassinosteroid- and Light-Regulated Cotyledon Opening in Arabidopsis in Darkness. Mol Plant 9: 113-125

Li T, Heuvelink E, van Noort F, Kromdijk J, Marcelis LFM (2014) Responses of two Anthurium cultivars to high daily integrals of diffuse light. Scientia Horticulturae 179: 306-313

Lian H, Xu P, He S, Wu J, Pan J, Wang W, Xu F, Wang S, Pan J, Huang J, Yang HQ (2018) Photoexcited CRYPTOCHROME 1 Interacts Directly with G-Protein beta Subunit AGB1 to Regulate the DNA-Binding Activity of HY5 and Photomorphogenesis in Arabidopsis. Mol Plant 11: 1248-1263

Lian HL, He SB, Zhang YC, Zhu DM, Zhang JY, Jia KP, Sun SX, Li L, Yang HQ (2011) Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. Genes Dev 25: 1023-1028

Liu B, Zuo Z, Liu H, Liu X, Lin C (2011) Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. Genes Dev 25: 1029-1034

Liu Y, Roof S, Ye Z, Barry C, van Tuinen A, Vrebalov J, Bowler C, Giovannoni J (2004) Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. Proc Natl Acad Sci U S A 101: 9897-9902

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25: 402-408

López Salmerón V (2013) ELF3 regulates cotyledon expansion and hypocotyl growth rhythmicity.

Lorrain S, Allen T, Duek PD, Whitelam GC, Fankhauser C (2008) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant J 53: 312-323

Lu SX, Knowles SM, Andronis C, Ong MS, Tobin EM (2009) CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL function synergistically in the circadian clock of Arabidopsis. Plant Physiol 150: 834-843

Ma D, Li X, Guo Y, Chu J, Fang S, Yan C, Noel JP, Liu H (2016) Cryptochrome 1 interacts with PIF4 to regulate high temperature-mediated hypocotyl elongation in response to blue light. Proc Natl Acad Sci U S A 113: 224-229

Mair A, Pedrotti L, Wurzinger B, Anrather D, Simeunovic A, Weiste C, Valerio C, Dietrich K, Kirchler T, Nagele T, Vicente Carbajosa J, Hanson J, Baena-Gonzalez E, Chaban C, Weckwerth W, Droge-Laser W, Teige M (2015) SnRK1-triggered switch of bZIP63 dimerization mediates the low-energy response in plants. Elife 4

Martin G, Rovira A, Veciana N, Soy J, Toledo-Ortiz G, Gommers CMM, Boix M, Henriques R, Minguet EG, Alabadi D, Halliday KJ, Leivar P, Monte E (2018) Circadian Waves of Transcriptional Repression Shape PIF-Regulated Photoperiod-Responsive Growth in Arabidopsis. Curr Biol 28: 311-318 e315

Martinez-Garcia JF, Gallemi M, Molina-Contreras MJ, Llorente B, Bevilaqua MR, Quail PH (2014) The shade avoidance syndrome in Arabidopsis: the antagonistic role of phytochrome a and B differentiates vegetation proximity and canopy shade. PLoS One 9: e109275

Martins MC, Hejazi M, Fettke J, Steup M, Feil R, Krause U, Arrivault S, Vosloh D, Figueroa CM, Ivakov A, Yadav UP, Piques M, Metzner D, Stitt M, Lunn JE (2013) Feedback inhibition of starch degradation in Arabidopsis leaves mediated by trehalose 6-phosphate. Plant Physiol 163: 1142-1163

Maruyama K, Takeda M, Kidokoro S, Yamada K, Sakuma Y, Urano K, Fujita M, Yoshiwara K, Matsukura S, Morishita Y, Sasaki R, Suzuki H, Saito K, Shibata D, Shinozaki K, Yamaguchi-Shinozaki K (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. Plant Physiol 150: 1972-1980

Más P, Kim W-Y, Somers DE, Kay SA (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in Arabidopsis thaliana. Nature 426: 567

Masri S, Cervantes M, Sassone-Corsi P (2013) The circadian clock and cell cycle: interconnected biological circuits. Curr Opin Cell Biol 25: 730-734

May P, Liao W, Wu Y, Shuai B, McCombie WR, Zhang MQ, Liu QA (2013) The effects of carbon dioxide and temperature on microRNA expression in Arabidopsis development. Nat Commun 4: 2145

McClung CR, Salome PA, Michael TP (2002) The Arabidopsis circadian system. Arabidopsis Book 1: e0044

McGee SL, Hargreaves M (2008) AMPK and transcriptional regulation. Frontiers in bioscience: a journal and virtual library 13: 3022-3033

McWatters HG, Kolmos E, Hall A, Doyle MR, Amasino RM, Gyula P, Nagy F, Millar AJ, Davis SJ (2007) ELF4 is required for oscillatory properties of the circadian clock. Plant Physiol 144: 391-401

Mi H, Muruganujan A, Thomas PD (2013) PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. Nucleic Acids Res 41: D377-386

Michalska J, Zauber H, Buchanan BB, Cejudo FJ, Geigenberger P (2009) NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. Proceedings of the National Academy of Sciences 106: 9908-9913

Millar AH, Whelan J, Soole KL, Day DA (2011) Organization and regulation of mitochondrial respiration in plants. Annu Rev Plant Biol 62: 79-104

Millar AJ, Short SR, Hiratsuka K, Chua N-H, Kay SA (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. Plant Molecular Biology Reporter 10: 324-337

Millenaar F, Lambers H (2003) The alternative oxidase: in vivo regulation and function. Plant Biology 5: 2-15

Mishra P, Panigrahi KC (2015) GIGANTEA - an emerging story. Front Plant Sci 6: 8

Miura K, Furumoto T (2013) Cold signaling and cold response in plants. Int J Mol Sci 14: 5312-5337

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song H-R, Carré IA, Coupland G (2002) LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Developmental cell 2: 629-641

Mizuno T, Nomoto Y, Oka H, Kitayama M, Takeuchi A, Tsubouchi M, Yamashino T (2014) Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in Arabidopsis thaliana. Plant Cell Physiol 55: 958-976

Mizuno T, Oka H, Yoshimura F, Ishida K, Yamashino T (2015) Insight into the mechanism of end-of-day far-red light (EODFR)-induced shade avoidance responses in Arabidopsis thaliana. Biosci Biotechnol Biochem 79: 1987-1994

Mockler T, Michael T, Priest H, Shen R, Sullivan C, Givan S, McEntee C, Kay S, Chory J (2007) The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. *In* Cold Spring Harbor symposia on quantitative biology, Vol 72. Cold Spring Harbor Laboratory Press, pp 353-363

Moe R, Heins RD (2000) Thermo-and photomorphogenesis in plants. Advances in floriculture research. Report: 52-64

Moe R, Heins RD, Erwin J (1991) Stem elongation and flowering of the long-day plant Campanula isophylla Moretti in response to day and night temperature alternations and light quality. Scientia horticulturae 48: 141-151

Mortensen LM, Moe R (1992) Effects of selective screening of the dayight spectrum, and of twilight on plant growth in greenhouses. Acta Horticulturae 305: 103-108

Mugford ST, Fernandez O, Brinton J, Flis A, Krohn N, Encke B, Feil R, Sulpice R, Lunn JE, Stitt M, Smith AM (2014) Regulatory properties of ADP glucose pyrophosphorylase are required for adjustment of leaf starch synthesis in different photoperiods. Plant Physiol 166: 1733-1747

Myster J, Moe R (1995) Effect of diurnal temperature alternations on plant morphology in some greenhouse crops—a mini review. Scientia Horticulturae 62: 205-215

Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua NH, Sakakibara H (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. Plant Cell 22: 594-605

Nakamichi N, Kusano M, Fukushima A, Kita M, Ito S, Yamashino T, Saito K, Sakakibara H, Mizuno T (2009) Transcript profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR arrhythmic triple mutant reveals a role for the circadian clock in cold stress response. Plant Cell Physiol 50: 447-462

Ng S, Giraud E, Duncan O, Law SR, Wang Y, Xu L, Narsai R, Carrie C, Walker H, Day DA, Blanco NE, Strand A, Whelan J, Ivanova A (2013) Cyclin-dependent kinase E1 (CDKE1) provides a cellular switch in plants between growth and stress responses. J Biol Chem 288: 3449-3459

Nieto C, Lopez-Salmeron V, Daviere JM, Prat S (2015) ELF3-PIF4 interaction regulates plant growth independently of the Evening Complex. Curr Biol 25: 187-193

Nozue K, Covington MF, Duek PD, Lorrain S, Fankhauser C, Harmer SL, Maloof JN (2007) Rhythmic growth explained by coincidence between internal and external cues. Nature 448: 358-361

Nukarinen E, Nagele T, Pedrotti L, Wurzinger B, Mair A, Landgraf R, Bornke F, Hanson J, Teige M, Baena-Gonzalez E, Droge-Laser W, Weckwerth W (2016) Quantitative phosphoproteomics reveals the role of the AMPK plant ortholog SnRK1 as a metabolic master regulator under energy deprivation. Sci Rep 6: 31697

Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, Farre EM, Kay SA (2011) The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature 475: 398-402

Oakenfull RJ, Davis SJ (2017) Shining a light on the Arabidopsis circadian clock. Plant Cell Environ 40: 2571-2585

Oh E, Zhu J-Y, Wang Z-Y (2012) Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nature cell biology 14: 802

Okamoto K, Onai K, Ezaki N, Ofuchi T, Ishiura M (2005) An automated apparatus for the real-time monitoring of bioluminescence in plants. Anal Biochem 340: 187-192

Oliveros JC (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. *In*, http://bioinfogp.cnb.csic.es/tools/venny/index.html

Osterlund MT, Hardtke CS, Wei N, Deng X-W (2000) Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature 405: 462-466

Osuna D, Usadel B, Morcuende R, Gibon Y, Bläsing OE, Höhne M, Günter M, Kamlage B, Trethewey R, Scheible WR (2007) Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. The Plant Journal 49: 463-491

Ouzounis T, Frette X, Rosenqvist E, Ottosen CO (2014) Spectral effects of supplementary lighting on the secondary metabolites in roses, chrysanthemums, and campanulas. J Plant Physiol 171: 1491-1499

Ow DW, Wood KV, DeLuca M, De Wet JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234: 856-859

Paltiel J, Amin R, Gover A, Ori N, Samach A (2006) Novel roles for GIGANTEA revealed under environmental conditions that modify its expression in Arabidopsis and Medicago truncatula. Planta 224: 1255-1268

Pantazopouloua CK, Bongers FJ, Küpers JJ, Reinen E, Das D, Evers JB, Anten NPR, Pierik R (2017) Neighbor detection at the leaf tip adaptively regulates upward leaf movement through spatial auxin dynamics. Proc Natl Acad Sci U S A 114: 7450-7455

Pantin F, Simonneau T, Muller B (2012) Coming of leaf age: control of growth by hydraulics and metabolics during leaf ontogeny. New Phytol 196: 349-366

Paparelli E, Parlanti S, Gonzali S, Novi G, Mariotti L, Ceccarelli N, van Dongen JT, Kolling K, Zeeman SC, Perata P (2013) Nighttime sugar starvation orchestrates gibberellin biosynthesis and plant growth in Arabidopsis. Plant Cell 25: 3760-3769

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG (1999) Control of Circadian Rhythms and Photoperiodic Flowering by the Arabidopsis GIGANTEA Gene. Science 285: 1579-1582

Park YJ, Lee HJ, Ha JH, Kim JY, Park CM (2017) COP1 conveys warm temperature information to hypocotyl thermomorphogenesis. New Phytol 215: 269-280
Patel D, Franklin KA (2009) Temperature-regulation of plant architecture. Plant Signal Behav 4: 577-579
Patil GG, Moe R (2009) Involvement of phytochrome B in DIF mediated growth in cucumber. Scientia horticulturae 122: 164-170
Paul MJ, Primavesi LF, Jhurreea D, Zhang Y (2008) Trehalose metabolism and signaling. Annu. Rev. Plant Biol. 59: 417-441
Pedmale UV, Huang SC, Zander M, Cole BJ, Hetzel J, Ljung K, Reis PAB, Sridevi P, Nito K, Nery JR, Ecker JR, Chory J (2016) Cryptochromes Interact Directly with PIFs to Control Plant Growth in Limiting Blue Light. Cell 164: 233-245
Pham VN, Kathare PK, Huq E (2018) Phytochromes and Phytochrome Interacting Factors. Plant Physiol 176: 1025-1038
Pierik R, de Wit M (2014) Shade avoidance: phytochrome signalling and other aboveground neighbour detection cues. J Exp Bot 65: 2815-2824
Pierik R, Djakovic-Petrovic T, Keuskamp DH, de Wit M, Voesenek LA (2009) Auxin and ethylene regulate elongation responses to neighbor proximity signals independent of gibberellin and della proteins in Arabidopsis. Plant Physiol 149: 1701-1712
Pilkington SM, Encke B, Krohn N, Hohne M, Stitt M, Pyl ET (2015) Relationship between starch degradation and carbon demand for maintenance and growth in Arabidopsis thaliana in different irradiance and temperature regimes. Plant Cell Environ 38: 157-171
Planas-Riverola A, Gupta A, Betegon-Putze I, Bosch N, Ibanes M, Cano-Delgado AI (2019) Brassinosteroid signaling in plant development and adaptation to stress. Development 146
Plaxton WC, Podestá FE (2006) The functional organization and control of plant respiration. Critical Reviews in Plant Sciences 25: 159-198
Polge C, Thomas M (2007) SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? Trends in plant science 12: 20-28
Ponnu J, Wahl V, Schmid M (2011) Trehalose-6-phosphate: connecting plant metabolism and development. Front Plant Sci 2: 70
Price J, Laxmi A, Martin SKS, Jang J-C (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. The Plant Cell 16: 2128-2150
Pucciariello O, Legris M, Costigliolo Rojas C, Iglesias MJ, Hernando CE, Dezar C, Vazquez M, Yanovsky MJ, Finlayson SA, Prat S, Casal JJ (2018) Rewiring of auxin signaling under persistent shade. Proc Natl Acad Sci U S A 115: 5612-5617
Pyl ET, Piques M, Ivakov A, Schulze W, Ishihara H, Stitt M, Sulpice R (2012) Metabolism and growth in Arabidopsis depend on the daytime temperature but are temperature-compensated against cool nights. Plant Cell 24: 2443-2469
Qiu Y, Li M, Kim RJ, Moore CM, Chen M (2019) Daytime temperature is sensed by phytochrome B in Arabidopsis through a transcriptional activator HEMERA. Nat Commun 10: 140
Quint M, Delker C, Franklin KA, Wigge PA, Halliday KJ, van Zanten M (2016) Molecular and genetic control of plant thermomorphogenesis. Nat Plants 2: 15190
Rademacher W (2017) Chemical Regulators of Gibberellin Status and Their Application in Plant Production. *In* Annual Plant Reviews online, pp 359-403

Ramon M, De Smet I, Vandesteene L, Naudts M, Leyman B, Van Dijck P, Rolland F, Beeckman T, Thevelein JM (2009) Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant Cell Environ 32: 1015-1032

Raschke A, Ibanez C, Ullrich KK, Anwer MU, Becker S, Glockner A, Trenner J, Denk K, Saal B, Sun X, Ni M, Davis SJ, Delker C, Quint M (2015) Natural variants of ELF3 affect thermomorphogenesis by transcriptionally modulating PIF4-dependent auxin response genes. BMC Plant Biol 15: 197

Rawat R, Takahashi N, Hsu PY, Jones MA, Schwartz J, Salemi MR, Phinney BS, Harmer SL (2011) REVEILLE8 and PSEUDO-REPONSE REGULATOR5 form a negative feedback loop within the Arabidopsis circadian clock. PLoS Genet 7: e1001350

Reed JW (2001) Roles and activities of Aux/IAA proteins in Arabidopsis. Trends in plant science 6: 420-425

Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. The Plant Cell 5: 147-157

Richau KH, Kaschani F, Verdoes M, Pansuriya TC, Niessen S, Stuber K, Colby T, Overkleeft HS, Bogyo M, Van der Hoorn RA (2012) Subclassification and biochemical analysis of plant papain-like cysteine proteases displays subfamily-specific characteristics. Plant Physiol 158: 1583-1599

Rockwell NC, Su YS, Lagarias JC (2006) Phytochrome structure and signaling mechanisms. Annu Rev Plant Biol 57: 837-858

Rosado D, Gramegna G, Cruz A, Lira BS, Freschi L, de Setta N, Rossi M (2016) Phytochrome Interacting Factors (PIFs) in Solanum lycopersicum: Diversity, Evolutionary History and Expression Profiling during Different Developmental Processes. PLoS One 11: e0165929

Ruan YL, Jin Y, Yang YJ, Li GJ, Boyer JS (2010) Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. Mol Plant 3: 942-955

Runkle ES, Heins RD (2001) Specific Functions of Red, Far Red, and Blue Light in Flowering and Stem Extension of Long-day Plants. Journal of the American Society of Horticultural Science 126: 275-282

Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Ma L, Hoecker U, Deng XW (2003) The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev 17: 2642-2647

Sairanen I, Novak O, Pencik A, Ikeda Y, Jones B, Sandberg G, Ljung K (2012) Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in Arabidopsis. Plant Cell 24: 4907-4916

Salome PA, McClung CR (2005) PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the Arabidopsis circadian clock. Plant Cell 17: 791-803

Salome PA, Weigel D, McClung CR (2010) The role of the Arabidopsis morning loop components CCA1, LHY, PRR7, and PRR9 in temperature compensation. Plant Cell 22: 3650-3661

Salter MG, Franklin KA, Whitelam GC (2003) Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature 426: 680

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science 318: 261-265
Schluepmann H, Pellny T, van Dijken A, Smeekens S, Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 100: 6849-6854
Schluepmann H, van Dijken A, Aghdasi M, Wobbes B, Paul M, Smeekens S (2004) Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation. Plant Physiol 135: 879-890
Schulz E, Tohge T, Zuther E, Fernie AR, Hincha DK (2015) Natural variation in flavonol and anthocyanin metabolism during cold acclimation in Arabidopsis thaliana accessions. Plant Cell Environ 38: 1658-1672
Scialdone A, Mugford ST, Feike D, Skeffington A, Borrill P, Graf A, Smith AM, Howard M (2013) Arabidopsis plants perform arithmetic division to prevent starvation at night. Elife 2: e00669
Scott IM, Clarke SM, Wood JE, Mur LA (2004) Salicylate accumulation inhibits growth at chilling temperature in Arabidopsis. Plant Physiol 135: 1040-1049
Seaton DD, Smith RW, Song YH, MacGregor DR, Stewart K, Steel G, Foreman J, Penfield S, Imaizumi T, Millar AJ, Halliday KJ (2015) Linked circadian outputs control elongation growth and flowering in response to photoperiod and temperature. Mol Syst Biol 11: 776
Seki M, Ohara T, Hearn TJ, Frank A, da Silva VCH, Caldana C, Webb AAR, Satake A (2017) Adjustment of the Arabidopsis circadian oscillator by sugar signalling dictates the regulation of starch metabolism. Sci Rep 7: 8305
Sergeeva LI, Claassens MMJ, Jamar DCL, van der Plas LHW, Vreugdenhil D (2012) Starch-related enzymes during potato tuber dormancy and sprouting. Russian Journal of Plant Physiology 59: 556-564
Sergeeva LI, De Bruijn SM, Koot-Gronsveld EA, Navratil O, Vreugdenhil D (2000) Tuber morphology and starch accumulation are independent phenomena: Evidence from ipt-transgenic potato lines. Physiologia plantarum 108: 435-443
Sergeeva LI, Keurentjes JJ, Bentsink L, Vonk J, van der Plas LH, Koornneef M, Vreugdenhil D (2006) Vacuolar invertase regulates elongation of Arabidopsis thaliana roots as revealed by QTL and mutant analysis. Proceedings of the National Academy of Sciences 103: 2994-2999
Sergeeva LI, Vonk J, Keurentjes JJ, van der Plas LH, Koornneef M, Vreugdenhil D (2004) Histochemical analysis reveals organ-specific quantitative trait loci for enzyme activities in Arabidopsis. Plant Physiol 134: 237-245
Seung D, Schreier TB, Burgy L, Eicke S, Zeeman SC (2018) Two Plastidial Coiled-Coil Proteins Are Essential for Normal Starch Granule Initiation in Arabidopsis. Plant Cell 30: 1523-1542
Shalit-Kaneh A, Kumimoto RW, Filkov V, Harmer SL (2018) Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. Proc Natl Acad Sci U S A 115: 7147-7152
Shapulatov U (2019) Exploring the molecular hub in plant elongation responses: regulation of PHYs and PIFs. Wageningen University
Shi L, Wu Y, Sheen J (2018) TOR signaling in plants: conservation and innovation. Development 145

Shin J, Sánchez-Villarreal A, Davis AM, Du Sx, Berendzen KW, Koncz C, Ding Z, Li C, Davis SJ (2017) The metabolic sensor AKIN10 modulates the Arabidopsis circadian clock in a light-dependent manner. Plant, cell & environment 40: 997-1008

Shor E, Paik I, Kangisser S, Green R, Huq E (2017) PHYTOCHROME INTERACTING FACTORS mediate metabolic control of the circadian system in Arabidopsis. New Phytol 215: 217-228

Shriram V, Kumar V, Devarumath RM, Khare TS, Wani SH (2016) MicroRNAs As Potential Targets for Abiotic Stress Tolerance in Plants. Front Plant Sci 7: 817

Sicher R (2011) Carbon partitioning and the impact of starch deficiency on the initial response of Arabidopsis to chilling temperatures. Plant Sci 181: 167-176

Singh D, Basu C, Meinhardt-Wollweber M, Roth B (2015) LEDs for energy efficient greenhouse lighting. Renewable and Sustainable Energy Reviews 49: 139-147

Smith H (1982) Light quality, photoperception, and plant strategy. Annual review of plant physiology 33: 481-518

Smith SM, Fulton DC, Chia T, Thorneycroft D, Chapple A, Dunstan H, Hylton C, Zeeman SC, Smith AM (2004) Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. Plant Physiol 136: 2687-2699

Somers DE, Devlin PF, Kay SA (1998) Phytochromes and Cryptochromes in the Entrainment of the *Arabidopsis* Circadian Clock. Science 282: 1488-1490

Song YH, Smith RW, To BJ, Millar AJ, Imaizumi T (2012) FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336: 1045-1049

Soy J, Leivar P, Gonzalez-Schain N, Martin G, Diaz C, Sentandreu M, Al-Sady B, Quail PH, Monte E (2016) Molecular convergence of clock and photosensory pathways through PIF3-TOC1 interaction and co-occupancy of target promoters. Proc Natl Acad Sci U S A 113: 4870-4875

Stavang JA, Lindgård B, Erntsen A, Lid SE, Moe R, Olsen JE (2005) Thermoperiodic stem elongation involves transcriptional regulation of gibberellin deactivation in pea. Plant physiology 138: 2344-2353

Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. Curr Opin Plant Biol 15: 282-292

Streb S, Zeeman SC (2012) Starch metabolism in Arabidopsis. Arabidopsis Book 10: e0160

Sun J, Qi L, Li Y, Chu J, Li C (2012) PIF4–mediated activation of YUCCA8 expression integrates temperature into the auxin pathway in regulating Arabidopsis hypocotyl growth. PLoS genetics 8: e1002594

Sun J, Qi L, Li Y, Zhai Q, Li C (2013) PIF4 and PIF5 transcription factors link blue light and auxin to regulate the phototropic response in Arabidopsis. Plant Cell 25: 2102-2114

Sun Q, Wang S, Xu G, Kang X, Zhang M, Ni M (2019) SHB1 and CCA1 interaction desensitizes light responses and enhances thermomorphogenesis. Nat Commun 10: 3110

Supek F, Bosnjak M, Skunca N, Smuc T (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One 6: e21800

Tantau H-J (1998) Energy saving potential of greenhouse climate control. Mathematics and computers in simulation 48: 93-101

Tarkowski LP, Van den Ende W (2015) Cold tolerance triggered by soluble sugars: a multifaceted countermeasure. Front Plant Sci 6: 203

Thain SC, Vandenbussche F, Laarhoven LJ, Dowson-Day MJ, Wang ZY, Tobin EM, Harren FJ, Millar AJ, Van Der Straeten D (2004) Circadian rhythms of ethylene emission in Arabidopsis. Plant Physiol 136: 3751-3761

Thalmann M, Santelia D (2017) Starch as a determinant of plant fitness under abiotic stress. New Phytol 214: 943-951

Thines B, Harmon FG (2010) Ambient temperature response establishes ELF3 as a required component of the core Arabidopsis circadian clock. Proc Natl Acad Sci U S A 107: 3257-3262

Thingnaes E, Torre S, Ernstsen A, Moe R (2003) Day and night temperature responses in Arabidopsis: effects on gibberellin and auxin content, cell size, morphology and flowering time. Ann Bot 92: 601-612

Thingnaes E, Torre S, Moe R (2008) The role of phytochrome B, D and E in thermoperiodic responses of Arabidopsis thaliana. Plant Growth Regulation 56: 53-59

Toivola J, Nikkanen L, Dahlstrom KM, Salminen TA, Lepisto A, Vignols HF, Rintamaki E (2013) Overexpression of chloroplast NADPH-dependent thioredoxin reductase in Arabidopsis enhances leaf growth and elucidates in vivo function of reductase and thioredoxin domains. Front Plant Sci 4: 389

Toledo-Ortiz G, Johansson H, Lee KP, Bou-Torrent J, Stewart K, Steel G, Rodriguez-Concepcion M, Halliday KJ (2014) The HY5-PIF regulatory module coordinates light and temperature control of photosynthetic gene transcription. PLoS Genet 10: e1004416

Tome F, Nagele T, Adamo M, Garg A, Marco-Llorca C, Nukarinen E, Pedrotti L, Peviani A, Simeunovic A, Tatkiewicz A, Tomar M, Gamm M (2014) The low energy signaling network. Front Plant Sci 5: 353

Tootle TL, Silver SJ, Davies EL, Newman V, Latek RR, Mills IA, Selengut JD, Parlikar BE, Rebay I (2003) The transcription factor Eyes absent is a protein tyrosine phosphatase. Nature 426: 299-302

Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol 31: 46-53

Turgeon R (1989) The sink-source transition in leaves. Annual review of plant biology 40: 119-138

Urban O, Klem K, Ač A, Havránková K, Holišová P, Navrátil M, Zitová M, Kozlová K, Pokorný R, Šprtová M, Tomášková I, Špunda V, Grace J (2012) Impact of clear and cloudy sky conditions on the vertical distribution of photosynthetic CO2uptake within a spruce canopy. Functional Ecology 26: 46-55

Usadel B, Blaesing OE, Gibon Y, Poree F, Hoehne M, Guenter M, Trethewey R, Kamlage B, Poorter H, Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. Plant, Cell & Environment 31: 518-547

van Dijken AJ, Schluepmann H, Smeekens SC (2004) Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant physiology 135: 969-977

Van Leeuwen W, Ruttink T, Borst-Vrenssen AWM, Van der Plas LHW, Van der Krol A, R. (2001) Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. Journal of Experimental Botany 52: 949-959

Wang F, Zhang L, Chen X, Wu X, Xiang X, Zhou J, Xia X, Shi K, Yu J, Foyer CH (2019) SlHY5 Integrates Temperature, Light, and Hormone Signaling to Balance Plant Growth and Cold Tolerance. Plant physiology 179: 749-760

Wang W, Bai M-Y, Wang Z-Y (2014) The brassinosteroid signaling network—a paradigm of signal integration. Current opinion in plant biology 21: 147-153

Wang Z-Y, Bai M-Y, Oh E, Zhu J-Y (2012) Brassinosteroid signaling network and regulation of photomorphogenesis. Annual review of genetics 46: 701-724

Webb AAR, Seki M, Satake A, Caldana C (2019) Continuous dynamic adjustment of the plant circadian oscillator. Nat Commun 10: 550

Weiss J, Egea-Cortines M (2009) Transcriptomic analysis of cold response in tomato fruits identifies dehydrin as a marker of cold stress. Journal of applied genetics 50: 311-319

Went FW (1944) Plant growth under controlled conditions. II. Thermoperiodicity in growth and fruiting of the tomato. American Journal of Botany 31: 135-150

Wingler A, Fritzius T, Wiemken A, Boller T, Aeschbacher RA (2000) Trehalose induces the ADP-glucose pyrophosphorylase gene, ApL3, and starch synthesis in Arabidopsis. Plant Physiology 124: 105-114

Xu F, Yuan S, Lin HH (2011) Response of mitochondrial alternative oxidase (AOX) to light signals. Plant Signal Behav 6: 55-58

Xu PB, Lian HL, Wang WX, Xu F, Yang HQ (2016) Pivotal Roles of the Phytochrome-Interacting Factors in Cryptochrome Signaling. Mol Plant 9: 496-497

Xu X, Paik I, Zhu L, Huq E (2015) Illuminating Progress in Phytochrome-Mediated Light Signaling Pathways. Trends Plant Sci 20: 641-650

Yakir E, Hilman D, Kron I, Hassidim M, Melamed-Book N, Green RM (2009) Posttranslational regulation of CIRCADIAN CLOCK ASSOCIATED1 in the circadian oscillator of Arabidopsis. Plant Physiol 150: 844-857

Yano R, Nakamura M, Yoneyama T, Nishida I (2005) Starch-related α-glucan/water dikinase is involved in the cold-induced development of freezing tolerance in Arabidopsis. Plant Physiology 138: 837-846

Yin R, Ulm R (2017) How plants cope with UV-B: from perception to response. Current Opinion in Plant Biology 37: 42-48

Yu X, Liu H, Klejnot J, Lin C (2010) The cryptochrome blue light receptors. The Arabidopsis Book/American Society of Plant Biologists 8

Zhang H, He H, Wang X, Wang X, Yang X, Li L, Deng XW (2011) Genome-wide mapping of the HY5-mediated gene networks in Arabidopsis that involve both transcriptional and post-transcriptional regulation. Plant J 65: 346-358

Zhang J, Yuan H, Yang Y, Fish T, Lyi SM, Thannhauser TW, Zhang L, Li L (2016) Plastid ribosomal protein S5 is involved in photosynthesis, plant development, and cold stress tolerance in Arabidopsis. J Exp Bot 67: 2731-2744

Zhang X, Huai J, Shang F, Xu G, Tang W, Jing Y, Lin R (2017) A PIF1/PIF3-HY5-BBX23 Transcription Factor Cascade Affects Photomorphogenesis. Plant Physiol 174: 2487-2500

Zhang Y, Mayba O, Pfeiffer A, Shi H, Tepperman JM, Speed TP, Quail PH (2013) A quartet of PIF bHLH factors provides a transcriptionally centered signaling hub that regulates seedling morphogenesis through differential expression-patterning of shared target genes in Arabidopsis. PLoS genetics 9: e1003244

Zhang Y, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RA, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. Plant Physiol 149: 1860-1871

Zhao X, Jiang Y, Li J, Huq E, Chen ZJ, Xu D, Deng XW (2018) COP1 SUPPRESSOR 4 promotes seedling photomorphogenesis by repressing CCA1 and PIF4 expression in Arabidopsis. Proceedings of the National Academy of Sciences 115: 11631-11636

Zhu D, Maier A, Lee JH, Laubinger S, Saijo Y, Wang H, Qu LJ, Hoecker U, Deng XW (2008) Biochemical characterization of Arabidopsis complexes containing CONSTITUTIVELY PHOTOMORPHOGENIC1 and SUPPRESSOR OF PHYA proteins in light control of plant development. Plant Cell 20: 2307-2323

Zhu JY, Oh E, Wang T, Wang ZY (2016) TOC1-PIF4 interaction mediates the circadian gating of thermoresponsive growth in Arabidopsis. Nat Commun 7: 13692

Summary

In horticulture, especially in the production of ornamental crops, it is important to grow compact-shaped plants. Achieving this year-round can be challenging, especially during cloudy and/or warm days because elongation of stems is stimulated under such conditions, resulting in spindly-shaped plants. Chemical plant growth retardants (PGRs) are used in horticulture to control plant elongation. However, due to increasing environmental and health safety concerns the use of chemical PGRs needs to be replaced by more sustainable methods for growth control. A commonly used alternative method for controlling plant growth without the use of PGRs is realizing a negative day-night temperature difference (-DIF regime: cold days/warm nights), instead of a 'normal' +DIF regime (warm days/cold nights). Although -DIF is effective for controlling growth in many plant species, a downside is that economic realization of -DIF is only possible during times of the year with moderate temperatures (e.g. early spring or fall in The Netherlands). Therefore, more alternative methods for controlling plant growth in greenhouses are needed. Aim of this thesis project was to increase our understanding of the molecular regulation of the physiological growth response to -DIF. Additionally, we aimed to find ways to manipulate the components behind this regulation by additional light treatments in order to enhance the effect of -DIF and/or identify alternative treatments for growing compact plants in greenhouses. **Chapter 1** provides an overview of the literature at the start of the project on light/temperature regulation of the main components involved in the growth response to -DIF: the growth-promoting PHYTCHROME INTERACTING FACTOR (PIF) transcription factors, the circadian clock and carbohydrate metabolism.

In order to study the transcriptional responses to light and temperature cues of components involved in growth regulation, we aimed to use firefly-luciferase (ffLUC) reporters for promoter activity of genes involved in plant growth (e.g. *PIF* and clock genes). For this purpose, LUMINATOR was developed. In **Chapter 2** I describe how LUMINATOR was built and used to semi-continuously monitor ffLUC activity of four-week-old Arabidopsis reporter plants for promoter activity of *PIF4*, *ELONGATED HYPOCOTYL 5* (*HY5*) and *GIGANTEA* (*GI*) under 12h light/12h dark cycles at constant 22°C. In addition, LUMINATOR was used to study transcriptional responses of *PIF4*, *HY5* and *GI* to one hour added light at the start and end of the photoperiod. Such short light pulse treatments triggered immediate and longer-lasting transcriptional responses, which were predictive for long-term plant growth responses of tomato to the added light treatment in SOLINATOR climate cabinets. Thus, LUMINATOR may be used to help predict plant growth responses to additional light treatments. LUMINATOR was also used to monitor changes in ffLUC reporter activity in response to sudden changes in temperature. However, comparing ffLUC profiles with mRNA levels of the endogenous genes shows that the effect of temperature on activity of the ffLUC enzyme itself make results difficult to interpret.

To obtain an overview of the processes that are affected by -DIF RNA sequencing (RNAseq) analysis was performed on total mRNA and miRNA of adult Arabidopsis

rosettes grown under +DIF (12h L 22°C/12h D 12°C) or -DIF (12h L 12°C/12h D 22°C) at two timepoints: end of day (ED) and end of night (EN). RNA was isolated from sink material (young sink leaves and petioles of older leaves) of Col-0 WT and the phytochrome B (PHYB) mutant *phyB-9*, which shows reduced sensitivity to -DIF. The results of the RNAseq analysis are described in **Chapter 3**. Analysis of differentially expressed mRNAs show that -DIF leads to differential expression of in total 2706 genes in WT. Gene Ontology (GO)-term enrichment analysis indicates that -DIF stimulates cold acclimation processes at ED and that -DIF leads to low energy status at EN. Cell-growth processes such as cell wall modification and water transport are downregulated at either ED or EN. Furthermore, overlap between genes that are differentially expressed under -DIF and PIF-target genes indicates reduced transcriptional activity of PIF proteins under -DIF, especially at EN. Differentially expressed genes under -DIF that are related to promotion of cell growth were often not differentially expressed at EN in the *phyB-9* mutant. This suggests that the transcriptional responses of these genes are causal for growth suppression under -DIF and that PHYB plays a role in mediating the transcriptional response to temperature at night. Analysis of differentially expressed miRNAs and their putative target mRNAs suggests that part of the transcriptional response to -DIF is through regulation of mRNA transcript stability by miRNAs.

Previously it was shown that -DIF results in altered clock-controlled leaf movement in Arabidopsis, suggesting the timing of the circadian clock is altered under -DIF. In **Chapter 4** it is shown that -DIF indeed leads to differential phase-shifts and increased amplitude in rhythmic expression of core clock genes. The altered clock gene expression under -DIF is associated with altered regulation of starch metabolism and results in a strong reduction of starch levels in source leaves and sucrose levels in sink leaves at the end of the night, leading to induction of marker genes for CH starvation. Since it is known that plant growth is suppressed in response to CH starvation, these results suggest that the induction of CH starvation at EN may be causal for growth suppression under -DIF.

Previous research and the results in Chapter 3 show that the plant growth response to -DIF is associated with reduced PIF activity. PIF activity is also regulated on a transcriptional level and protein level by light quality. Thus, PIF activity may be (further) reduced by changing light quality by providing additional red (R) or blue (B) light. In **Chapter 5** LUMINATOR and ffLUC reporter plants are used to study the transcriptional response of *PIF3*/*4*/*5*, the PIF-antagonist *HY5* and the PIF-induced target gene *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) to one-hour additional B or R light at dawn and dusk. Results show that additional R light does not have a strong effect on *PIF* or *HY5* transcription, but *IAA29* transcription is reduced throughout the night in response to added R light at dusk, suggesting reduced PIF protein activity. Additional B light suppresses *PIF* and *IAA29* transcription but stimulates *HY5* transcription in the morning. Combined, these results on transcriptional responses to added light treatments indicate that one-hour additional R or B light at dawn and dusk suppresses PIF activity and may potentially be used to suppress plant growth. Indeed, stem growth in tomato plants could be suppressed by providing additional R/B light at dawn and dusk in SOLINATOR climate cabinets and/or greenhouses. However, the effectiveness of the additional light treatments depends on the light intensity of the pulses and the daily light integral (DLI).

Furthermore, results presented in Chapter 6 show that the timing of the additional R/B light determines whether the added light has an effect on plant growth. In agreement with the transcriptional responses to the additional light treatments, growth responses of tomato to additional R light are gated to the end of the day, while growth responses to additional B light are gated to the start of the day.

Besides discussing the gating of growth responses to additional R/B light, in **Chapter 6** I also summarize the results described in the experimental chapters of this thesis and try to relate the results to the currently available literature. In addition, I provide an updated version of the model for the regulation of PIF4 in Chapter 1 and discuss how the altered regulation of the clock and carbohydrate metabolism may explain for reduced PIF activity and growth under -DIF. Finally, a number of suggestions for potential protocols for growing compact plants in greenhouses are provided, and several recommendations for using LUMINATOR in combination with (new) ffLUC reporters in order to help identify novel sustainable protocols for regulating plant growth and development.

Samenvatting

Het produceren van compacte planten van hoge kwaliteit is een grote uitdaging voor de Nederlandse glastuinbouw. Dit is met name het geval gedurende perioden van lage lichtintensiteit, zoals in de herfst of winter. Vandaag de dag maakt men regelmatig gebruik van chemische groeiremmers om strekking tegen te gaan, maar door groeiende bezorgdheid over duurzaamheid en veiligheid van deze middelen zoekt men naar alternatieve methoden. Het realiseren van een negatief verschil in dag-nacht temperatuur (-DIF; koude dag/warme nacht) in plaats van 'normale' +DIF condities (warme dag/koude nacht) wordt al ingezet om strekking te remmen. Echter, het realiseren van -DIF in de kas is niet altijd mogelijk of rendabel. Daarom is het belangrijk dat er naar meer duurzame manieren wordt gezocht waarmee strekking kan worden geremd in de kas. Het project waarvan de resultaten beschreven staan in dit proefschrift heeft als doel om de moleculaire mechanismen die de plantengroei reguleren onder -DIF condities beter te begrijpen. Daarnaast wordt er gezocht naar manieren om de factoren die deze mechanismen beïnvloeden te reguleren met additionele lichtbehandelingen om zo het effect van -DIF te versterken en/of alternatieve methoden te vinden voor het groeien van compacte planten in de kas. In **Hoofdstuk 1** wordt een overzicht gegeven van de literatuur aan het begin van het project over licht-/temperatuur-regulatie van de belangrijkste componenten die een rol spelen in de remming van groei onder -DIF condities: de groei-stimulerende PHYTCHROME INTERACTING FACTORs (PIF) transcriptiefactoren, de biologische klok en zetmeel metabolisme.

Om in detail de activiteit van genen die betrokken zijn bij de regulatie van plantengroei (PIF- en klok-genen) te bestuderen onder verschillende licht- en temperatuurcondities is gebruik gemaakt van firefly luciferase (ffLUC) reporter planten voor promoter activiteit van deze genen. In **Hoofdstuk 2** staat beschreven hoe het LUMINATOR systeem is gemaakt en gebruikt voor het analyseren van ffLUC-activiteit van vier weken oude Arabidopsis reporterplanten voor promoter activiteit van *PIF4*, *ELONGATED HYPOCOTYL 5* (*HY5*) en *GIGANTEA* (*GI*) onder 12u dag/12u nacht cycli bij constant 22°C. Daarnaast is LUMINATOR gebruikt voor het bestuderen van het effect van één uur additioneel licht in de ochtend en avond op de activiteit van *PIF4*, *HY5* en *GI*. Deze additionele lichtpulsen waren al genoeg om genactiviteit voor een langere periode te beïnvloeden. De veranderingen in genactiviteit bleken daarnaast voorspellend voor de groei van jonge tomatenplanten onder een vergelijkbare lichtbehandeling in SOLINATOR klimaatkasten. Deze resultaten laten zien dat LUMINATOR, in combinatie met ffLUC reporter planten, gebruikt kan worden voor het voorspellen van het effect van additionele lichtbehandelingen op plantengroei. LUMINATOR kan ook worden gebruikt voor het analyseren van veranderingen in genactiviteit in reactie op temperatuursveranderingen. Echter, de temperatuurgevoeligheid van het ffLUC enzym zelf maakt de interpretatie van LUMINATOR resultaten van proeven waarbij gewerkt wordt met temperatuursveranderingen erg moeilijk.

Om een overzicht te krijgen van de processen die worden beïnvloed door -DIF is een RNA sequencing (RNAseq) analyse uitgevoerd op al het mRNA en miRNA van

Arabidopsis rozetplanten gegroeid onder +DIF (12 uur licht bij 22°C/12 uur donker bij 12°C) of -DIF (12 uur licht bij 12°/12 uur donker bij 22°C). Dit is gedaan op twee tijdstippen: aan het einde van de dag (ED) of aan het einde van de nacht (EN). Het RNA is geïsoleerd van sink materiaal (jonge sink bladeren en bladstelen van oudere bladeren) van Col-0 wild-type (WT) en van de fytochroom B (FYB) mutant *phyB-9*. Deze mutant is minder gevoelig voor het effect van -DIF op groei. De analyse van de differentieel tot expressie komende mRNAs in **Hoofdstuk 3** laat zien dat -DIF leidt tot veranderingen in expressie van in totaal 2706 genen in de WT. Gene Ontology (GO)-term enrichment analyse laat zien dat –DIF aan het einde van de dag processen stimuleert die helpen bij het acclimatiseren aan kou en dat -DIF leidt tot een lage energiestatus aan het einde van de nacht. Processen die celgroei stimuleren, zoals celwand-modificatie en water transport, worden onderdrukt aan het einde van de dag of nacht. Bovendien laat vergelijking van genen die differentieel tot expressie komen onder -DIF met genen die gereguleerd worden door PIFs zien dat de activiteit van PIF eiwitten is onderdrukt onder -DIF. Dit is voornamelijk aan het einde van de nacht. Genen die differentieel tot expressie komen onder -DIF en betrokken zijn bij het stimuleren van celgroei komen vaak niet differentieel tot expressie in de *phyB-9* mutant aan het einde van de nacht. Dit betekent dat lagere activiteit van deze genen mogelijk verantwoordelijk is voor de onderdrukking van groei onder -DIF en dat FYB een belangrijke rol speelt in de regulatie van de genactiviteit door temperatuur gedurende de nacht. Veranderingen in expressie van verschillende miRNAs en hun target-mRNAs wekt de suggestie dat de veranderingen in genexpressie onder -DIF deels veroorzaakt worden door veranderingen in expressie van miRNAs.

Het was al bekend dat -DIF de klok-gereguleerde bladbewegingen beïnvloedt in Arabidopsis. Dit wekt de suggestie dat de biologische klok is verstoord onder -DIF. In **Hoofdstuk 4** staat beschreven hoe -DIF inderdaad leidt tot veranderingen in de fase en amplitude van ritmische expressie van klokgenen. Ook veranderd de regulatie van zetmeel metabolisme onder -DIF, wat resulteert in een sterke reductie in zetmeel in source bladeren en een sterke reductie van sucrose in sink bladeren. Dit leidt tot activatie van markergenen voor een hongertoestand. Het is bekend dat een hongertoestand ertoe leidt dat fysiologische processen die groei stimuleren worden onderdrukt. Dus de hongertoestand onder -DIF aan het einde van de nacht is mogelijk (deels) de oorzaak voor het remmen van groei onder -DIF.

Eerder onderzoek en de resultaten in dit proefschrift laten zien dat het onderdrukken van groei onder -DIF condities gerelateerd is aan verminderde activiteit van PIFs. Naast temperatuur worden PIFs ook gereguleerd door lichtkwaliteit. Activiteit van PIFs kan mogelijk dus (verder) worden onderdrukt door het veranderen van de lichtkwaliteit door middel van het toevoegen van extra licht met een specifieke golflengte. In **Hoofdstuk 5** zijn LUMINATOR en ffLUC reporter planten gebruikt voor het bestuderen van het effect van één uur extra blauw (B) of rood (R) licht in de ochtend of avond op activiteit van *PIF3*/*4*/*5*, de PIF-antagonist *HY5* en het door PIFs geactiveerde *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*). De resultaten laten zien dat extra R licht geen duidelijk effect heeft op *PIF3*/*4*/*5* of *HY5*, maar *IAA29* wordt sterk onderdrukt gedurende de hele nacht in reactie op het extra R licht aan het einde van de dag. Extra B licht onderdrukt

PIF3/*4*/*5* en *IAA29*, maar stimuleert *HY5* in de ochtend. Deze resultaten leiden tot de voorspelling dat één uur extra R of B licht in de ochtend en avond mogelijk kan worden gebruikt om plantengroei te remmen. De extra R of B lichtbehandelingen blijken inderdaad de strekkingsgroei in jonge tomatenplanten te kunnen remmen in SOLINATOR en/of kassen. Echter, de effectiviteit van de lichtbehandelingen blijkt wel sterk afhankelijk van de lichtintensiteit van het extra licht en/of de dagelijkse lichtsom. Daarnaast blijkt uit resultaten in Hoofdstuk 6 dat het tijdstip van het toedienen van extra licht bepalend is voor het effect op de groei. Zoals verwacht op basis van de responsen van *PIF3*/*4*/*5*, *HY5* en *IAA29* op het extra R of B licht is R licht alleen aan het einde van de dag effectief om groei te remmen, terwijl B licht alleen aan het begin van de dag effectief is.

Naast het bediscussiëren van het tijdstip-afhankelijke effect van de extra R/B licht behandelingen worden in **Hoofdstuk 6** ook de resultaten van alle experimentele hoofdstukken bediscussieerd in relatie tot de huidige literatuur. Daarnaast wordt er een update gegeven van het model voor PIF4 regulatie, gebaseerd op artikelen die gepubliceerd zijn tijdens dit project. De bevindingen die in een aantal van deze artikelen beschreven zijn laten zien dat de onderdrukking van PIF activiteit onder -DIF mogelijk direct gerelateerd is aan de verstoorde activiteit van klokgenen en het in hongertoestand geraken van de plant aan het einde van de nacht onder -DIF condities. Tot slot worden nog een aantal suggesties gedaan voor potentiële protocollen voor het groeien van compacte planten in de kas en voor het gebruik van LUMINATOR in combinatie met (nieuwe) reporters in de zoektocht naar nieuwe duurzame methoden voor het reguleren van groei en ontwikkeling van planten in de kas.

Acknowledgements

Although stressful at times, doing my PhD at the Laboratory of Plant Physiology (PPH) has been a great experience. I feel that I have learned a lot over the last 5-6 years and, in the end, I am very proud of this thesis and all the work it represents. However, this thesis would not have been possible without the help and support of many other people. Here I would like to express my gratitude to some of the people that were directly or indirectly involved in my PhD work.

First of all, I would like to thank my supervisor and co-promoter Sander van der Krol for giving me the opportunity to do a PhD in his group and guiding me throughout the process. Sander, I really enjoyed our scientific meetings in which we discussed literature and constructed schematic models and I think your ideas and input were essential for successfully completing this project. You also taught me a lot about doing scientific research. For example, how valuable information can be extracted from details in data that might not look very ‘exciting’ at first. But besides all your professional guidance and scientific input you were also just a very nice person to talk with and you were always supportive and understanding.

I would also like to thank my promoter Christa Testerink for her feedback on the manuscripts and input during finalizing this thesis. Also many thanks to the members of my thesis committee that were willing to read and judge my thesis: Luisa Trindade, Kaisa Kajala, Corine de Groot and Martijn van Zanten.

About six months after I started my PhD at PPH, Umidjon Shapulatov started his PhD project that matched with the work described in this thesis. Umid, it was a real pleasure to work with you. You helped me out a lot with my experimental work and taught me to be more confident in the lab. Besides a great colleague you have also become a good friend over the years and I enjoyed our activities outside work and hope to stay in contact with you in the future.

Gonda, our PhDs started around the same time and, although our projects were not connected in any way, we also became friends. I want to thank you for always being supportive and willing to talk and for taking me out to have beers now and then.

Mark (Levisson), I am grateful you are willing to be my paranymph. I asked you because I have always very much enjoyed your company in the lab and during social activities. I think you have a great sense of humor and you were always willing to help others out.

Many results that are presented in this thesis would not have been there without the work of several technicians of PPH. Thank you Marielle, Jacqueline, Diaan and Lidiya. Not only for your experimental work, but also for teaching me new lab skills and for the nice talks and the support during my PhD. I would also like to thank the students that helped me doing experiments: Robin, Xiao, Casper, Monique and Jolien. I very much enjoyed working with you all!

I would also like to thank (former) staff members of PPH: Rina, Margaret, Harro, Leonie, Iris, Henk, Richard, Wilco and Dick for their help and support during my time at the

department and for their input during the annual PhD meetings. An important reason for why I had such a great time during my PhD was the nice atmosphere at the PPH department. Besides the staff members of PPH, this was also thanks to many other nice (former and new) colleagues at PPH and all the fun activities that were organized by the group such as the multi-culti parties, participation in the Veluweloop and WE-days, and the PhD trip to Germany and Switzerland. For that I want to thank all of you!

The compact plants project was a collaboration between PPH and the Horticulture and Product Physiology (HPP) group. I would like to thank Wim van Ieperen for his input and support during the project and overseeing the experiments that took place at HPP and that I think were essential for the success of this project. Habtamu and Elias, I would also like to thank you guys for your input and all the experimental work you did. Maarten, without you there would probably not have been such a fancy new LUMINATOR. Your work made many of the experiments described in this thesis possible and working together with you on developing LUMINATOR was one of my favorite parts of the project. Thank you for everything!

I would also like to thank all the companies and their representatives that supported the compact plants project (listed in Table 1). These played an important role in testing ideas that were generated during this thesis work and their active participation in the user-meetings were essential to keep thinking about how our fundamental research finds its application in potential new growth protocols for greenhouses. Special thanks to the company representatives that formed the steering committee: Arjan Stolte (ASP-Quality Support), William Barbier (Florensis), Leon Vrijland (PanAmerican Seed), Ronald Clemens and Riny Westdijk (ICL Fertilizers). Thank you all for your guidance and advice during this project.

Last but not least: I would like to thank my friends and family for their support during my PhD. Jona, Micha, Jean-Pierre en Bernhard, ik wil jullie bedanken voor jullie begrip en support de afgelopen jaren en de leuke dingen die we hebben gedaan. Kevin, mijn grote broer, bedankt voor alle adviezen die je me hebt gegeven tijdens mijn studies en PhD en voor het helpen met bedenken van propositions. Ik vind het heel leuk dat jij dadelijk paranimf zal zijn bij mijn verdediging. Linda, mijn lieve zus, bedankt voor alle steun de afgelopen tijd en je lieve woorden. Mam en Pap, ik wil jullie bedanken voor al jullie steun, geduld en begrip. Niet alleen gedurende de afgelopen jaren tijdens mijn PhD, maar ook daarvoor. Door jullie heb ik mijzelf kunnen ontwikkelen en dit kunnen bereiken. Heel erg bedankt voor alles.

Table 1: Companies participating in the STW project Compact Plants

ASP - Quality Support	PanAmerican Seed
Beekenkamp Group	Plantenkwekerij Gitzels
Bejo Zaden	Plantenkwekerij Valstar
Dekker Chrysanten	Plantenkwekerij Van der Lugt
Dümmen Orange	Rijk Zwaan
Florensis	Schneider Youngplants
Gipmans Plants	Schoneveld Breeding
Globe Plant	Selecta One
Grow Group	Syngenta Seeds
ICL Fertilizers	Takii Europe
Noordam Plants	Westlandse Plantenkwekerij - WPK

About the Author

Mark Daniël van Hoogdalem was born in The Hague on December 10th, 1987. He started his BSc in Biology at Utrecht University in 2006. During his studies he got interested in (molecular) plant sciences and its application in industry. Therefore, he started the MSc program Science and Business management at Utrecht University after obtaining his BSc degree in 2011. During this MSc program he did not only gain experience in conducting experimental research in the field of molecular plant physiology, but also learned about business and economics. During the first year of this program he performed a 9-month research project at the group Molecular Plant Physiology at Utrecht University under the supervision of Dr. Marcel Proveniers. Here he studied how metabolic signals regulate meristem activity in Arabidopsis seedlings. In the second year of the program he followed the 6-month course program Fundamentals of Business and Economics (FBE) and did a thesis project at the Europe China Institute of Nyenrode Business University under the supervision of Prof. Dr. Haico Ebbers. During this project, which was supported by Capgemini Consulting, he identified challenges and opportunities for companies that want to invest in the western region of China through economic data analysis and conducting interviews with managers of companies located in Chengdu, China. After obtaining his MSc in 2014, Mark started his PhD at the Laboratory of Plant Physiology under the supervision of Dr. Sander van der Krol. The project focused on the molecular mechanisms that regulate plant cell elongation in response to light and temperature cues and using insights into these mechanisms to develop new methods for suppressing plant elongation in greenhouses using light and/or temperature treatments. More background information and findings of this project are described in this thesis.

Publications

Umidjon Shapulatov, **Mark van Hoogdalem**, Marielle Schreuder, Harro Bouwmeester, Ibrokhim Y. Abdurakhmonov & Alexander R. van der Krol. (2018) Functional intron-derived miRNAs and host-gene expression in plants. *Plant Methods 14*, 83

Lennard C. van der Woude, Giorgio Perrella, L. Basten Snoek, **Mark van Hoogdalem**, Ondrej Novák, Marcel van Verk, Heleen N. van Kooten, Lennert E. Zorn, Rolf Tonckens, Joram A. Dongus, Myrthe Praat, Evelien A. Stouten, Marcel C.G. Proveniers, Elisa Vellutini, Eirini Patitaki, Umidjon Shapulatov, Wouter Kohlen, Sureshkumar Balasubramanian, Karin Ljung, Alexander R. van der Krol, Sjef Smeekens, Eirini Kaiserli & Martijn van Zanten (2019) HISTONE DEACETYLASE 9 stimulates auxin-dependent thermomorphogenesis in Arabidopsis thaliana by mediating H2A.Z depletion. *Proceedings of the National Academy of Sciences 116* (50), 25343-25354.

Mark van Hoogdalem, Maarten Wassenaar, Habtamy Giday, Umidjon Shapulatov, Jacqueline Busscher-Lange, Marielle Schreuder, Casper van den Abeele, Wim van Ieperen & Sander van der Krol (2019) LUMINATOR: monitoring transcriptional responses to light pulses to predict long-term plant elongation responses. (*under review*)

Mark van Hoogdalem, Umidjon Shapulatov, Lidiya Sergeeva, Jacqueline Busscher-Lange, Marielle Schreuder, Diaan Jamar, Xiao Han & Alexander R. van der Krol (2019) Physiological disruption of photoperiod-controlled starch mobilization reduces growth of sink leaves in Arabidopsis. (*in preparation*)

The research described in this thesis was financially supported by the Netherlands Organization of Scientific Research (NWO) domain Applied and Technical Sciences (TTW; project number 13149).

Cover design by Mark van Hoogdalem

Printed by Proefschriftmaken.nl

Propositions

1. Pushing plants into a temporary CH starvation mode is an effective way to control growth.
   (this thesis)

2. Luciferase reporter lines related to growth, in combination with a sophisticated detection system like LUMINATOR, provides the most efficient and flexible method for screening light treatments to control plant growth.
   (this thesis)

3. Because most processes in the plant are under direct or indirect clock control (Sanchez & Kay 2016, *Cold Spring Harb Perspect Biol*, Vol. 8, pp.1-16), sample annotation requires inclusion of harvest time or time-window.

4. The current academic evaluation and reward system discourages pursuing high-risk research projects and thereby hampers breakthrough scientific progress.

5. The subject of P-hacking (Nuzzo 2014. *Nature*, Vol. 506, pp. 150-152) is an essential part of any scientific training or education program.

6. The idea that you can achieve anything if only you work very hard for it may be encouraging but not realistic.

7. A successful PhD candidate is (potentially) a good Lego builder, as both require similar skills.

Propositions belonging to the thesis, entitled:

From lab to greenhouse: Molecular mechanisms of physiological control of plant growth

Mark van Hoogdalem

Wageningen, 18 February 2020