ANTIPHASE LIGHT AND TEMPERATURE CYCLES DISRUPT RHYTHMIC PLANT GROWTH

THE ARABIDOPSIS JETLAG

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences (EPS).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 2 June 2014
at 16 p.m. in the Aula

Ralph Bours
Antiphase light and temperature cycles disrupt rhythmic plant growth: the Arabidopsis jetlag
166 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2014)
With references, with summaries in Dutch and English.

ISBN 978-94-6173-957-5

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On doing a PhD…

“Wisdom is a thing vast and grand. She demands all the time that one can consecrate to her”

- Lucius Annaeus Seneca

1

GENERAL INTRODUCTION

THE EVOLUTION AND ADAPTATION OF PLANTS TO DIURNAL LIGHT AND TEMPERATURE

Cyanobacteria were among the first autotrophic organisms to evolve on earth and they thrived in the ancient oceans. These prokaryotes were the first photosynthetic organisms and can be considered as the evolutionary origin of all plant life [1]. More than 1.5 billion years ago they facilitated the evolution of unicellular photosynthetic eukaryotes in the Proterozoic oceans [2]. Aquatic unicellular photosynthetic eukaryotes are often referred to as phytoplankton, and although far outnumbered by cyanobacteria, they are responsible for the majority of the flux of organic matter to higher trophic levels [3]. Due to the earth's rotation, the amount of light, and consequently temperature, in the top water layer of the ocean fluctuates diurnally [4]. As photosynthesis in phytoplankton depends on light, during the photoperiod, it is restricted to the top layer. In addition to photosynthesis, also mineral nutrients are required for growth, but nitrate and phosphate are often more abundant in deeper water layers. In addition, many species of phytoplankton prefer to maintain an optimal temperature for growth, which may be found at a specific depth [5]. Optimal growth conditions are thus distributed over different depths in the ocean and to accommodate different growth requirements, some phytoplankton developed diurnal vertical migration cycles. By manipulating cellular density or through active motion they vertically migrate to more favourable water layers in response to diurnal changes in light and temperature conditions [6].

In the mid-Palaeozoic era, between about 480 and 360 million years ago, aquatic photosynthetic organisms began to colonise land and this marked the origin and early evolution of land plants [7]. Land plants (from now on referred to as 'plants') are sessile organisms and therefore differ from aquatic phytoplankton and animals in that they cannot migrate away from unfavourable environmental conditions. This implies that plants need to deal with, and adjust to whatever challenges the environment presents through plasticity and adaptation [8]. Light and temperature are two of the strongest environmental factors influencing plant architecture [9] but vice versa adjustment in the plants architecture may help the plant to optimally cope with its local (micro)environment. This may involve structural adaptations for optimal light acquisition for photosynthesis, or the opposite: structural adaptations aimed at limiting light exposure when irradiation levels are too high [10]. Such adjustments in plant architecture may involve elongation responses (e.g. shade avoidance), differential growth responses (e.g. photomorphogenesis) [11] and leaf hyponastic movement [12].

LIGHT PERCEPTION AND SIGNALLING

Light is perceived through different types of photoreceptors, which are light sensitive proteins that can be activated by light of specific wavelengths. Two important families of photoreceptors are the CRYPTOCROME (mostly blue light activated) and PHYTOCHROME proteins (mostly red light activated, far-red light inactivated) [13]. Sunlight, composed of

many different wavelengths, contains (among others) blue, red, and far-red light. High radiance levels of red and blue light inhibit plant elongation by activation of the phytochrome (PHY) and cryptochrome (CRY) photoreceptors [14]. The CRY are photolyase-like flavoproteins that regulate various responses such as blue light inhibition of cell elongation, guard cell development and stomatal opening [15]. In the model plant Arabidopsis (*Arabidopsis thaliana*) two cryptochromes, CRY1 and CRY2 were known [16] and in 2006 a third one, CRY3, was identified [17].

PHY proteins are reversibly photochromic biliproteins that absorb maximally in the red and far-red light spectra [18]. The family of identified PHY receptors in Arabidopsis consists of five members, PHYA, PHYB, PHYC, PHYD, and PHYE [19]. Upon absorbance of red light, PHY undergoes a conformational change from the inactive (Pr) to the active (Pfr) form [20]. The active PHY Pfr form migrates into the nucleus, where among other signalling functions, it stimulates the degradation of growth stimulating basic helix-loop-helix (bHLH) transcription factors called PHYTOCHROME INTERACTING FACTOR (PIFs) [14]. Irradiation with far-red light converts the Pfr form back to the inactive Pr form, hence interrupting the growth suppressing effect of Pfr [21]. Also in darkness the Pfr form reverts back to Pr through a process referred to as dark reversion [22].

The growth regulating functions of PHY and CRY play an essential role in plant neighbour detection and resource competition. When plants are growing in a dense canopy of neighbouring plants they have to compete for light to maintain optimal photosynthesis for growth. As neighbouring plants absorb red light for photosynthesis the ratio of red to far-red light decreases in a canopy. Plants gauge this red:far-red ratio with PHY and hence can detect neighbour competition [23]. A high far-red:red light ratio inactivates PHY to the Pr form and stimulates rapid elongation growth aimed at outgrowing neighbour shading. These rapid elongation responses include hypocotyl, petiole and internode elongation and upward leaf movement (hyponasty). Such rapid adaptations in response to neighbour shade are collectively referred to as shade avoidance (SA) [11]. PHYB was the first photoreceptor identified as essential for the SA response [24-26]. Later, from several studies it became clear that even *phyB* null mutants display residual SA suggesting that other light signalling components are involved [27-29]. Studies using natural variation [30], double mutants and SA screens of mutagenised *phy* single and double mutants showed that PHYD and PHYE are also redundantly involved in the SA response [31, 32]. PHYA may act more antagonistically, regulating the severity of the SA response depending on irradiance levels [33, 34]. As mentioned above, activated PHY migrates into the nucleus where it degrades PIFs. Increased far-red:red ratios thus stabilise PIF protein levels and particularly PIF4 and PIF5 are redundantly essential for eliciting the growth stimulation of the SA response [35].

Unavoidably, SA driven elongation frequently occurs in the dense crops often used in agriculture, which can decrease crop quality. Experiments with several spectral filters applied to greenhouse glass have been performed with the aim to minimise the SA response [36]. Indeed filtering out far-red light reduced stem elongation in commercially important crops

such as chrysanthemum [37] and antirrhinum [38]. This is undoubtedly related to altering the input signal (ratio of red:far-red light) of the PHY-PIF growth signalling cascade. Several of these filters are commercially available but their overall applicability has been disputed to be limited by their short shelf life and cost effectiveness.

In addition to PHY also the CRY photoreceptors regulate elongation responses [39]. Similar to red light, plants also absorb blue light for photosynthesis and thus also the amount of blue light decreases in dense plant canopies, allowing plants to detect neighbours also by decreased blue light [23]. The use of spectral filters that selectively remove blue wavelengths from sunlight indeed results in pronounced stem elongation in multiple species [40]. In addition, increased leaf hyponasty in tobacco (*Nicotiana tabacum*) plants subjected to reduced blue light irradiance, has been observed [41]. Indeed, CRY2 mediates blue light-dependent inhibition of Arabidopsis hypocotyl elongation. Only when exposed to reduced blue light intensity, *CRY2* over-expressors had constitutive short hypocotyls, while *cry2* loss-of-function plants displayed increased hypocotyl length compared with wild-type [42]. Combined, it can be concluded that a reduction in blue light quantity can elicit responses similar to far-red triggered SA.

TEMPERATURE PERCEPTION AND SIGNALLING

In contrast to light perception and signalling, the way in which temperature is perceived and translated into growth adjustments by plants is not well understood. Attempts to isolate components directly acting as thermo-sensors have so far yielded no clear candidates, and temperature receptors in plants are sofar unknown [43]. In general, temperature affects the speed of biochemical reactions and thus affects many different processes [44]. Temperature may therefore be 'sensed' at multiple points and may not necessarily depend on a dedicated receptor and signalling pathway. When the rate of biochemical processes is somehow coupled to transcriptional responses this would allow for a mechanism by which temperature can adjust growth. For instance, the fluidity of biological membranes depends on their temperature, and it has been hypothesised that in yeast and cyanobacteria the membrane fluidity may be the primary temperature sensing mechanism [43]. The hypothesis that this may also be the case in higher plants led to experiments that aimed to influence temperature responses by genetically [45] or chemically modifying cell membrane properties. Dimethyl sulfoxide (DMSO) artificially rigidifies the cell membrane and treatment with this chemical indeed induced the expression of genes specific for a cold response in *Brassica napus* cell cultures [46]. However, the experimental data underlying the claims that alterations in membrane fluidity are a universal mechanism for temperature perception are generally not very convincing [47].

Another example of how temperature could affect gene transcription, and thus explain the response to temperature, was the discovery that Arabidopsis nucleosome unpacking and thus DNA accessibility and translation are temperature dependent [48]. Histones are the chief

protein components of chromatin. DNA wraps around histone proteins forming nucleosomes, which play a crucial role in gene regulation and transcription [16]. In Arabidopsis, H2A.Z nucleosome occupancy decreased with increasing temperatures, thus regulating gene expression by increasing DNA accessibility for transcription. Under normal growth temperatures, genotypes that are unable to incorporate H2A.Z into nucleosomes display the same adaptations as plants exposed to high temperature (27°C), such as early flowering and rapid elongation. In addition, the 'H2A.Z deficient' plants express genes that are normally only expressed in response to heat [48]. This indicates that heat responsive genes may be more abundant in H2A.Z containing nucleosomes.

In another example of indirect perception of temperature, the induction of flowering in response to high temperature in Arabidopsis was demonstrated to depend on DNA binding of the bHLH transcription factor PIF4. PIF4 binds the promoter of the flowering promoting gene *FLOWERING LOCUS T* (*FT*) in a temperature dependent manner, thus regulating the floral transition in response to increasing temperature [43]. Because PIF4 has also been implicated in heat induced elongation responses [49], the temperature sensitive DNA binding of PIF4 could provide an additional factor by which the effect of temperature is regulated. Another way by which temperature may affect cellular processes is protein unfolding. At higher temperatures the folding of proteins may become limiting for correct function, providing another indirect mechanism by which temperature can affect growth. Specific protein folding chaperone proteins may help the plant cope with such consequences of heat stress. Indeed the HEAT SCHOCK PROTEIN 101 (HSP101) plays a crucial role in temperature tolerance in Arabidopsis. *hsp101* mutants were more severely affected by heat and plants over-expressing *HSP101* were more tolerant than wild-type [50].

TEMPERATURE INTERACTS WITH LIGHT SIGNALLING IN THE CONTROL OF PLANT DEVELOPMENT

Previous work demonstrated that there is significant overlap between the response of plants to temperature and light (reviewed in [9]). Loss of photoreceptor activity frequently results in an altered response to temperature. This was first observed in seeds, for which the temperature range that permits germination strongly depends on the presence of red and far-red light [9]. Far-red light acts similar to low temperatures, inhibiting germination, while red light induces germination similar to the induction of germination by optimal temperature [51]. These results indicated that PHYB was responsible for these temperature effects on germination [52, 53]. Later it was demonstrated that PHYB is essential for germination of Arabidopsis seeds at a wide range of temperatures [54].

Similarly, high temperatures trigger excessive elongation of Arabidopsis internodes and the magnitude of this response depends on both PHYB and CRY1 photoreceptors. In loss-of-function mutants of these photoreceptors the response is much stronger. By inhibiting the elongation response to heat, PHYB and CRY1 buffer the detrimental effect of high temperature on biomass accumulation [55]. Another response of Arabidopsis rosette plants

to heat is the upward movement of the leaves, hyponasty. A sudden increase in temperature induces differential petiole growth, which results in an increase of the leaf angle relative to the soil [12]. Similar to internode elongation, PHYB is a negative regulator of heat induced hyponasty. Heat induced changes in rosette compactness were shown to facilitate evaporative cooling of Arabidopsis. Thus, by adjusting its architecture in response to environmental conditions the plant is able to actively regulate its leaf surface temperature [10]. It should be noted that these photoreceptor dependent temperature responses probably depend on active photoreceptors, which require light activation. Therefore, modulation of the high temperature response by the photoreceptors is expected to occur during the light period, which normally coincides with the period of higher temperatures.

THE CIRCADIAN CLOCK INTEGRATES ENVIRONMENTAL LIGHT AND TEMPERATURE CUES

Optimal plant growth requires the temporal coordination of all the different processes required for growth. In plants, the circadian clock allows for the anticipation of predictable rhythmic environmental changes [56]. The circadian clock has been studied extensively in Arabidopsis [57]. The intrinsic output of the clock is generated by multiple interlocking feedback loops of oscillating expression of core clock genes and has a rhythmicity of approximately 24 hours. The clock can be divided into three such expression loops, a central loop, a morning loop and an evening loop (Fig. 1A). The central loop contains the morning-expressed MYB transcription factors *CIRCADIAN CLOCK ASSOCIATED1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)* that negatively regulates the evening-expressed *TIMING OF CAB EXPRESSION1 (TOC1)* which, in turn, transcriptionally downregulates *CCA1* and *LHY.* This central loop interlocks with the so called 'morning loop' which includes the *PSEUDO-RESPONSE REGULATORS PRR5*, *PRR7*, and *PRR9* (that repress the expression of *CCA1* and *LHY*). In addition, the central loop also interacts with an 'evening' loop, containing *GIGANTEA* (*GI*) and *ZEITLUPE* (*ZTL*), which feedback to repress *TOC1* [58] (Fig. 1.1A) (for recent reviews see [57, 59-61]).

All the clock components show oscillating expression under constant conditions albeit with different phases for their peak activities. For their sustained rhythmic activity they do however depend on entrainment by environmental signals. The Arabidopsis circadian clock can be entrained by light and temperature cycles [62, 63]. A temperature cycle with a 4°C difference in temperature is sufficient to entrain the clock [64]. Temperature cycle entrainment of mutant populations under continuous light, and subsequent observation of circadian leaf movement after transfer to conditions with continuous light and temperature, identified genes important for temperature entrainment in Arabidopsis. In this way *PRR7* and *PRR9* were shown to be redundantly required for temperature entrainment. The double mutant failed to maintain robust rhythmicity after temperature entrainment, but behaved like wild-type after light entrainment [65]. Later, it was shown that also *EARLY FLOWERING 3* (*ELF3*), an evening-specific repressor of light signals to the clock, is essential for temperature

entrainment. As it was shown that *elf1-3* null mutants were unable to maintain circadian rhythms after entrainment to only temperature cycles [66].

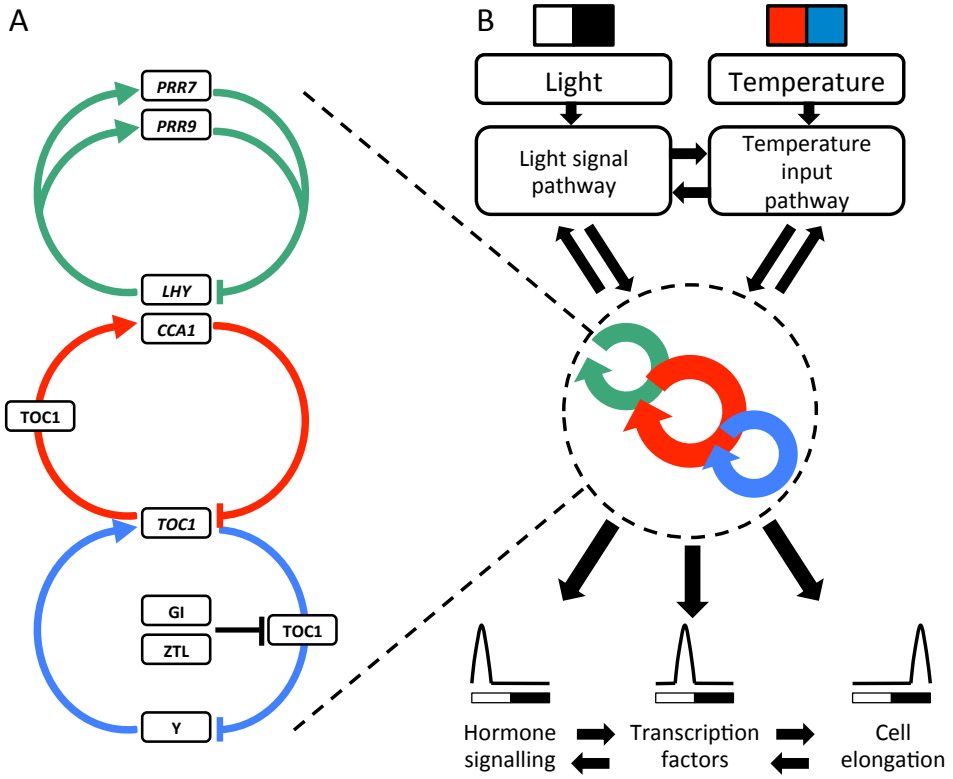

Figure 1.1A Simplified model for the central oscillator. Three interlocking feedback loops, each represented by a colour, regulate each other's expression. In the central loop (red) *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), repress *TIMING OF CAB EXPRESSION 1* (*TOC1*) transcription in the morning. As the day progresses, expression of *LHY* and *CCA1* decreases, allowing *TOC1* expression to increase. *TOC1* then closes the loop by indirectly reactivating the expression of *LHY* and *CCA1*. The 'morning' loop (green) involves *PSEUDO-RESPONSE REGULATORS 7* and *9* (*PRR7* and *PRR9*), and *LHY/CCA1*. The 'evening' loop implicates TOC1 and Y, an unknown component identified by mathematical modelling and whose activity is partially explained by *GIGANTEA* (*GI*). In addition, GI interacts with ZEITLUPE (ZTL) leading to TOC1 degradation.

Figure 1.1B Circadian regulation of rhythmic growth. The circadian clock, depicted with coloured arrows, generates 24-hour rhythms. Light and temperature signals provide entrainment for the clock, which through appropriate signalling pathways determines the output depending on the time of day. Light and temperature input pathways often overlap, and in addition receive circadian feedback, depicted by the double arrows. Ultimately the clock coordinates various output processes, such as hormone signalling, expression patterns of transcription factors and processes required for cell elongation to occur in an orchestrated manner to allow optimal plant growth in response to the environment. Note that in addition to having an independent signalling pathway, temperature could affect the clock by modifying the light signalling pathways, and vice versa. Square icons indicate light (white), dark (black) and high (red) and low (blue) temperatures *(Figure adapted with permission* [61]).

Because all biochemical processes are temperature sensitive and the clock consists of a network of biochemical interactions this would infer that the function of the clock is slowed down at lower temperatures and sped up at higher temperatures. However, because the clock needs to coordinate processes over the 24-hour day cycle regardless of temperature, mechanisms exist that buffer the temperature effects on the clock output over a wide temperature range. These phenomena are referred to as temperature compensation [67, 68]. A quantitative genetic approach indicated a role for the clock components *GI* [69] and other evening loop genes, such as *ZTL* in temperature compensation in Arabidopsis [68, 69].

An interaction between temperature and the clock was also demonstrated by heat shock experiments that can suppress the plant elongation response. For instance, it was shown that while moderately warm constant ambient temperatures tend to oppose light signals in the control of plant elongation, a brief heat shock during the night enhances the inhibition of hypocotyl growth. This effect in Arabidopsis seedlings was dependent on light perceived by PHYB. The daily heat shock resulted in a transient increase in the expression of *PRR7* and *PRR9* and in an increase in the amplitude of the rhythms of *LHY* and *CCA1* expression. In turn, these rhythms gated the hypocotyl response to red light, in part by changing the expression of *PIF4* and *PIF5* [70].

THE CIRCADIAN CLOCK ORCHESTRATES DIURNAL GROWTH

To determine if the clock plays a role in a plant growth related process, the process is characterised under continuous conditions after proper entrainment of the clock [71]. Under natural diurnal conditions, the clock plays an important role in controlling the phase of growth and cell elongation [69, 72] (Fig. 1.1B). The clock enables to set peak activities of processes that have to occur in an obligate sequential order, and thus helps to give an optimal response [73]. For photosynthesis, the clock allows all the necessary photosynthetic machinery to be produced just before dawn, to thus effectively utilise the very first light of the day [56]. Plant cell walls are extremely rigid, which limits cell expansion, and first require loosening, so water uptake can cause cell expansion, and subsequently refortification after expansion [74]. Because cell wall loosening should occur before water uptake these processes need to be tightly coordinated. Several of the key steps in cell elongation were shown to be under circadian control and therefore still occur in an orchestrated fashion under continuous light and temperature (Fig. 1.1B). Under continuous light and temperature, cell elongation peaks late in the subjective day until early in the subjective night [72]. The clock controls cell elongation in part by regulating the rhythmic transcription of growth stimulating transcription factors. For instance PIF4 and PIF5 protein levels accumulate at dusk, allowing rhythmical seedling elongation in response to short day light cycles [75].

PLANT GROWTH RESPONSES TO DIURNAL TEMPERATURE DIFFERENCES

In natural stands, presence of daylight (photoperiod) and temperature fluctuate diurnally. Typically, the photoperiod (day) coincides with warm temperatures and the dark period (night) with low temperatures. In horticulture, the natural light/temperature difference is often referred to as positive day/night difference (+DIF). The difference between day and night temperatures has a strong influence on plant growth. Collectively, responses to diurnally fluctuating temperatures are referred to as thermoperiodism [76]. Plants grown under conditions with a positive temperature difference, i.e. higher day temperature and lower night temperature (+DIF) display more stem elongation than plants grown at a constant average temperature [76]. When a negative temperature difference is created, and the temperature of the photoperiod is (artificially held) colder than that of the dark period (-DIF), plant growth in many species is strongly inhibited (Supplemental Table S1.1). For this reason -DIF regimes are often applied in greenhouses to counteract excessive elongation and to keep crop plants compact. -DIF has been effectively applied to reduce internode elongation in various crop species [77-79]. Also in the model plant Arabidopsis, -DIF has an inhibitory effect on elongation [80] and reduces leaf hyponasty (Fig. 1.2).

Figure 1.2 Two week old Arabidopsis rosette plants were exposed to either +DIF or -DIF conditions for two more weeks. The -DIF treatment results in reduced leaf lengths, which in combination with reduced leaf angles gives the plant a compact stature. Square icons indicate light (white), dark (black) and high (red) and low (blue) temperatures.

In Arabidopsis, cell elongation in the inflorescence was reduced with decreasing day temperatures [80]. In leaves, a 10°C -DIF resulted in a 20% decrease in leaf length compared with +DIF [81]. Thermoperiodic responses are known to be species specific and some species were reported to be insensitive to -DIF (Supplemental Table S1.1). Despite the frequent application in horticultural practice and therefore its economic importance, full mechanistic understanding of the growth control by -DIF is limited. Insights into the effect of -DIF on plant growth processes as available at the onset of this research project are summarised in Fig. 1.3.

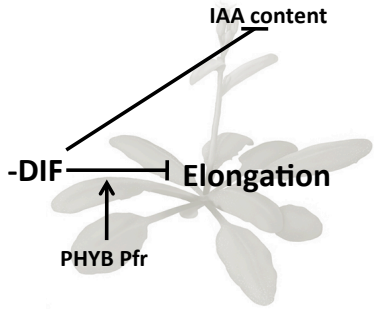

Figure 1.3 Components known to be involved in the response of Arabidopsis (*Arabidopsis thaliana*) to -DIF at the onset of this thesis (beginning 2009). The inhibitory effect of -DIF on elongation depends on PHYB. *phyB* mutants or plants illuminated with far-red light show a reduced sensitivity to -DIF. In contrast to other species, -DIF did not affect the level of active gibberellins, but did result in a reduced auxin content. The mechanism by which reduced auxin affects elongation was not further investigated [80].

Increased gibberellin catabolism under -DIF is for example described for Pea (*Pisum sativum*) [82] but could neither be detected in Begonia (*Begonia x hiemalis*) [80], nor in Arabidopsis [83]. The -DIF growth response in Arabidopsis was however associated with reduced auxin levels in the inflorescence stem [80]. The photoreceptor PHYB has been shown to be involved in the growth inhibition under -DIF. For example, additional red light enhanced the -DIF response while additional far-red light almost abolished the reduction in stem elongation in various species [84, 85]. In accordance with these observations, *phyB* mutants in Arabidopsis [86] and cucumber (*Cucumis sativus*) [87] are insensitive to -DIF treatment.

-DIF TREATMENTS IN HORTICULTURE

Horticulture at higher latitudes suffers from short light periods and reduced light intensities in winter, resulting in lower plant quality during this season. Even if the light intensity during the photoperiod is sufficient to sustain growth, many plant species may show undesirable elongation, leading to spindly shaped, and therefore unmarketable plants, especially in densely packed plant cultivation systems. Thus, the economic pressure to make efficient use of greenhouse space and grow plants at high densities is counteracted by undesirable effects such as excessive stem elongation, strong leaf hyponasty and long petioles, which have a negative effect on crop value. The desired plant size and architecture in horticulture is usually referred to as ‘compact’. The demands for compactness differ between crop species and individual market requirements, and are an important issue for various members of the horticultural production chain, such as growers of young plants, final product growers, transporters and retail in the pot and bedding plants, vegetables, and cut-flower industry.

Plant growers can reduce plant growth by applying growth retarding chemicals, mainly gibberellin biosynthesis inhibitors [88]. However, environmental concerns and new legislation for the use of chemicals in horticulture [89] increased the need for more sustainable physiological treatments to control plant architecture [88]. The technical advances in greenhouse climate control now allow manipulation of many environmental conditions. These developments, in combination with the mostly empirically derived insights that plant height may be controlled by changing the temperature cycle, made -DIF of interest for the horticulture industry. Especially in the more northern latitudes, during winter, the days are naturally cold and heating of the greenhouse is more economical during the dark periods. At night the greenhouse can be insulated using screens. Many crops respond favourably to -DIF (Supplemental Table S1.1) and the concept became a popular approach in greenhouses across North America, Japan, and Europe [77].

THE TTI-COMPACT PROJECT

Greenhouse horticulture is of importance for the Dutch economy as it significantly (19%) contributes to the approximately €15 billion surplus on the national trade balance (2010) [90]. The solid international position of Dutch horticulture is in part due to a strong tradition in the development of innovative greenhouse plant growing systems, combining knowledge of plant genetics and physiology with greenhouse climate control. Maintaining a leading position requires year-round production of high quality plants, which at present is hard to achieve without regularly spraying chemical growth retarding chemicals to reduce elongation growth in dense crop systems during the winter. Diurnal temperature treatments are an attractive alternative for chemical elongation control. They have great practical use, but the mechanism by which they influence plant growth is poorly understood, hampering further optimisation. The objective of the TTI-Compact project (this thesis) was therefore to identify the processes that are affected by these temperature treatments and regulate plant elongation.

By studying plant growth and its regulation on a physiological and genetic level we aimed to provide a fundamental basis for the understanding of growth reduction, enabling its use in a more (cost) effective way. Not only the efficiency and costs of -DIF like regimes may be improved, but also molecular insight may deliver marker genes that could be used for breeding more -DIF sensitive crop species. Combined with optimised climate control protocols, such improvements should reduce future use of chemical growth regulators and could potentially also result in energy saving. The importance of the project is reflected by the intellectual involvement and financial support of 37 (inter)national companies active in the horticultural sector. See Supplemental Table S1.2 for a complete list of companies that participated intellectually and financially in this project.

NEW PERSPECTIVES ON -DIF

-DIF could affect the internal synchronisation of growth processes in the plant

All components known to be involved in mediating the effect of -DIF on the growth of the model species Arabidopsis at the start of this research at the start of 2009 are summarised in Fig. 1.3. It was already speculated that alternating temperature cycles (like -DIF), and light quality, may affect the synchronisation of growth processes in the plant [91]. Based on the importance of the endogenous circadian clock, which depends on both light and temperature for entrainment and thus optimal coordination of growth processes with the environment (Fig. 1.1B), we postulated the following hypothesis: the conflicting entrainment resulting from the -DIF antiphase light and temperature cycles could directly affect the plant circadian oscillator. Alterations in clock function could subsequently result in altered synchronisation of clock controlled growth related output processes. If true, this could be causal for the reduced elongation growth observed in various species under -DIF (Supplemental Table S1.1). In this thesis we set out to test this hypothesis. We exploited the available resources available in Arabidopsis, which combined with the custom development

of novel techniques, enabled us to monitor clock function and rhythmic growth processes under different diurnal light and temperature cycles (+DIF and -DIF).

OUTLINE OF THE THESIS

Using Arabidopsis, our research aimed to understand the mechanisms underlying the -DIF response. To determine when -DIF affects elongation during the diurnal cycle, we developed an infrared imaging system that allowed the semi-continuous quantification of leaf growth and movement throughout the diurnal day (**chapter 2**). The OSCILLATOR system is subsequently used to study the response of rosette plants to -DIF compared to the +DIF control. Results show that -DIF mainly affects growth during the cold photoperiod, resulting in a phase shift and decreased amplitude of leaf movement. Ethylene production shifts in response to -DIF and the sensitivity and local production of this hormone becomes limiting for leaf growth movement under -DIF. This response depends on the photoreceptor PHYB (**chapter 3**). Next we study how ethylene links to reduced cell elongation using Arabidopsis hypocotyls as a model system. We demonstrated that auxin biosynthesis is reduced during -DIF and this disrupts an auxin-ethylene signalling cascade, which regulates downstream transcription of PIF3 (**chapter 4**). Because -DIF differentially affects circadian output processes, we also investigated whether -DIF directly affects the circadian oscillator. To this aim, rhythmic activity of core clock genes are compared between +DIF and -DIF using luciferase reporter plants. -DIF differentially affects the amplitude and phase of clock genes, resulting in reduced clock robustness. In addition, *GIGANTEA* is identified as essential for this response and loss of *gi* results in more robust clock activity and restores elongation growth under -DIF. Combined, we conclude that -DIF affects plant growth by differentially affecting the circadian coordination of growth processes. This is further illustrated by the inability of plants exposed to -DIF to adjust starch degradation rates to the anticipated dawn, resulting in carbohydrate starvation (**chapter 5**). In the last chapter, (**chapter 6**) we discuss the connections between the processes studied and the altered circadian clock and expand upon the future implications of the obtained results. In addition we speculate on how our findings may assist breeding crops for more favourable responses and can help further optimising DIF like treatments.

SUPPLEMENTAL DATA

Supplemental Table S1.1 Selection of plants according to DIF sensitivity and flowering day length

Plant species name	DIF sensitivity	
	Strong or medium effect	Little or no effect
Short day flowering plants		
Aster novi-belgii L.		[92-94]
Begonia x cheimantha	[93, 95]	
Begonia x hiemalis Fortsch.	[79, 93, 96-98]	
Chrysanthemum L. sp.	[96, 99-103]	
Chrysanthemum morifolium	[93]	
Chrysanthemum paludosum 'North Pole'	[93]	
Dahlia Cav.	[104]	
Chrysanthemum indicum (*Dendranthema*)	[93]	
Dendranthema grandiflora Ramat	[77, 95, 100, 105-109]	
Euphorbia pulcherrima Wild ex. Klotzseh	[93, 96, 98, 108, 110-113]	
Kalanchoe blossfeldiana v. Poelin	[93, 95, 114, 115]	[96, 98]
Magnolia soulangiana	[93, 116]	
Poinsettia	[93, 98, 105, 117]	
Sandersonia aurantiaca	[93, 118]	
Xanthium pensylvanicum L.	[93, 119]	
Long day flowering plants		
Antirrhinum majus L. as bedding plant	[93, 120, 121]	[122]
Antirrhinum majus L. as cut flower	[79, 93, 120, 121]	
Arabidopsis thaliana Ler	[80]	
Brassia L.	[79, 123]	
Brassica oleracea var. acephala	[93]	
Calendula officinalis Marigold	[94]	
Campanula	[91, 93, 98, 124]	
Campanula isophylla Moretti	[84, 91, 98, 125, 126]	
Dianthus L. sp.	[124, 127]	
Fuchsia	[93, 119]	
Fuchsia x hybrida Hort. Ex Vilm.	[101, 128-132]	
Matthiola incana	[93, 120, 121]	
Snapdragon	[93, 133]	
Verbena bonariensis	[93, 134]	

Plant species name	DIF sensitivity	
	Strong or medium effect	Little or no effect
Neutral day flowering plants		
Cucumis sativus	[93, 135-138]	
Cucurbita L.	[139]	[94]
Cyclamen persicum Mill.	[140]	[141]
Gerbera jamesonii H. Bolus ex Hook. F.	[142]	[143]
Hycinthus L.		[94]
Impatiens walleriana Hook. F.	[93, 120, 121, 131, 144]	
Lilium longiflorum Thunb.	[128, 145-147]	
Lycopersicum Mill.	[93, 102, 108, 136, 137, 139]	
Narcissus L.		[94]
Passiflora caerulea L.		[148]
Pelargonium L' Her. ex Ait. sp. cuttings		[97, 131, 149]
Pelargonium L' Her. ex Ait. sp. seed	[150, 151]	
Petunia x hybrida Hort Vilm.- Andr.	[97, 152, 153]	[97]
Platycodon grandiflorus	[82]	
Rosa L.		[154] [97]
Salvia splendens F. Sellow ex Roem. & Schult	[97]	
Streptpcarpus nobilis C. B. Clarke	[93, 119]	
Tagetes patula cv. 'Bonanza Yellow'	[93]	
Tulipa L.		[94]
Viola wittrockiana Gams.	[93, 155, 156]	
Zinnia	[93, 133]	
Many foliage plants		[157]

Supplemental Table S1.2 List of companies that contributed financially and/or intellectually to the TTI Compact project (this thesis)

Company expertise	Company name
Project Coordinator: Arjan Stolte	ASP - Quality Support (no project partner)
Ornamental and fruit breeders	Combinations
	Dekker Chrysanten
	Goldsmith Seeds Europe BV (Syngenta flowers)
	Kieft Seed BV (PanAmerican Seed)
	Schoneveld Twello BV
	Syngenta Seeds BV
	Takii Europe BV
	Varinova Europe BV
	ABZ Seeds BV
Vegetable breeders	Bejo Seeds BV
	ENZA Seeds BV
	Nunhems BV (Bayer CropScience)
	Rijk Zwaan BV
	Monsanto Holland BV
Young plants production	Beekenkamp Plants BV
	Brabant Plant Plantenkwekerij BV
	Florensis BV / Ball Holland BV
	Grow Group BV
	Gipmans Plants BV
	Plantenkwekerij Gitzels BV
	Plantenkwekerij Grootscholten BV
	Plantenkwekerij Valstar BV
	Plantenkwekerij van der Lugt BV
	Plantenkwekerij van der Plas BV
	Raes Plant Production NV
	Schneider Youngplants BV
	Selecta Klemm GmbH & Co. KG
	Westlandse plantenkwekerij WPK
Pot plants production	Fides BV
	Formosa International
	Martha Plant VOF
	Plantenkwekerij Leo Ammerlaan BV
	Plant Planet BV
	Plantenkwekerij Arie Baas
Fertiliser / Plant nutrition	Everris (formerly Scotts International) BV
Growth substrates	Van Der Knaap Group BV
Others	LTO Groeiservice

On leaf movement…

“Each separate movement is produced by the same energy that moves the sum of things”

- Hermes

2

OSCILLATOR: A SYSTEM FOR ANALYSIS OF DIURNAL LEAF GROWTH USING INFRARED PHOTOGRAPHY COMBINED WITH WAVELET TRANSFORMATION

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PUBLISHED IN:

Plant Methods 2012, 8:29
doi:10.1186/1746-4811-8-29

ABSTRACT

Quantification of leaf movement is an important tool for characterising the effects of environmental signals and the circadian clock on plant development. Analysis of leaf movement is currently restricted by the attachment of sensors to the plant or dependent upon visible light for time-lapse photography. The study of leaf growth movement rhythms in mature plants under biological relevant conditions, e.g. diurnal light and dark conditions, is therefore problematic. Here we present OSCILLATOR, an affordable system for the analysis of rhythmic leaf growth movement in mature plants. The system contains three modules: (1) infrared time-lapse imaging of growing mature plants, (2) measurement of projected distances between leaf tip and plant apex (leaf tip tracking growth-curves), and (3) extraction of phase, period, and amplitude of leaf growth oscillations using wavelet analysis. A proof-of-principle is provided by characterising parameters of rhythmic leaf growth movement of different Arabidopsis (*Arabidopsis thaliana*) accessions as well as of *Petunia hybrida* and *Solanum lycopersicum* plants under diurnal conditions. The amplitude of leaf oscillations correlated to published data on leaf angles, while amplitude and leaf length did not correlate, suggesting a distinct leaf growth profile for each accession. Arabidopsis mutant accession Landsberg *erecta* displayed a late phase (timing of peak oscillation) compared to other accessions and this trait appears unrelated to the *ERECTA* locus. OSCILLATOR is a low cost and easy to implement system that can accurately and reproducibly quantify rhythmic growth of mature plants for different species under diurnal light/dark cycling.

BACKGROUND

The movement displayed by plants has long fascinated people and it is believed that ancient tribes used rhythmic leaf movements to schedule their prayers [158]. The first documented attempt to elucidate whether the rhythm of movement was inherent to the plant or the result of external stimuli was performed by de Mairan in 1729. He observed that the rhythmic leaf movements of his 'sensitive plant' (*Mimosa pudica*) continued even in continuous darkness [158, 159]. Indeed, leaf movements of many species are controlled by the endogenous circadian clock [71] and in the past decade, plant circadian clock research has frequently used leaf movements of Arabidopsis (*Arabidopsis thaliana*) seedlings as a marker of clock output which can be easily compared between different genotypes [160-162].

ANALYSIS OF RHYTHMIC GROWTH IN SEEDLINGS

Various systems have been described for the analysis of leaf movement in Arabidopsis seedlings [160, 161]. These systems are characterised by sequential imaging of seedlings from the side. The position of the cotyledon or the first and second real leaf tip is then recovered from the time series images using for example NKTRACE [163], METAMORPH software, or custom made programs [164]. Subsequently, the quantified leaf position curves are often

analysed using Biological Rhythms Analysis Software System (BRASS). Analysis of the leaf tip plots includes Fast Fourier Transform-Non Linear Least Squares (FFT-NLLS) analysis [165, 166]. FFT-NLLS provides the average phase and amplitude of cyclic processes, based on the best fitted sinusoidal curve over multiple days [166] and thus does not capture the daily changes in phase and amplitude upon transition to a different growth condition or during development.

ANALYSIS OF RHYTHMIC GROWTH IN MATURE PLANTS

Methods developed for the analysis of circadian movements or upward leaf reorientation (hyponastic growth) in mature plants include physical attachment of sensors to the plant [167], photoelectric devices developed for measuring leaf movements in space independent of direct contact [168], and strings attached to a rotation resistance transducer glued to the leaf [169]. Time-lapse photography is another commonly used method (e.g. [164, 170, 171]). Imaging from the side makes it difficult to quantify leaf movements of mature plants as the dense whirl of (rosette) leaves may obscure a clear view of single leaves. For this reason, leaves obscuring the petiole base of the tracked leaf need to be removed. This procedure was previously used to quantify hyponastic growth in Arabidopsis and removal of leaves was reported not to influence the movement of the tracked leaves [164]. Moreover, in order to correct for diurnal and circadian effects on leaf movement, the angles of treated and control plants were subtracted in these experiments [164]. Another disadvantage of time-lapse photography is that it commonly depends on standard cameras, which require light visible to the human eye. It is therefore only suitable for continuous light experiments. To simulate night conditions, non-photosynthetic green light was used to image Arabidopsis leaf growth during the dark period [172]. Similarly infrared (IR) imaging has been used to measure the kinetics of Arabidopsis leaf reorientation in response to light quality [171], the response kinetics of Arabidopsis seedlings to ethylene [173], and the growth rate of Arabidopsis hypocotyls in diurnal conditions [75]. Alternatively, images taken at the beginning and end of the day period were used to analyse the diurnal leaf growth and movement of developing Arabidopsis rosette leaves [174]. This approach gives an average growth rate for the light and dark period and can therefore not be used to determine phase, period, or amplitude in the 2leaf growth movement. Here we developed a monitoring system based on infrared photography called OSCILLATOR. Our system allows continuous, high resolution growth analysis of mature rosette plants, independent of the presence of visible light. It thus enables measurement under biological relevant diurnal photoperiods. We positioned the IR sensitive cameras above the plants. Although imaging from above does not always allow for exact quantification of leaf length due to leaf hyponasty, a top view avoids the problem of rosette leaves obscuring each other. In addition it still allows reliable extraction of leaf movement parameters (phase, period, amplitude) in model species such as Arabidopsis, *Petunia hybrida* (petunia) and *Solanum lycopersicum* L. (tomato) plants. By tracking the movement of the leaf tip of specific leaves over time (typically 7 days), growth movement curves were obtained

from which phase and amplitude were extracted using wavelet analysis. This processing method allows for reliable measurement of daily phase and amplitude, which are convenient parameters to quantify the effect of mutant genes or physiological treatments on growth. To validate the system, we determined the natural variation for diurnal leaf growth movement in several Arabidopsis accessions. We identified in L*er* ERECTA independent variation as a regulator of the phase of leaf movement.

RESULTS AND DISCUSSION

The OSCILLATOR system for continuous analysis of plant growth under continuing diurnal light/dark cycles contains three modules: (1) data acquisition in the experimental setup, (2) image processing, and (3) extraction of phase, period, and amplitude using wavelet analysis (Fig. 2.1).

(1) DATA ACQUISITION IN THE EXPERIMENTAL SETUP

The hardware of the system consists of a climate controlled growth cabinet fitted with two IR LED light units (890 nm) and two modified single-lens reflex (SLR) cameras with the IR filter removed. The cameras are fitted to a sliding frame to allow easy positioning above the plants (Fig. 2.1A). With two cameras per cabinet the full surface area of the growth cabinet could be monitored (Fig. 2.1B) with minimal 'visual angle-to-object' effects. Cameras are controlled using NIKON Camera Control software run on dedicated laptops. Arabidopsis rosette plants (32 days old) are placed on an irrigation mat, which is saturated with tap water every 3 days. After 7 days of acclimation in the growth cabinet images are taken every 20 minutes over a period of up to 16 days.

(2) IMAGE PROCESSING

From the obtained digital image stack of multiple plants, single plant frames were cropped using ImageJ freeware (Fig. 2.1C). For Arabidopsis, for each individual plant, the 11^{th} and 12^{th} real leaves (≈5-7 mm long at t=0) were selected in the first frame and used for measurement of the projected distance between leaf tip to rosette centre throughout the image stack (Fig. 2.1C). This can be achieved in two ways

1: ImageJ 'manual tracking' plugin allows manual tracking of the leaf tip position.

2: ImageJ 'particle tracker' [175] allows measurement of the position of a small dot of inert paint placed on the leaf tip at the start of imaging (this did not affect growth).

Both methods provide Microsoft Excel compatible files containing the leaf tip coordinates in pixels (Fig. 2.1C). The rosette centre coordinates were similarly determined in the first frame. For Arabidopsis these remained fixed throughout the experiment. The projected distance in pixels between leaf tip and rosette centre was calculated and converted into millimetres

according to a scale marker (placed at average plant height) included in the images. This distance is plotted against time and represents projected leaf growth (Fig. 2.1D).

(3) EXTRACTION OF PHASE, PERIOD, AND AMPLITUDE USING WAVELET ANALYSIS

In the projected length curve the vertical rhythmic leaf movements are identified as oscillations in the curve. For the extraction of these oscillations the individual curves are imported into R freeware and fitted with a best fit 2° polynomial regression line representing average projected growth rate (R^2>0.85) (Fig. 2.1D). The regression curve was subtracted from the leaf tip movement curve, providing a residual oscillation curve. Decreasing distance between tip and centre indicates upward leaf growth movement. Because we want to use the maximum hyponastic leaf position as a marker for the phase of leaf movement, the residual oscillations were inverted to allow maximum upright leaf position to correspond to maximum peak height (compare Figs. 2.1D and E). We confirmed that the peak of oscillations indeed corresponds to the highest leaf angle: for one set of plants the absolute lengths of the tracked leaves were measured daily at the end of the photoperiod (Supplemental Fig. S2.1A). Based on the measured projected and absolute leaf lengths, for each day, the leaf angle was calculated. A comparison between the smoothed projected leaf tip oscillations and the calculated leaf angle confirmed that peak oscillations match maximal leaf angles (Supplemental Fig. S2.1B). On the transition from day to night period a 'bump' is observed in the projected leaf growth oscillations. This is caused by a temporary relapse in the upward leaf movement on the light to dark transition (Fig. 2.1E). In order to obtain an objective phase and amplitude of leaf movement, the raw projected oscillation plots of individual leaves were smoothed by removal of harmonic noise using wavelet analysis based on WAVECLOCK [176] (Fig. 2.1F). Wavelet analysis provides an alternative for the commonly used FFT-NLLS method and allows for an accurate day-to-day estimation of phase and amplitude [176]. With wavelet analysis we therefore get an accurate description of adaptations in phase and amplitude throughout development. Smoothed projected oscillation curves of 10 to 12 leaves from 5 to 6 individual plants were averaged and plotted with standard errors for each time point (Fig. 2.1F).

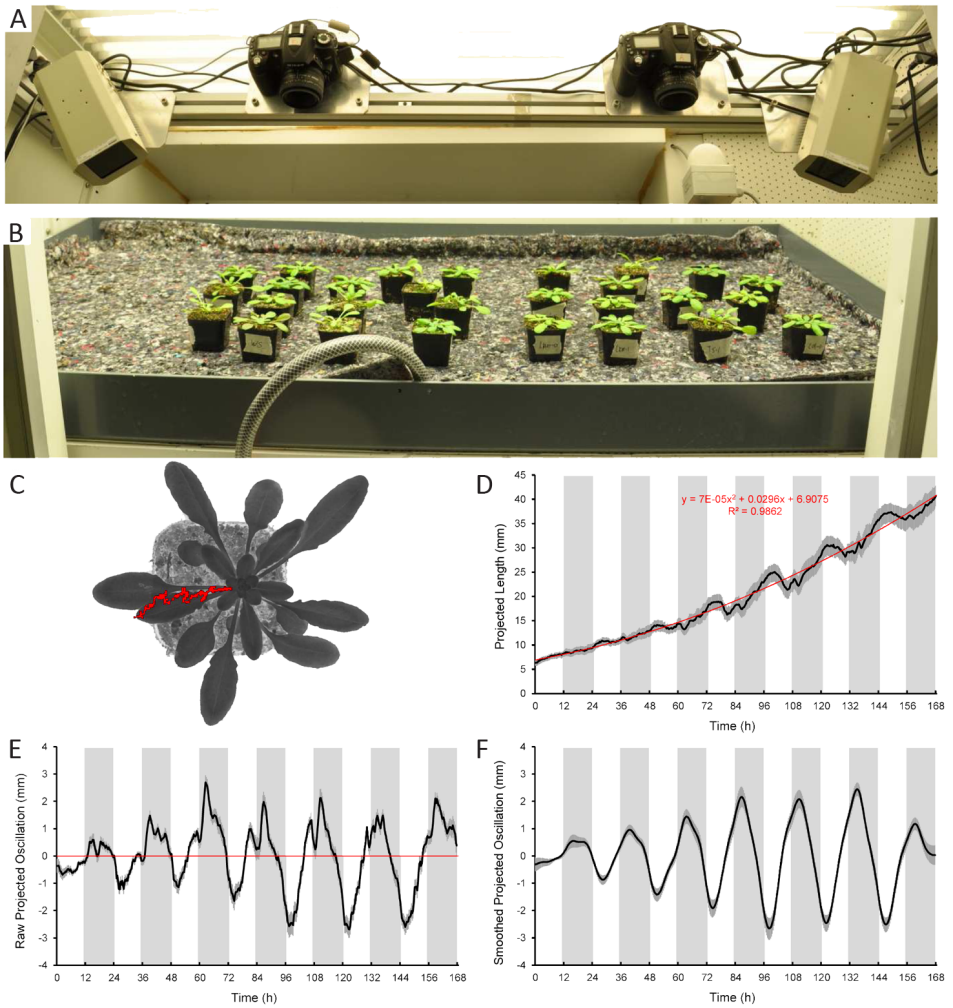


Figure 2.1 Experimental setups and the procedure of leaf growth and movement analysis. (**A**) Single-lens reflex (SLR) cameras are mounted to an aluminium frame inside a growth cabinet, IR illumination is provided from LED lights (far left and far right). (**B**) The camera frame is suspended above a tray containing randomised plants. (**C**) ImageJ plugins (Supplemental File S2.1) allow tracking of the leaf tip throughout a virtual image stack, save the coordinates, and project the trajectory. (**D**) The distance in mm from the leaf tip to the rosette centre is calculated, averaged, and plotted against time. A best fit 2° polynomial regression line (red) is fitted to individual leaf curves and subtracted from the data. (**E**) The result is the residual from the regression line, here depicted as raw projected oscillations. Note: originally decreasing distance between tip and centre indicated upward leaf movement. For clarity the residual projected oscillations were inverted to allow maximum upright leaf position to correspond to maximum peak height. (**F**) Harmonic noise is removed from individual leaf growth movement plots using wavelet analysis resulting in smoothed projected leaf oscillation curves. All data represent averages of 10 leaves: for 5 plants, 2 leaves per plant were tracked and the analysis was performed with these 10 leaves. Because of the high density, the standard errors were plotted for each data point and depicted as shading.

CHARACTERISTICS OF LEAF GROWTH MOVEMENT IN ARABIDOPSIS ROSETTE PLANTS

OSCILLATOR was used to characterise growth of 32 day old Arabidopsis Columbia-0 (Col-0) rosette plants. For each individual plant, the 11th and 12th real leaves (≈5-7 mm long at t=0) were analysed throughout 7 days. During this period, the projected lengths increased from ≈7 mm to ≈42 mm. Oscillations initially increased with progressing development, but decreased after ≈144 hours (h) and leaves were no longer moving after 9 days 24 h (Supplemental Fig. S2.1C). For characterisation of the phase and amplitude of leaf growth movement in subsequent experiments, we therefore chose the developmental window of 7 days during which robust oscillations were observed (Supplemental Fig. S2.1C). It was previously shown that low levels of IR illumination did not influence seedling development [75]. Nevertheless, we compared projected leaf lengths between plants grown with IR lights (allowing night measurements) and plants grown without IR (day only measurements). Results show an overlap between the IR and non-IR day measurements and final projected leaf length did not differ under these two conditions (Supplemental Fig. S2.1A). Therefore, we conclude that also in our system the supplemental IR light does not influence leaf growth movement.

NATURAL VARIATION IN LEAF GROWTH MOVEMENTS

Previously, natural variation in the angle of the petiole of Arabidopsis accessions was determined at a fixed time of the day [177]. In this assay the petiole angle of the different accessions varied between 15.3 degrees in Warschau-1 (Wa-1) to 52.0 degrees in Meloy Ornes [177]. For characterisation of the leaf growth movement we selected six accessions with varying petiole angles, including the common 'laboratory accessions' Col-0, Landsberg *erecta*-1 (L*er*-1*)*, and Wassilewskija-2 (Ws-2) (Supplemental Fig. S2.2). For each accession the projected leaf lengths and extracted leaf growth oscillations were determined (Fig. 2.2). Results show distinct differences between leaf movement amplitude in the different accessions. Average±SE amplitudes varied among the dataset from 1.89±0.23 mm (Wa-1) to 6.83±0.56 mm Tossa del Mar-1 (Ts-1) (Figs. 2.2H, J, and 2.3A). To verify whether variation in leaf petiole angle relates to leaf movement amplitude under diurnal conditions the correlation between reported leaf angles and the amplitudes obtained through OSCILLATOR was determined. Indeed the correlation between amplitude of leaf movement and initial petiole angles [177] was very strong (R^2=0.88) (Fig. 2.3B). Differences in leaf length in the different accessions could contribute to differences in the measured amplitude. To examine the correlation between leaf length and amplitude these parameters were compared for all accessions. However, no significant correlation between the two was found (Supplemental Fig. S2.1D). This indicates that variation in amplitude does not result from variation in leaf length and each accession has a distinct leaf growth profile. The period of leaf movement did not differ significantly and was ≈24 h for all accessions, which may be expected under the diurnal entrainment regime (Fig. 2.3C). The phase of leaf movement varied somewhat in time but was most stable between 2 and 6 days where the strongest amplitudes were observed

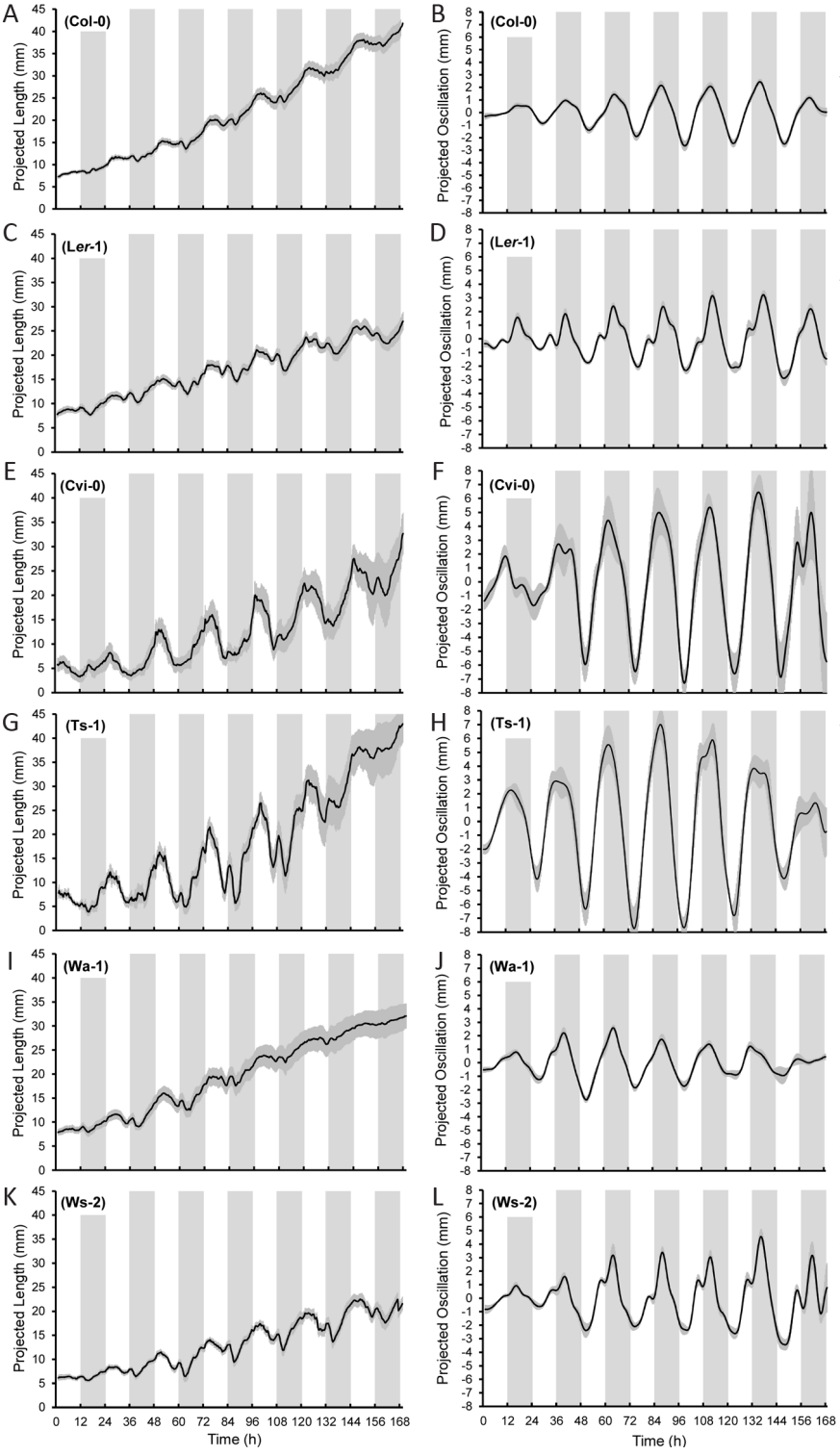


Figure 2.2 Natural variation in projected leaf lengths and projected leaf oscillations for selected Arabidopsis accessions. Projected lengths of selected accessions are depicted in the left column (**A**,**C**,**E**,**G**,**I**,**K**) and the inverted and smoothed projected oscillations in the right column (**B**,**D**,**F**,**H**,**J**,**L**). For all accessions two leaves per plant were analysed and in total eight leaves (four plants) were used for analysis (n=8) except for Cvi-0 where for one plant only one suitable leaf was tracked (n=7). Error bars represent SE.

(Supplemental Fig. S2.3). The average phase for this period differed between the accessions (p=0.018). The phase of L*er-1* (16.9 h) was significantly later than those of Ts-1 (14.5 h, p=0.016), Ws-2 (15.2 h, p=0.039), and Wa-1 (15.7 h, p=0.048) (Fig. 2.3D).

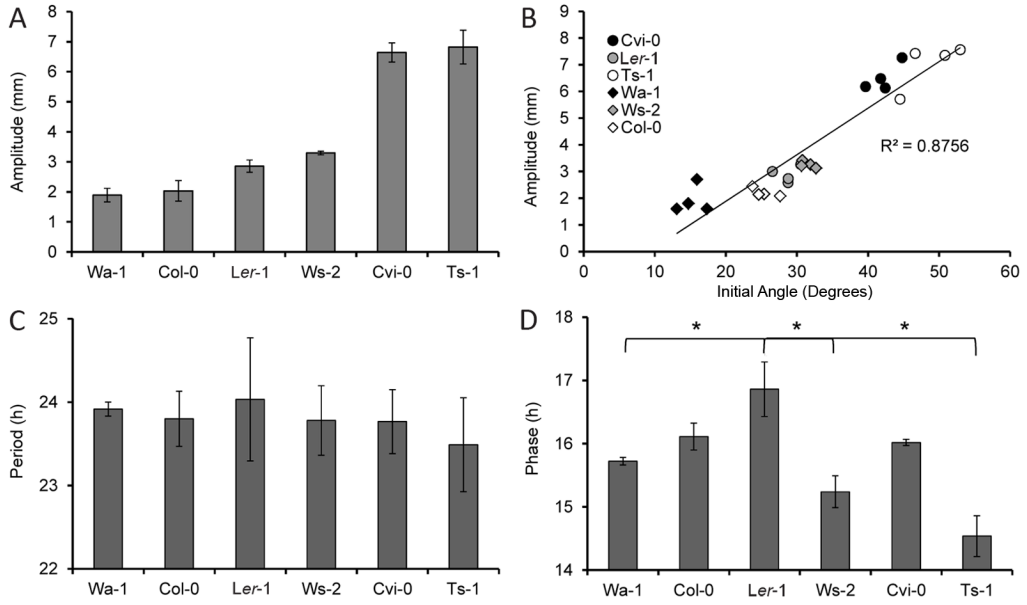


Figure 2.3 Natural variation of diurnal leaf growth oscillations in Arabidopsis for selected accessions. (**A**) Average amplitudes (days 2 to 6), (**B**) correlation between reported angle [177] and measured average amplitude, (**C**) average period (days 2 to 6), and (**D**) average phase of smoothed projected oscillations (days 2 to 6). N=8 leaves, except for Cvi-0 n=7, from four plants per accession. Error bars represent SE. One-way ANOVA was used to identify significant differences between the accessions. Individual contrasts were then identified in a post-hoc Tukey test. *p-value<0.05.

THE LATE PHASE OF L*ER*-1 LEAF OSCILLATIONS APPEARS UNRELATED TO THE *ERECTA* LOCUS

We investigated whether the late phase of the L*er-1* relates to the null allele of the *ERECTA* (*ER*) locus in this accession. *ER* encodes a leucine-rich repeat receptor-like Ser/Thr kinase, and L*er* carries a missense mutation within the conserved region of the kinase domain [178]. Therefore, *ER* activity differs between Landsberg *erecta* and the original Landsberg wild-type line (Lan-0) from which L*er* was isolated [179]. *ER* has previously been reported to control ethylene induced leaf hyponasty [180, 181]. However, it was not investigated whether *ER* affects the phase of leaf movement. Leaf oscillations of L*er-1* were compared to those of Lan-0 (Fig. 2.4A) and results show that the observed late phase of L*er-1* under diurnal light and temperature cycles is also present in Lan-0 (Fig. 2.4B-C). This indicates that in both accessions genetic variation independent of ER is responsible for this phenotype.

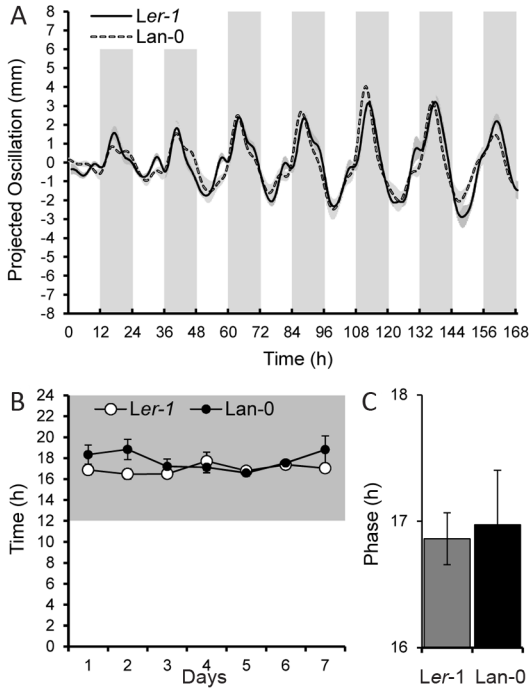


Figure 2.4 The ERECTA locus does not determine phase of leaf oscillations. (**A**) Comparison of smoothed projected oscillations between L*er-1* and Lan-0. (**B**) Timing of peak oscillations (phase) depicted per period, grey area indicates night. (**C**) No significant differences were observed for the average phase (between day 2 and day 6) of L*er-1* and Lan-0. Error bars represent SE (n=8).

LEAF GROWTH MOVEMENT IN PETUNIA AND TOMATO PLANTS

The OSCILLATOR system was developed and optimised for Arabidopsis. To test if the system can be used for other species without major modification 32 days old plants of two additional model species; petunia (Fig. 2.5A) and tomato (Fig. 2.5B) were analysed. In its vegetative stage petunia has a rosette structure (Fig. 2.5A) and therefore growth analysis could be measured using OSCILLATOR without any modification. Fig. 2.5C shows the projected leaf length measured for the petunia plants. From these curves the projected oscillations were extracted (Fig. 2.5D). Tomato plants displayed strong circumnutations (variable apex position in time). This made tracking of the central meristem necessary for calculation of the projected distance. After correction for centre displacement clear diurnal rhythms in leaf growth movement could be extracted (Fig. 2.5D). Both petunia (V26) and tomato (MoneyMaker) displayed a phase±SE of 18.0±0.3 h and 18.3±1.2 h respectively, which is about 2 h later than the phase of Arabidopsis (e.g. 16.1±0.2 h for Col-0). Results indicate that each species displays a unique leaf growth movement pattern, which can be considered the integrated result of the effects of light, temperature, and the endogenous circadian oscillator on plant development.

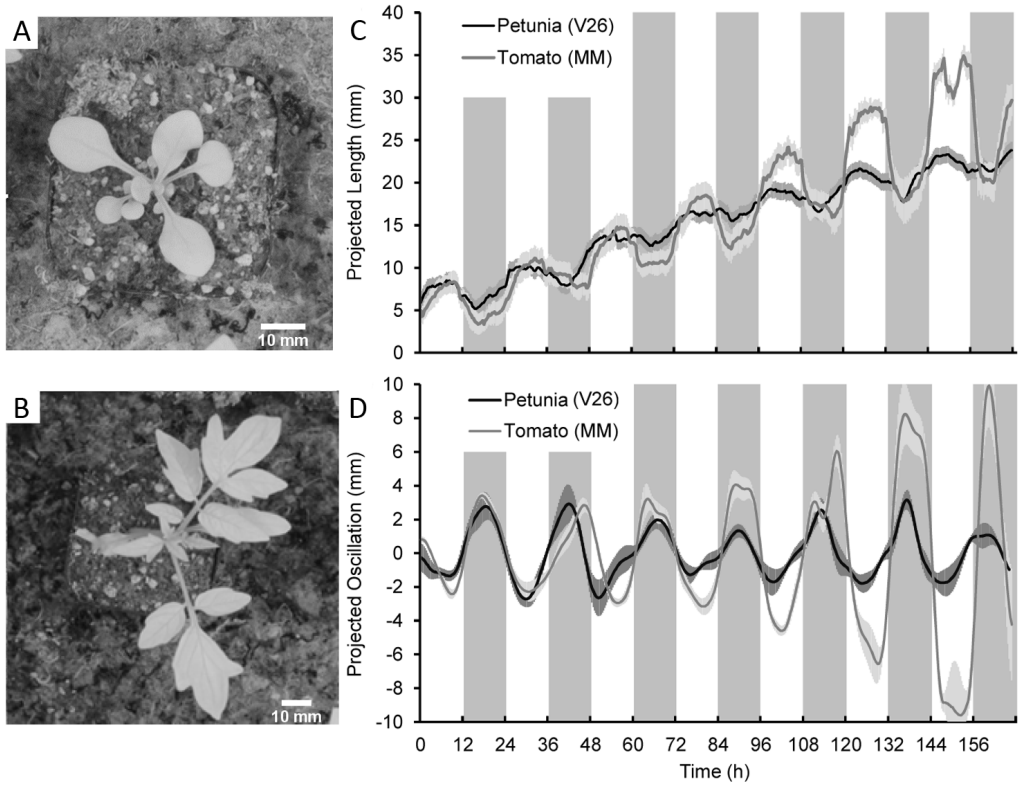


Figure 2.5 OSCILLATOR can be used for different species. (**A-B**) Thirty-two day old (**A**) petunia, and (**B**) tomato plants at the start of imaging. (**C**) Projected lengths were measured using OSCILLATOR. (**D**) From the projected lengths the projected oscillations were extracted, inversed, and smoothed. Error bars represent SE, (n=8).

CONCLUSIONS

The natural variation we identified within six Arabidopsis accessions matched previous described observations, demonstrating the validity of our system. Variation among natural accessions has been studied under continuous light conditions before [162, 182]. OSCILLATOR now provides the possibility to study leaf growth and movement under various diurnal conditions, which more closely mimic the natural environment. Characterisation of leaf growth movement by phase, period, and amplitude allows easy comparison between different genotypes and treatments. Our system also opens up the perhaps even more intriguing possibility to evaluate plant behaviour under gradual changing photoperiods (mimicking seasonal transitions). Diurnal rhythms in leaf growth and movement are directly related to plant growth and help repositioning of leaves relative to the light and could contribute to increased photosynthetic capacities [177, 183, 184]. Furthermore, leaf growth movement and related changes in rosette compactness have been shown to facilitate cooling

and allow adaptation to increasing ambient temperatures [10]. Combined, the results show that OSCILLATOR can be used to extract parameters of leaf growth movement that can be used to characterise different genotypes. OSCILLATOR provides plant scientists with a relatively cheap, reliable, and non-invasive tool to accurately dissect diurnal growth rhythms of various plant species under continuing day/night cycles.

METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of *Arabidopsis thaliana* accessions were provided by M. van Zanten (Laboratory of Molecular Plant Physiology, Utrecht University, The Netherlands) and J. Keurentjes (Laboratory of Plant Genetics, Wageningen University, The Netherlands). Seeds of *Petunia hybrida* (V26) were donated by T. Gerats (Laboratory of Plant Genetics, Radboud University Nijmegen, The Netherlands). Seeds of tomato (*S. lycopersicum* L.) cultivar; MoneyMaker were obtained from W. Kohlen (Max Planck Institute for Plant Breeding Research, Cologne, Germany). All experiments were performed in automated climate controlled WEISS (http://www.wkt.com) cabinets (12 h/12 h light/dark cyles). Relative humidity was kept constant at 60% (v/v) and photosynthetic active radiation (PAR) was 150 µmol m^2 s^{-1} from white fluorescents tubes (PHILIPS, type T5, colour code: 840). Ambient temperature cycles for growth were 22°C (photoperiod) and 12°C (dark period) with a temperature ramp of 0.33°C/min. Measurements showed that soil temperature lagged≈20 minutes behind ambient air temperature. Plants were grown in fertilised peat/perlite based soil in square (5 cm x 5 cm x 5 cm) plastic pots with different genotypes placed at random positions in the growth cabinet. Plants were placed on an irrigation mat, which was watered automatically to saturation through porous tubing from a basin containing tap-water every 3 days at the start of the photoperiod. After 20 days plants were watered once with half strength Hoagland-nutrient solution instead of water. Five days later plants were transferred to a second climate cabinet for imaging with similar conditions and an IR camera system with IR lights. Plants were allowed to acclimate for 7 days before the onset of imaging. Thus, at the start of imaging plants were 32 days old and the Arabidopsis accessions all had 13-14 true leaves.

PLANT GROWTH IMAGING AND IMAGE DATA ANALYSIS

The pipeline for imaging and image analysis as used by OSCILLATOR is summarised in Supplemental Fig. S2.4. To enable automated leaf tip tracking the 11^{th} and 12^{th} real leaves were marked with inert paint before the start of imaging although for manual tip tracking this is not necessary (tip tracking will be explained in more detail later). The 11^{th} and 12^{th} leaf were analysed and the leaf length varied from 5-8 mm. Imaging was with SLR NIKON D90 digital camera's with a NIKON AF 50 mm f/1.8 lens. Cameras were powered by net adaptors (http://www.nikon.com). To enable night photography, cameras were custom modified by

MAXMAX (http://www.maxmax.com) for removal of the internal IR filter to allow detection of IR light. Sufficient IR illumination per cabinet was provided by two continuous burning LED lights (890 nm, 12 W, KÖNIG electronics, (http://www.konigelectronic.com). Each camera was connected to a dedicated laptop with active USB 2.0 repeater cables and controlled with time-lapse photography software (NIKON Camera Control Pro 2; http://www.nikon.com). Camera settings were fixed; f-stop = f/8, exposure-time = 1/5 s, ISO speed = 200. Field of view for each camera was 16 rosette plants. Photographs were taken every 20 minutes and stored as individual images. Sequential images were imported as virtual stacks into ImageJ (http://rsbweb.nih.gov/ij/) and the image stack was subsequently cropped into individual plant image stacks. For cropping of single plant image stacks, the desired areas can be selected in the first frame and cropping of all the stacks is further automatic (Supplemental Fig. S2.4B). The resulting multiple single plant 'virtual stacks' were manually saved as individual plant image stacks and named as appropriate. Each individual plant image stack was used for leaf tip tracking, using either the manual tracking (Supplemental File S2.1 http://rsbweb.nih.gov/ij/plugins/track/track.html) or the automated MOSAIC particle tracking plugin (Supplemental File S2.1 http://www.mosaic.ethz.ch/Downloads/ParticleTracker). The manual tracking plugin allows semi-automated selection of leaf tip coordinates throughout the stack (Supplemental Fig. S2.4C). To facilitate this procedure Standard Mouse Auto Clicker 2.8 (Supplemental File S2.1) can be used to automate screen mouse clicks at specified intervals and any location on the screen. Alternatively, when selected leaves are marked with a small paint dot at the start of the experiment, automated tracking of the leaf tip with the particle tracker MOZAIC plugin can be used. However, the particle recognition occasionally fails in single frames, resulting in gaps in the leaf tip tracks. This then requires manual correction, which can be labour intensive. Therefore, in this work we used the manual tracking plugin. In combination with Standard Mouse Auto Clicker 2.8 (Supplemental File S2.1) set at 1 click per 0.2 second a typical stack of 500 images is manually processed in 100 seconds. Both the manual and automated tracking plugins provide output format compatible with Microsoft Excel version 2010 (http://office.microsoft.com), which can be individually named and saved by the user for each individual leaf track (e.g. Plant1.1.xls etc.; Supplemental Fig. S2.4D).

EXTRACTION OF PARAMETERS OF LEAF GROWTH OSCILLATIONS WITH THE OSCILLATOR SCRIPT

To determine the relative leaf movement, for each image the distance between leaf tip and plant centre needs to be calculated. In our experiments the Arabidopsis and petunia rosette centres were static throughout the image stack and thus were provided by the single manually determined (x,y) coordinates of the rosette apical centre. For tomato the apex positions varied throughout the stack and apex position was determined using 'manual tracking plugin' (Supplemental File S2.1). The leaf tip and apex coordinates of all individual leaves of a single genotype (two leaves per plant, four plants per genotype) are subsequently

combined in the OSCILLATOR_input.csv file (Supplemental File S2.1; Supplemental Fig. S2.4E). For each genotype, the OSCILLATOR_input.csv file was placed in designated folders each containing the OSCILLATOR SCRIPT (Supplemental File S2.1; Supplemental Fig. S2.4F). For each folder the script was activated in R version 2.13.1 (http://www.r-project.org/), which generates the following outputs:

- Projected length for individual leaves and averaged projected length;
- The distance between the leaf tip and the plant apex was calculated by the OSCILLATOR.R script based on the following equation:

Distance = $\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$;

- The resulting distance (projected leaf length) is provided for single leaves in output file project_length.csv;
- In addition the average is plotted against time as a graph with SE depicted as shading (Projectedlength.jpeg).

RAW PROJECTED OSCILLATIONS FOR INDIVIDUAL LEAVES AND AVERAGED RAW PROJECTED OSCILLATIONS

Subsequently a best fit 2° degree polynomial trend line is automatically calculated for each individual projected length curve and the residual values are subtracted from this line. The resulting residuals were inverted to allow maximum upright leaf position to correspond to maximum peak height. These raw projected oscillations are provided as data in output file AvgRawOscillation.csv. In addition the average is plotted against time with SE as shading in AvgRawOscillations.jpeg (Supplemental Fig. S2.4G).

SMOOTHED PROJECTED OSCILLATIONS FOR INDIVIDUAL LEAVES AND SMOOTHED AVERAGED PROJECTED OSCILLATIONS

The raw oscillations of individual leaves are then smoothed using WAVECLOCK script [176] imbedded in the OSCILLATOR script (Supplemental File S2.1). The smoothed projected oscillations are provided for single leaves in the file IndividualSmoothedOscillation.csv and as an average with SE in the file AvgSmoothedOscillations.csv. In addition the average is plotted against time with SE depicted as shading in AvgSmoothedOscillation.jpeg (Supplemental Fig. S2.4G).

PHASE AND AMPLITUDE OF INDIVIDUAL LEAVES AND AVERAGED PHASE, AMPLITUDE, AND PERIOD

The peak values for the smoothed projected oscillations of individual leaves for all periods are used to calculate the phase and amplitude. The phase and amplitude data are provided for single leaves in file phaseDays.csv and amplitudeDays.csv respectively and are then averaged and plotted against time with SE in graph RplotphaseMinMax.jpeg and

RplotampMinMax.jpeg respectively (Supplemental Fig. S2.4G). Period information is plotted as a wavelet scalogram (Wave1.png) [176].

Given all materials and software in place it will take an experienced user approximately 1 h to complete a single genotype set (four plants, eight leaves) analysed throughout a stack of imaging representing 7 days of development as typically performed in this study. All data presented is the typical result of at least two independent experiments, each based on at least seven individual leaf track analysis.

STATISTICAL ANALYSES

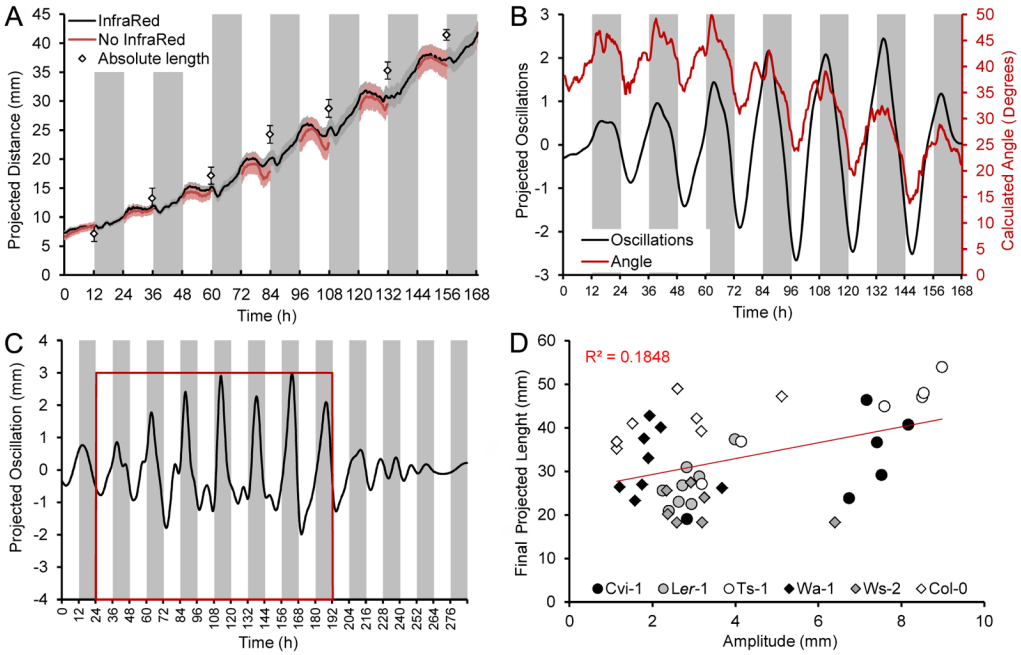
Statistical differences between accessions were determined using one-way ANOVA. Individual differences were then identified using a post-hoc Tukey test (p-value<0.05). All analysis were performed using SAS 9.2 (http://www.sas.com/).

ACKNOWLEDGEMENTS

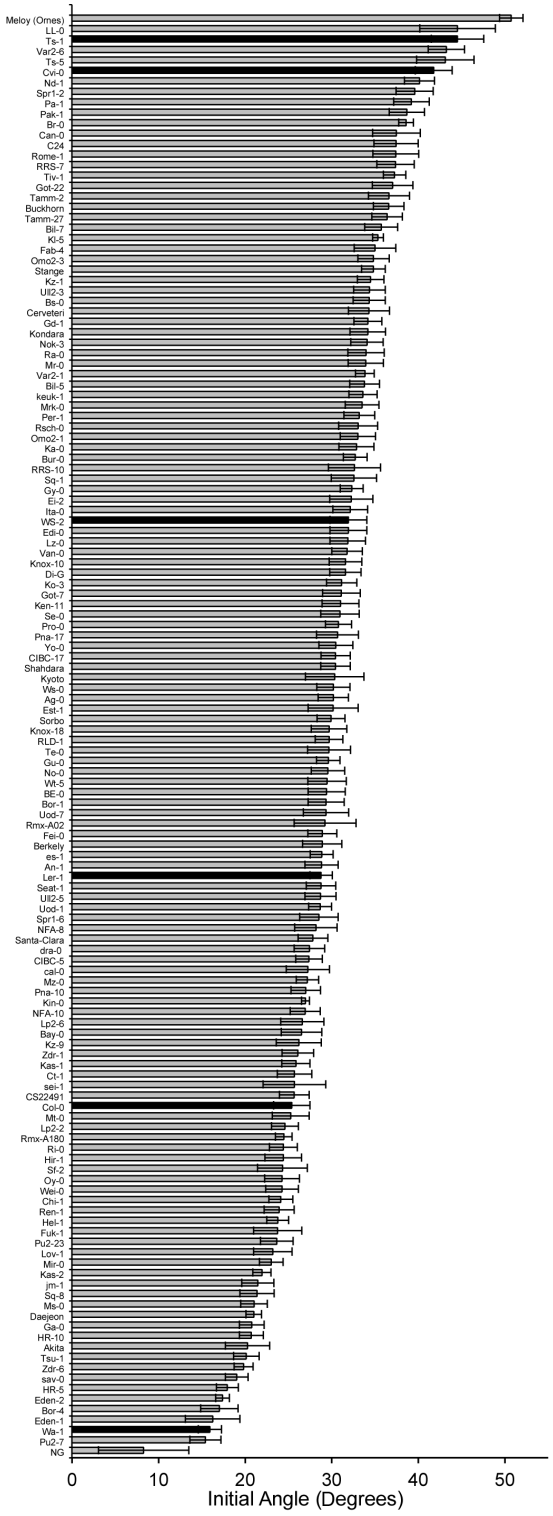
This research is funded by the Top Technological Institute Green Genetics (TTI-GG) (Project: 2CFL009RP). We are indebted to: R. Pierik (Utrecht University), M. van Zanten (Utrecht University), J. Rienks (Wageningen University), N. Zagari (Wageningen University), and W. Kohlen (Max-Planck Institute, Cologne) for providing essential feedback on the method and manuscript. We thank T. Stoker and G. Stunnenberg (Wageningen University) for maintaining the Weiss cabinets.

SUPPLEMENTAL DATA

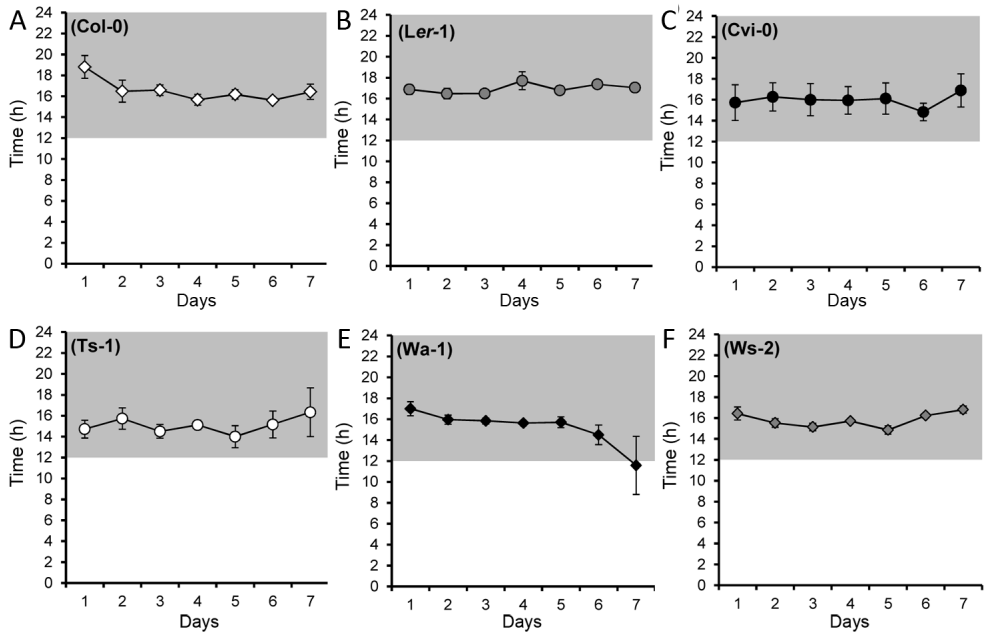
Supplemental File S2.1 OSCILLATOR package: Plugins, scripts, and example file. All plugins and scripts needed for OSCILLATOR are provided and combined in File S1.zip. All the components of this file are described in more detail in Supplemental Fig. S2.4 and the methods section. In addition, an example input file (OSCILLATOR_input) is included. Plugins needed for image processing in ImageJ and Standard Mouse Auto Clicker 2.8 are freeware and have been included for completeness but were not developed by the authors. This file can be downloaded from: http://www.plantmethods.com/content/8/1/



Supplemental Figure S2.1 Validation experiments. (**A**) Additional IR light does not affect leaf growth movement of Col-0. Standard Errors (SE) are depicted as shading (n=8). Absolute length was measured at the end of each photoperiod (white dots). Error bars represent SE; n=8. (**B**) Leaf angles were calculated from the absolute leaf lengths and their phase corresponds to the phase of the smoothed projected oscillations of Col-0 (n=8). (**C**) Col-0 smoothed projected oscillations increase gradually and decrease again during development. The red block shows the timeframe during which all further experiments were performed (n=8). (**D**) Final projected leaf lengths were plotted against the averaged amplitudes of individual leaves of six different accessions, (n=8 for all accessions except for Cvi-1, n=7).

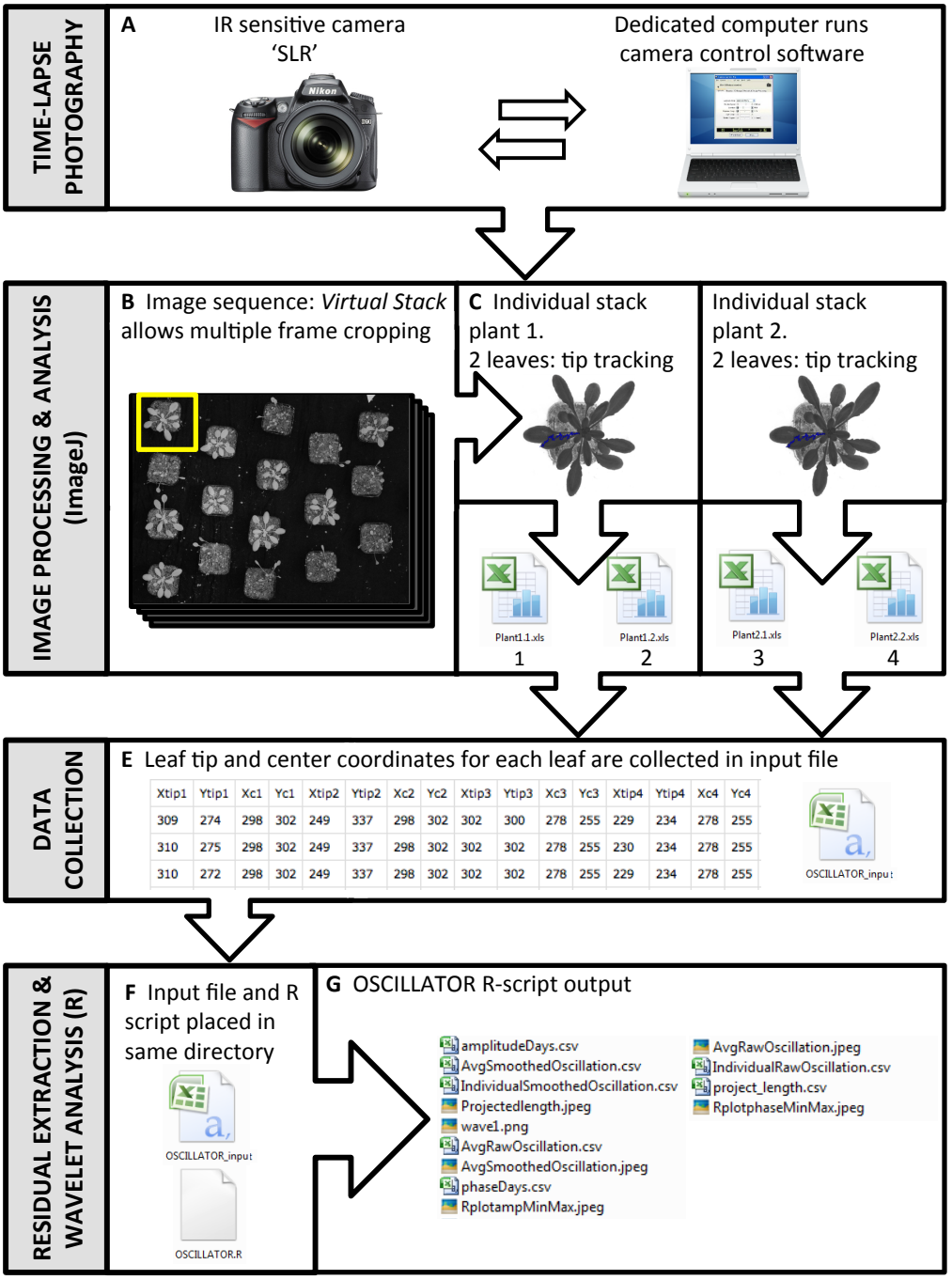


Supplemental Figure S2.2 Natural variation in initial petiole angle as previously reported [181]. Values represent absolute angles (degrees) relative to the horizontal of Arabidopsis accessions measured at a fixed time point [181]. Black bars indicate selected accessions screened with OSCILLATOR (Adapted and reproduced with permission). Error bars represent SE (n(petioles)≥8).



Supplemental Figure S2.3 Timing of peak oscillations (phase) for selected accessions depicted per period (day). Grey colour indicates night. For all accessions n=8 leaves except for Cvi-0 (n=7). Error bars represent SE.

Supplemental Figure S2.4 (see next page) Schematic presentation of the different steps of OSCILLATOR. (**A**) Hardware consisting of cameras that are connected to a dedicated laptop are controlled by NIKON camera control software. (**B**) Sequential images were imported as virtual stacks into ImageJ. For cropping of single plant slices the desired area needs to be selected in the first frame and cropping is automatic throughout the stack. The resulting single plant 'virtual stacks' were then saved as 'image sequence'. (**C**) Leaf tip tracking was performed with the manual tracking plugin that allows semi-automated selection of leaf tip coordinates throughout the stack. Alternatively, if selected leaves are marked with a small paint dot at the start of the experiment this allows the particle tracker MOZAIC plugin to track the dot throughout the virtual image stack. Both plugins are provided in Supplemental File S2.1. (**D**) The output of the leaf tip tracking plugins is provided as Microsoft Excel files containing the X and Y values for each image (frame) which can be named and saved as appropriate. (**E**) Centre coordinates are determined for each plant (Xc, Yc) and are combined with the leaf tip track coordinates (x,y) in the input file (OSCILLATOR input.csv). (**F**) The OSCILLATOR input.csv file is placed in a dedicated folder together with the OSCILLATOR SCRIPT. This folder directory is set in R software and the OSCILLATOR script is run (source("OSCILLATOR.R")). (**G**) As the script runs output files are provided in the folder containing the script and input file. For each step the data is provided for individual leaves and as average including SE. In addition these averages±SE are plotted against time and provided as JPEG files.



Supplemental Figure S2.4 (see legend on previous page)

On scientific writing…

“Consider the lilies, how they grow; they neither toil nor spin…”

- Luke

3

ANTIPHASE LIGHT AND TEMPERATURE CYCLES AFFECT PHYB-CONTROLLED ETHYLENE SENSITIVITY AND BIOSYNTHESIS; LIMITING LEAF MOVEMENT AND GROWTH OF ARABIDOPSIS

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PUBLISHED IN:

Plant Physiology 2013 Oct;163(2):882-95.
doi: 10.1104/pp.113.221648

ABSTRACT

In the natural environment, days are generally warmer than the night, resulting in a positive day/night temperature difference (+DIF). Plants have adapted to these conditions, and when exposed to antiphase light and temperature cycles (cold photoperiod/warm night (-DIF)) most species exhibit reduced elongation growth. To study the physiological mechanism of how light and temperature cycles affect plant growth, we used infrared imaging to dissect growth dynamics under +DIF and -DIF in the model plant Arabidopsis (*Arabidopsis thaliana*). We found that -DIF altered leaf growth patterns, decreasing the amplitude and delaying the phase of leaf movement. Ethylene application restored leaf growth in -DIF conditions, and constitutive ethylene signalling mutants maintain robust leaf movement amplitudes under -DIF, indicating that ethylene signalling becomes limiting under these conditions. In response to -DIF, the phase of ethylene emission advanced 2 h, but total ethylene emission was not reduced. However, expression analysis on members of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase ethylene biosynthesis gene family showed that *ACS2* activity is specifically suppressed in the petiole region under -DIF conditions. Indeed, petioles of plants under -DIF had reduced ACC content, and application of ACC to the petiole restored leaf growth patterns. Moreover, *acs2* mutants displayed reduced leaf movement under +DIF, similar to wild-type plants under -DIF. In addition, we demonstrate that the photoreceptor PHYTOCHROME B restricts ethylene biosynthesis and constrains the -DIF induced phase shift in rhythmic growth. Our findings provide a mechanistic insight into how fluctuating temperature cycles regulate plant growth.

INTRODUCTION

In nature, during the day (light), temperatures are usually higher than during the night (dark). Correspondingly, most plants show optimal growth under such synchronous light and temperature cycles. Increasing the difference between day and night temperature (+DIF) results in increased elongation growth in various species, a phenomenon referred to as thermoperiodism [76]. The opposite regime, when the temperature of the day (DT) is lower than that of the night (NT), is called -DIF (negative DT/NT difference). Under -DIF conditions, elongation growth of stems and leaves of various plant species is reduced [77, 78, 80]. Arabidopsis (*Arabidopsis thaliana*) plants grown under -DIF (DT/NT 12°C/22°C), displayed a reduction in leaf elongation of approximately 20% compared with the control (DT/NT 22°C/12°C) [80]. -DIF is frequently applied in horticulture to produce crops with a desirable compact architecture without the need for growth retarding chemicals [79]. Despite the economic importance of the application of such temperature regimes in horticulture, the mechanistic basis of the growth reduction under -DIF is still poorly understood.

Previously, it was demonstrated that -DIF affects phytohormone signalling in plants. In pea (*Pisum sativum*) for instance, the -DIF growth reduction correlated with increased catabolism of the phytohormone gibberellic acid (GA) [82]. In contrast to pea, active GA levels

did not decrease in response to -DIF in Arabidopsis [80]. On the other hand, the -DIF growth response in Arabidopsis was associated with reduced auxin levels [80]. The photoreceptor PHYTOCHROME B (PHYB) has been shown to be important for the response to -DIF as *phyB* mutants of Arabidopsis [86] and cucumber (*Cucumis sativus*) [185] are insensitive to -DIF.

In this work, the growth related movement of mature Arabidopsis rosette leaves was analysed under +DIF (control) and -DIF conditions. Under -DIF the amplitude of leaf movement was decreased and the phase of movement was later, compared to +DIF plants. The altered leaf growth patterns observed in -DIF could be restored by application of ethylene. -DIF reduced the expression of *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE2 ACC SYNTHASE2* (*ACS2*) in the petiole, which correlated with reduced 1-aminocyclopropane-1-carboxylic acid (ACC) levels and decreased amplitude and delayed phase of leaf movement. Our results indicate that local ACC activity plays an important biological role, despite the fact that ethylene is a gaseous and fast diffusing hormone. In addition, we demonstrate that in the *phyB-9* mutant, the phase of leaf movement is almost fully temperature entrained. Finally, ethylene levels and sensitivity are increased in *phyB-9*, suggesting a role for PHYB in constraining temperature-induced shifts in the phase of leaf movement and dampening of leaf movement amplitude by controlling ethylene production and sensitivity.

RESULTS

LEAF MOVEMENT AND ELONGATION GROWTH ARE REDUCED DURING THE COLD PHOTOPERIOD

To characterise the growth of Arabidopsis plants in response to antiphase light and temperature cycles (-DIF), we used the OSCILLATOR growth monitoring system, which enables accurate analysis of phase, amplitude, and period of growth related leaf movements [186]. Plants were pregrown under +DIF for four weeks, after which temperature cycles were either kept identical (control) or reversed to the opposite -DIF regime. The plants that were exposed to -DIF showed a visually lower leaf angle compared to the +DIF plants (Fig. 3.1A), suggesting a difference in diurnal leaf movement rhythm. Top-view images of the plants were recorded during one week of growth under +DIF and -DIF conditions (Supplemental File S3.1) and used for the measurement of projected leaf lengths (Supplemental Fig. S3.1A) from which the projected oscillations were extracted (Fig. 3.1B). These projected oscillations were analysed for peak amplitude and for the timing of the peak amplitude relative to the onset of the photoperiod, here referred to as the 'phase' of the leaf movement rhythms (Fig. 3.1C). This analysis confirmed that the visually lower leaf angle observed under -DIF (Fig. 3.1A) corresponds to reduced leaf movement amplitude relative to +DIF (Fig. 3.1D). Moreover, also the phase of movement shifted under -DIF compared with the +DIF. Under +DIF conditions, the phase of movement for small emerging leaves during day 1 is approximately 19 h (where t=0 is dawn and t=12 h is dusk). After 2 days, when the leaves matured, the phase of leaf

movement stabilised at around t=16 h (Fig. 3.1E). In contrast, in response to -DIF, the phase of leaf movement shifted from t=19 h during the first day to t=21 h, which persisted throughout the next 6 days. Under -DIF the timing of the peak amplitude thus shifts to late night, whereas the lowest leaf position is measured at mid-day. Consequently, the phase of movement of leaves developing under -DIF is delayed by 5 h compared with the +DIF (Fig. 3.1E).

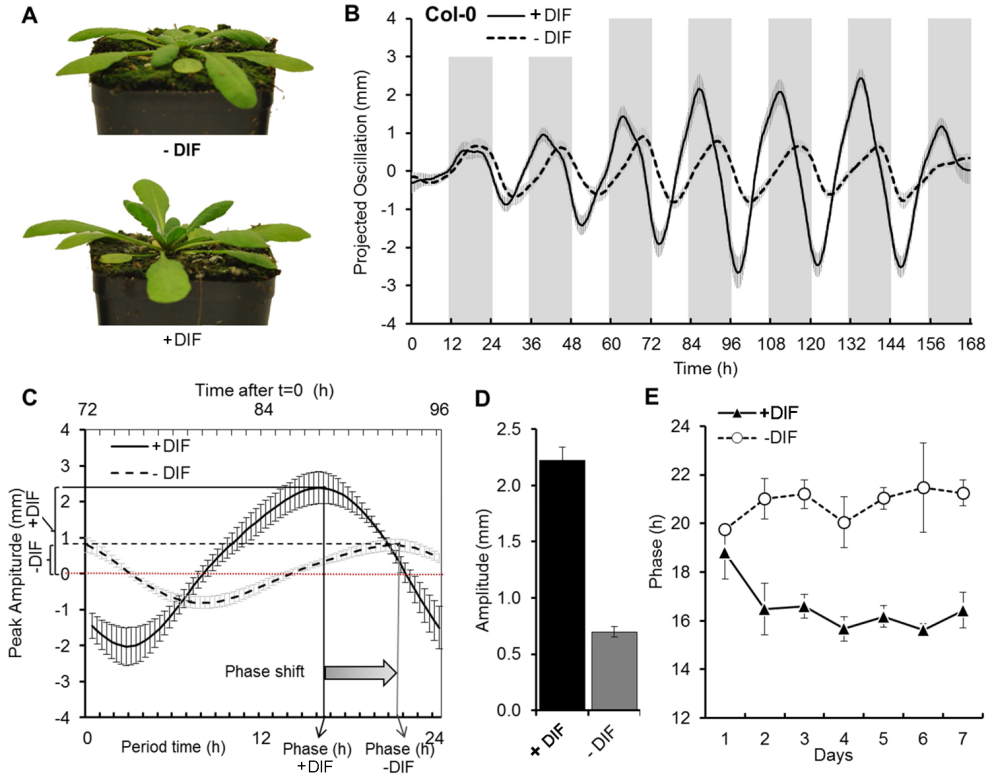


Figure 3.1 Rhythmic growth and movement of leaves under +DIF and -DIF conditions. (**A**) Two 36 day old Arabidopsis (Col-0) rosette plants exposed to -DIF (top) or +DIF (+DIF; bottom) conditions as photographed at the end of the fourth photoperiod. (**B**) Projected oscillations for leaves developing 7 days under +DIF (solid line) or -DIF (dashed line) conditions. Standard Errors (SE) are depicted per 20 min time point as shading, n=8 leaves. Grey areas indicate the dark period. (**C**) Close-up of (B) for day 4, depicting an example of amplitude, phase, and phase shift for +DIF leaves compared to -DIF. Error bars represent SE; n=8. (**D**) Average amplitudes of days 2 to 6 calculated from the projected oscillations (B) for leaves developing under +DIF (black bar) or -DIF (grey bar) conditions. Error bars represent SE; n=8. **(E**) The phase of oscillations for leaves developing under -DIF conditions (dashed line, white circles), shifts compared with the +DIF (solid line, black triangles). Error bars represent SE; n=8.

Taken together, under -DIF, upward movement is largely restricted to the warm night, whereas under +DIF conditions the largest part of the upward leaf movement occurs during the warm day (Fig. 3.1B). This suggests that under alternating diurnal light and temperature cycles, temperature, rather than light, contributes most to the leaf movement of Arabidopsis.

Analysis of the projected leaf length curves showed that the reduced leaf movement during the day under -DIF coincides with decreased (projected) leaf elongation (Supplemental Fig. S3.1A), suggesting a relationship between leaf movement and elongation. Measurement of absolute lengths of leaves developed under -DIF confirmed reduced elongation (Supplemental Fig. S3.1B).

ETHYLENE AFFECTS THE AMPLITUDE AND PHASE OF DIURNAL LEAF MOVEMENT

It is previously demonstrated that the gaseous plant hormone ethylene is involved in upward leaf movement (hyponastic growth) in response to different stresses in Arabidopsis [180, 181, 187]. Application of the ethylene perception inhibitor silver thiosulphate (STS) to plants growing under control +DIF conditions reduced leaf movement amplitude by approximately 50%, compared with mock treatment (Fig. 3.2A-B). No additional inhibitory effect on amplitude was noted when STS was applied to leaves developing under -DIF (Supplemental Fig. S3.2A-B). The ethylene insensitive mutants *ethylene response1-1* (*etr1-1*) and *ethylene insensitive2-1* (*ein2-1*) showed a strongly reduced leaf movement amplitude under control +DIF (Fig. 3.2A, C), as well as under -DIF conditions (Supplemental Fig. S3.2A, C). The amplitude of leaf movement in these mutants under +DIF conditions is similar to that of wild-type plants under -DIF (Fig. 3.1D) or wild-type plants treated with STS under +DIF conditions (Fig. 3.2A). STS application to leaves developing under +DIF conditions initially shifted the phase of leaf movement from t=16 h (mock treatment) to t=20 h (STS) (Fig. 3.2D; Supplemental Fig. S3.2D). This mimics the phase shift induced by -DIF for untreated plants (Fig. 3.2D). Three days after the start of the treatment, the phase of STS-treated leaves under +DIF conditions changed back to 18 h, indicating that the effect of a single STS application is transient (Supplemental Fig. 3.2D), which is expected, as novel synthesis of ethylene receptors renders the plant sensitive again. Under -DIF, STS application only caused a small phase shift from t=21 h (mock) to t=22 h (STS) (Fig. 3.2D). In the ethylene insensitive mutants *etr1-1* and *ein2-1* the estimated phase did not shift between the +DIF control and -DIF conditions and was around t=20-22 h (Fig. 3.2D). Thus, the estimated phase of leaf movement of these ethylene insensitive mutants resembled the late phase of wild-type leaf movement under -DIF (Fig. 3.2D). The differences between the STS treatment and the ethylene-insensitive mutants (Fig. 3.2D) may arise in part due to ethylene-independent effects of STS on auxin [188].

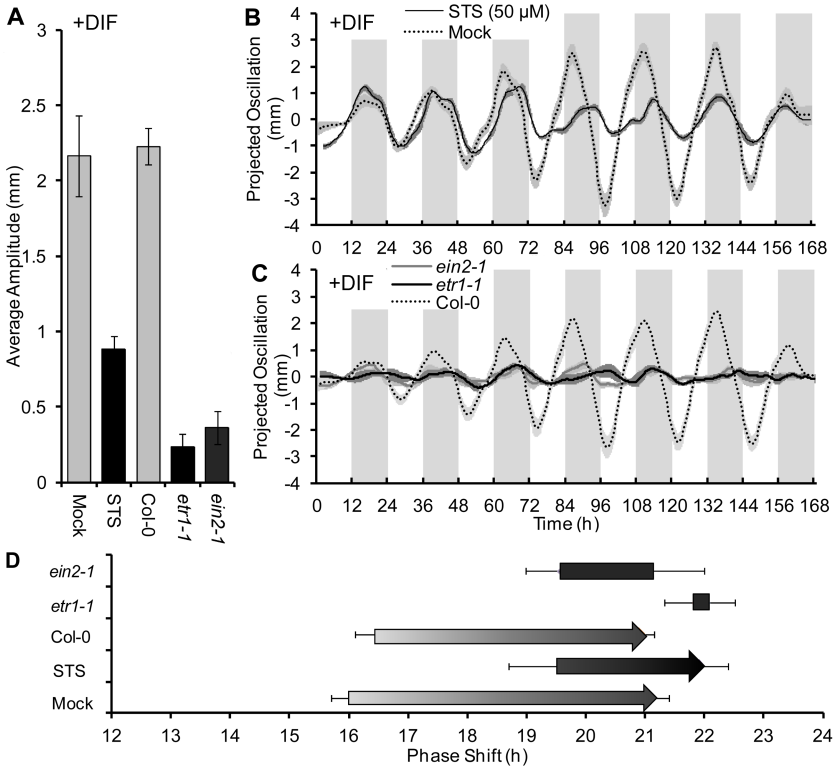


Figure 3.2 Ethylene signalling controls (+DIF) amplitude and phase of diurnal leaf movement. (**A**) Average amplitudes (days 2 to 6) calculated from the projected oscillations (in B and C) for leaves treated with mock (grey bar) or 50 µM STS (black bar), and for Col-0 (light grey bar), *etr1-1* (black bar), and *ein2-1* (dark grey bar) developing under +DIF conditions. Error bars represent SE; n=8. (**B**) Projected oscillations of Col-0 leaves under +DIF conditions treated with 50 µM STS (solid line) compared to mock (dashed line). Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Projected oscillations for leaves of *etr1-1* (solid black line), and *ein2-1* (solid grey line) compared to Col-0 wild-type (black, dashed line) under +DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**D**) Phase shifts of leaf movements between +DIF and -DIF conditions for leaves treated with mock or 50 µM STS and for Col-0, *ein2-1*, and *etr1-1*. Significant phase shifts ($p<0.05$) are indicated with arrows and non-significant shifts are indicated with bars. Each arrow depicts direction and strength of the shift in phase: the start of the arrow indicates average phase during days 2 to 6 under +DIF conditions and the arrowhead indicates the average phase for -DIF (days 2 to 6). Error bars represent SE; n=8.

ETHYLENE AND ETHYLENE SIGNALLING RESTORE LEAF GROWTH AND MOVEMENT UNDER -DIF

Because our results indicate that ethylene signalling is involved in controlling the amplitude, phase, and elongation response of leaves to diurnal temperature cycles, we reasoned that ethylene signalling or ethylene levels may be limiting for leaf movement under -DIF. To test this hypothesis, plants were sprayed with different concentrations of the ethylene-releasing compound ethephon (2-chloroethylphosphonic acid) at the start of the first -DIF photoperiod (t=0 h), and leaf movement was subsequently monitored over a period of 7 days. Consistent with our hypothesis, a single ethephon application was sufficient to restore leaf movement amplitude under -DIF, in a dose-responsive manner, to a level comparable to untreated +DIF

plants (Fig. 3.3A-B). Ethephon application only marginally enhanced the amplitude of leaf movement under +DIF conditions, and this effect became visible only 3 days after treatment (Supplemental Fig. S3.3A-B). Subsequently, we tested whether the loss-of-function mutant *etr1-7* and the triple mutant *etr1-6 etr2-3 ein4-4*, which both have constitutive ethylene signalling phenotypes [189, 190], are insensitive to the inhibitory effect of -DIF on leaf movement and growth. Indeed, both mutants showed a robust and persistent amplitude of leaf movement under -DIF conditions, comparable to that of 0.5 mM ethephon-treated wild-type plants under -DIF (Fig. 3.3A, C; Supplemental Fig. S3.3C) or wild-type plants under +DIF conditions (Fig. 3.2A). The single application of 0.5 mM ethephon to wild-type plants resulted in an almost biphasic growth related movement of the treated leaves. After processing of the data in OSCILLATOR, we used the highest peak value as a phase indicator. The results indicate a modest phase shift of leaf oscillation in response to ethephon treatment under both -DIF (from mock t=21 h to ethephon t =22 h) (Fig. 3.3A, D) and +DIF conditions (from mock t=16 h to ethephon t=15 h) (Fig. 3.3D; Supplemental Fig. S3.3B). The phase of leaf movement of the constitutive ethylene signalling mutants *etr1-7* and *etr1-6 etr2-3 ein4-4* was similar to that of the wild-type (t=16 h) under +DIF conditions and, in contrast to wild-type, did not shift between +DIF and -DIF (Fig. 3.3B, D). Taken together, these data indicate that ethylene stimulates both the amplitude and phase of leaf movement under -DIF.

Quantification of the leaf length of ethephon-treated plants showed that ethylene can also partly restore the inhibited leaf elongation under -DIF (Supplemental Fig. S3.3E-F). In contrast, ethephon treatment of plants developing under +DIF conditions did not affect leaf elongation (Supplemental Fig. S3.3E-F), suggesting that ethylene is only limiting for leaf elongation under -DIF.

These results may implicate reduced ethylene action under -DIF. Therefore, we measured real-time ethylene emissions of wild-type Columbia-0 (Col-0) plants developing under +DIF and -DIF conditions (Fig. 3.4A). Ethylene accumulated in the headspace showed diurnal oscillations under both +DIF and -DIF conditions (Fig. 3.4A). The absolute amount of ethylene produced per gram of shoot fresh weight, however, was not reduced for plants grown under -DIF compared with the +DIF (Fig. 3.4A). However, since -DIF grown plants have an approximately 40% reduction of fresh weight, total emissions per plant are reduced under -DIF. The phase of peak ethylene emission for +DIF plants was at t=6 h, while the phase of plants developing under -DIF was at 2 h and thus shifted 4 h backward compared with the +DIF (Fig. 3.4A). Subsequently, ACS reporter plants were analysed to determine the effect of -DIF on local ethylene production in plants.

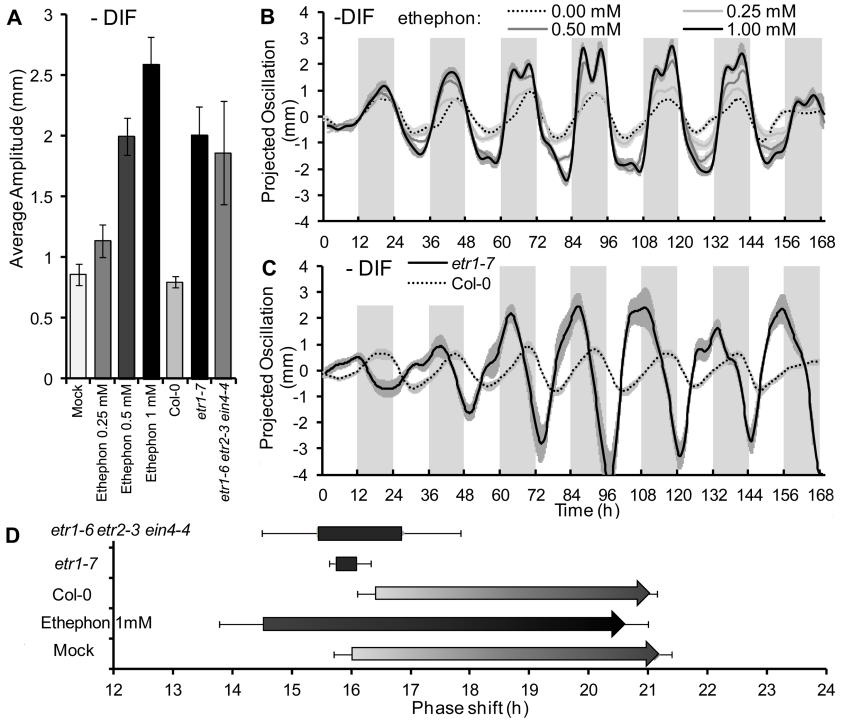


Figure 3.3 Ethylene restores growth and movement under -DIF. (**A**) Average amplitudes of days 2 to 6, calculated from the projected oscillations (in B and C) of leaves treated with mock (white bar), increasing concentrations of ethephon (grey to black bar), and Col-0 (light grey bar), etr1-7 (black bar), and etr1-6 etr2-3 ein4-4 (grey bar) developing under -DIF conditions. Error bars represent SE; n=8. (**B**) Ethephon dose-response of projected oscillations for Col-0 leaves developing on plants sprayed with increasing ethephon concentrations (0.25-1 mM; grey to black lines) compared to mock (0.0 mM; dashed line) at the start (t=0 h) of -DIF treatment. Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Projected oscillations of leaves of the constitutive ethylene signalling mutant etr1-7 (black line) compared to Col-0 wild-type (dashed line) under -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**D**) Phase shifts of leaf movements between +DIF and -DIF conditions for leaves treated with mock or 1 mM ethephon and for Col-0, etr1-7, and etr1-6 etr2-3 ein4-4. Significant phase shifts ($p<0.05$) are indicated with arrows and non-significant shifts are indicated bars. Each arrow depicts direction and strength of the shift in phase: the start of the arrow indicates average phase during days 2 to 6 under +DIF conditions, and the arrowhead indicates the average phase for -DIF (days 2 to 6). Error bars represent SE; n=8.

ACS2 ACTIVITY IS ALTERED UNDER -DIF

The rate of ethylene synthesis is dependent on the production of the biological precursor ACC by ACS enzymes [191]. The Arabidopsis *ACS* gene family contains eight functional members [192], which can form a multitude of functional homodimers and heterodimers [193]. Furthermore, different *ACS* members display unique spatial expression patterns [194] and may respond differentially to -DIF. To test if and which *ACS* gene(s) are differentially expressed in -DIF versus +DIF conditions, we compared GUS presence in seven different promoter *ACS::GUS* reporter lines [194]. In five of these lines (*ACS4, ACS5, ACS6, ACS9, and*

ACS11), GUS expression did not change in response to -DIF conditions (Supplemental Fig. S3.4A). However, the *ACS2:GUS* and *ACS8:GUS* reporter plants showed markedly different expression patterns under -DIF conditions (Fig. 3.4B-C).

In +DIF conditions, *ACS2* promoter activity was visible in the proximal side of the leaf, the petiole, and midrib veins, while in leaves developed under -DIF, the activity in veins and petioles was strongly reduced or absent (Fig. 3.4B). The *ACS8* promoter showed increased *GUS* expression in the transverse edges of the blade (Fig. 3.4C). *ACS8* was shown to be negatively regulated by ethylene [195] and the increased activity under -DIF might indicate that ethylene-mediated repression of *ACS8* is relieved. Because *ACS2* showed differential activity in the petiole we tested whether the activity of *ACS2* in the petiole relates to a function in leaf movement. To this aim we analysed the diurnal leaf movement of two independent *ACS2* loss-of-function alleles (*acs2-1* and *acs2-2*). Indeed, both mutants showed reduced leaf movement amplitude under +DIF and -DIF conditions (Fig. 3.4D-E). The phase of leaf movement under +DIF conditions was delayed in the two mutants compared with the wild-type under control +DIF and was comparable to the phase of wild-type plants under -DIF (Fig. 3.4F). In response to -DIF, the phase in the *acs2* mutants did not change in contrast to the 5 h phase shift in wild-type leaves under -DIF (Fig. 3.4F). Analysis of the projected and absolute leaf lengths confirmed that both mutants had reduced leaf lengths under both conditions (Fig. S3.4B-E). These results indicate that ACC production by ACS2 in the petiole strongly contributes to leaf elongation and the phase and amplitude of diurnal leaf movement and that the effects of -DIF on growth are mediated through effects on ACS2 activity.

In order to relate ACS2 activity to local ACC levels, petioles of both wild-type and *acs2-1* plants developed in +DIF and -DIF conditions were analysed for ACC content. In wild-type plants, the ACC levels in the petioles were reduced in response to -DIF. Compared with the wild-type, the *acs2-1* mutant had reduced ACC levels under +DIF conditions, and these levels were not further affected by -DIF conditions, indicating that -DIF is not affecting other ACS activities in the petiole (Fig. 3.4G). Taken together, we conclude that *ACS2* is the main -DIF responsive ACS gene in the petiole.

These results suggest that ethylene is able to exert local growth effects even though ethylene is expected to diffuse rapidly throughout the leaf tissue. We tested whether the site of application of ACC is important for leaf movement responses by placing lanolin paste containing ACC (0.5%) at either the base of the petiole or the centre of the leaf blade. ACC paste applied to the petiole restored both the amplitude and the phase of leaf movement under -DIF, whereas ACC applied to the blade only minimally affected leaf movement patterns in these conditions (Fig. 3.4H and I respectively, Supplemental Fig. S3.4F-G). Combined, these results indicate that local, tissue specific *ACS2*-dependent ACC production in the petiole contributes to leaf elongation and regulates both the amplitude and phase of leaf movement in response to diurnal light and temperature cycles.

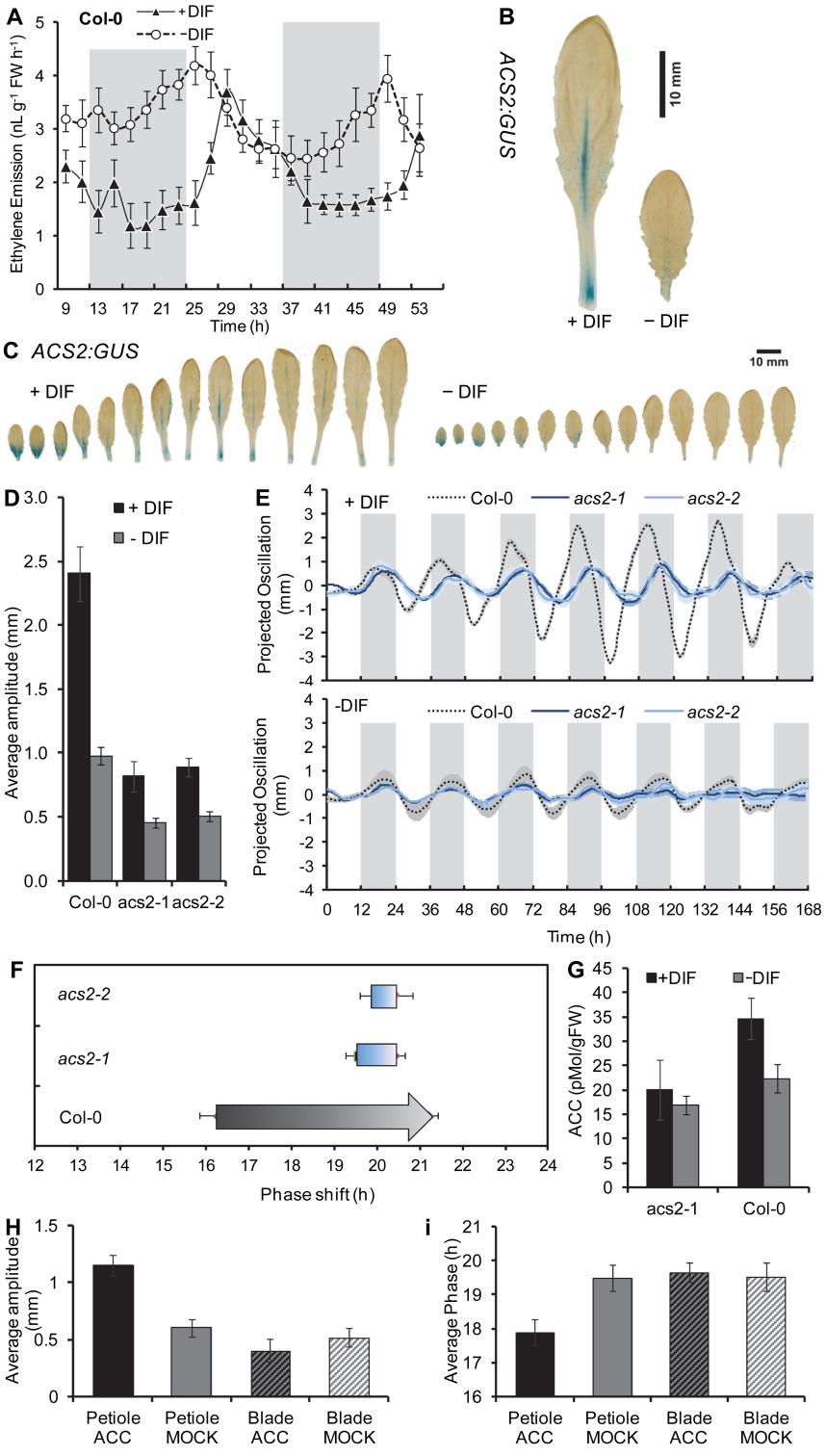

A
Col-0
+DIF
-DIF
Ethylene Emission (nL g-1 FW h-1)
Time (h)
B
ACS2:GUS
10 mm
+ DIF
– DIF
C
ACS2:GUS
+ DIF
– DIF
10 mm
D
+ DIF
- DIF
Average amplitude (mm)
Col-0 acs2-1 acs2-2
E
+ DIF
Col-0
acs2-1
acs2-2
Projected Oscillation (mm)
-DIF
Time (h)
F
acs2-2
acs2-1
Col-0
Phase shift (h)
G
+DIF
-DIF
ACC (pMol/gFW)
acs2-1
Col-0
H
Average amplitude (mm)
Petiole ACC
Petiole MOCK
Blade ACC
Blade MOCK
i
Average Phase (h)
Petiole ACC
Petiole MOCK
Blade ACC
Blade MOCK

Figure 3.4 (see previous page) Ethylene biosynthesis in the petiole is reduced under -DIF conditions. (**A**) Diurnal ethylene emissions of Col-0 rosette plants growing under +DIF (solid line, black triangles) or -DIF (dashed line, white circles) conditions. Error bars represent SE; n ≥ 10. Grey areas indicate the dark period. (**B**) Typical *ACS2* promoter activity pattern obtained using histochemical GUS staining in leaves of equal age developed under +DIF or - DIF conditions on 4 week old plants. (**C**) Promoter activity analysis of *ACS2* using histochemical GUS staining in leaves developed during 10 days of +DIF or - DIF conditions in 4 week old plants. (**D**) Average amplitudes of *acs2-1* and *acs2-2* leaves developing under +DIF (black bars) or -DIF (grey bars) conditions compared with the Col-0 wild-type, calculated from days 2 to 6 of the projected oscillations (in E). Error bars represent SE; n=8. (**E**) Projected oscillations of *acs2-1* (dark blue line) and *acs2-2* (light blue line) leaves compared with the Col-0 wild-type (black dashed line) under +DIF (top) and -DIF (bottom) conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**F**) Phase shifts of leaf movement between +DIF and -DIF conditions for *acs2-1*, *acs2-2*, and Col-0 wild-type leaves. A significant phase shift ($p<0.05$) is indicated with the arrow, and non-significant shifts are indicated with bars. The arrow depicts direction and strength of the shift in phase: the start of the arrow indicates the average phase during days 2 to 6 under +DIF conditions, and the arrowhead indicates the average phase for -DIF (days 2 to 6). Error bars represent SE; n=8. (**G**) ACC content of *acs2-1* and Col-0 wild-type petioles harvested on the peak of ethylene emission (in A) under -DIF or +DIF conditions. Error bars represent SE; n=8. FW, fresh weight. (**H**) Average amplitudes of days 2 to 6 calculated from the projected oscillations (Supplemental Fig. S3.4F-G) of leaves treated with 0.5% ACC (dark bars) or mock (light bars) lanolin paste (approximately 1 mg) on the petiole (solid bars) or the leaf blade (dashed bars) under -DIF conditions. Error bars represent SE; n=8. (**I**) Average phase of days 2 to 6 calculated from the projected oscillations (Supplemental Fig. S3.4F-G) of leaves treated with 0.5% ACC (dark bars) or mock (light bars) lanolin paste (approximately 1mg) on the petiole (solid bars) or the blade (dashed bars) under -DIF conditions. Error bars represent SE; n =8.

-DIF REDUCES ETHYLENE SENSITIVITY OF ARABIDOPSIS

Besides differences in ethylene production, sensitivity to the hormone may play a role in controlling leaf movement under -DIF. Therefore, we assayed the effect of -DIF on ethylene sensitivity using the classical seedling triple-response assay [196]. Seedlings were germinated for a full 2 days (48 h) in either +DIF or -DIF conditions and then kept 5 d in the dark, while the temperature cycling was maintained. In these conditions, seedlings entrained to +DIF light and temperature cycles before etiolating displayed the typical dose-dependent triple response to ACC, which includes short, thickened hypocotyls (embryonic stems), and apical hook curvature with closed cotyledons (Fig. 3.5A). In contrast, in seedlings exposed to -DIF conditions, the inhibition of hypocotyl elongation in response to ACC was absent up to a reasonably high concentration of ACC (1 µM), and only at the highest tested ACC concentrations a strong inhibition of hypocotyl elongation was induced. As a negative control, we used the *ein2-1* ethylene insensitive mutant, which did not respond to any of the treatments (Fig. 3.5B). Combined, our results suggest that limiting ACC levels in the petiole, possibly enhanced by reduced ethylene sensitivity, results in reduced leaf movement and elongation under -DIF.

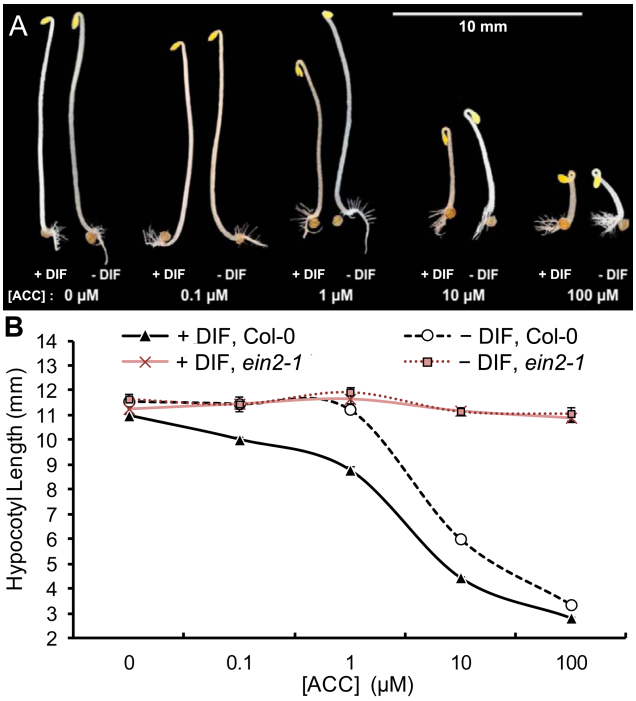


Figure 3.5 -DIF reduces ethylene sensitivity of Arabidopsis. (**A**) Responses of 5 day-old etiolated Col-0 seedlings to various concentrations of ACC. After 48 h of germination under +DIF or -DIF conditions, seedlings were kept in the dark with temperature cycles for 72 h. (**B**) Hypocotyl length of 5 day-old Col-0 wild-type (black line) or ein2-1 (red line) seedlings in response to various concentrations of ACC after 48 h germination under +DIF or -DIF conditions. Seedlings were then kept in the dark with temperature cycles for 72 h on various ACC concentrations. Error bars represent SE; n≥60.

PHYB MODULATES ETHYLENE LEVELS AND SIGNALLING TO CONTROL RHYTHMIC DIURNAL LEAF GROWTH

It has been reported that the Arabidopsis photoreceptor *PHYB* mutant *phyB-1* is insensitive to growth inhibition under -DIF; however, the underlying mechanism for this insensitivity remained unclear [86]. In agreement with the work of Thingnaes et al. (2008), elongation of *phyB-9* mutant leaves was not reduced by our -DIF treatment (Supplemental Fig. S3.5A). Given that ethylene becomes limiting for leaf movement under -DIF (Fig. 3.3), we investigated a possible link between *PHYB* and ethylene. First, the leaf movement patterns in the *phyB-9* null mutant [22] under +DIF and -DIF conditions were compared with the leaf movement of wild-type plants (Fig. 3.6A-B). This revealed that the leaf movement amplitude of *phyB-9* was strongly increased compared with the wild-type in both conditions (Fig. 3.6C). Under +DIF conditions, the phase of *phyB-9* leaf movement was advanced compared with the wild-type (t≈13 h and t=16 h respectively). In response to -DIF, the phase of the *phyB-9* leaf oscillations shifted approximately 9 h (from t≈13 h to t≈22 h under -DIF), compared with an approximately 5 h shift in wild-type plants (Fig. 3.6D). This early phase of *phyB-9* under +DIF conditions is similar to that of wild-type plants treated with ethephon (Fig. 3.3D).

Given that some features of leaf movement of the *phyB-9* mutant resemble those of ethylene treated plants (Supplemental Fig. S3.5B), ethylene production could be increased in the *phyB-9* mutant. Therefore, ethylene emissions from *phyB-9* plants under the contrasting day/night temperature difference conditions were measured. Peak ethylene evolution was

indeed about two-fold higher in *phyB-9* than in wild-type plants under both +DIF and -DIF conditions (Fig. 3.6E and F respectively). The phase of ethylene emission in the *phyB-9* mutant, however, was comparable with the wild-type, including the typical phase shift between +DIF and -DIF conditions (Fig. 3.6E-F). Because of the specific role of *ACS2* in ethylene production for leaf movement (Fig. 3.4), we next introduced *ACS2::GUS* in the *phyB-9* background by crossing and examined *ACS2* promoter activity. *ACS2::GUS* activity strongly increased in leaves of the *phyB-9* mutant compared with the wild-type under +DIF conditions, while (in contrast to wild-type) -DIF did not reduce the *ACS2::GUS* activity in the *phyB* mutant (compare Figs. 3.6G and 3.4C). This indicates that *PHYB* suppresses *ACS2* expression and that diurnal light/temperature control of this repression is an important regulator of leaf movement and growth. Because -DIF reduced ethylene sensitivity in wild-type, we next tested the ethylene sensing capacity in *phyB-9* with a triple response assay. Compared with the wild-type, the *phyB-9* mutant displayed an enhanced triple response under +DIF conditions. In contrast to wild-type, under -DIF the triple response of *phyB-9* was similar to control +DIF (Fig. 3.5H; Supplemental Fig. S3.5C-D), indicating that increased ethylene sensitivity is apparent in *phyB-9* mutants independent of the day/night temperature difference conditions. Combined, the results suggest that the insensitivity of *phyB-9* to -DIF relates to both increased ethylene production and enhanced ethylene sensitivity.

To further test the role of ethylene signalling in the response of the *phyB-9* mutant to the day/night temperature difference phenotypes, we constructed a *phyB-9 ein2-1* double mutant (Supplemental Fig. S3.6A), because *ein2-1* confers insensitivity to ethylene [196]. In response to -DIF the *phyB-9 ein2-1* double mutant displayed visually lower leaf angles compared to *phyB-9* (Fig. 3.7A). The amplitude of leaf movement of *phyB-9 ein2-1* under both +DIF and -DIF conditions was decreased compared with *phyB-9*, but was higher than in the *ein2-1* mutant (Fig. 3.7). This confirms that the *phyB-9* leaf movement phenotype is in part caused by increased ethylene signalling. The stronger leaf movement of the *phybB9 ein2-1* double mutant compared to the *ein2-1* single mutant, however, suggests that additional *EIN2* independent mechanisms may also operate in the regulation of leaf movement in the *phyB-9* background. The absolute lengths of *phyB-9 ein2-1* leaves were strongly reduced in response to -DIF compared with +DIF conditions (Fig. S3.6B). This is in contrast to the *phyB-9* single mutant, in which leaf length is not reduced by -DIF (compare Supplemental Figs. 3.5C and 3.6B).

In conclusion, the leaf movement and growth responses to -DIF directly relate to ACC production in the petiole, which is mostly mediated by *ACS2* activity and depends on ethylene signalling capacity through *EIN2*, both of which act downstream of the photoreceptor *PHYB*.

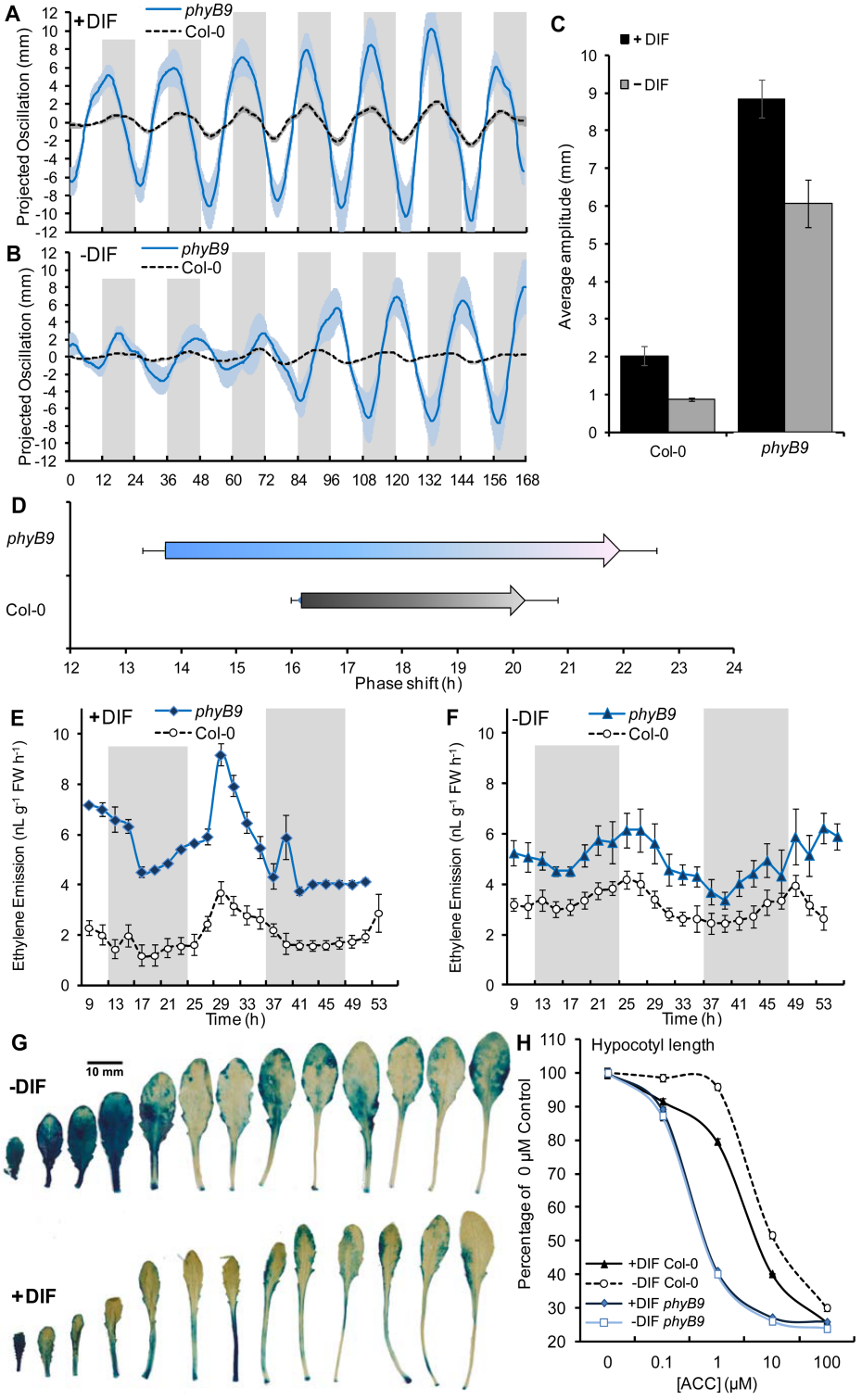

A
+DIF
phyB9
Col-0
Projected Oscillation (mm)
B
-DIF
phyB9
Col-0
Projected Oscillation (mm)
0 12 24 36 48 60 72 84 96 108 120 132 144 156 168
C
+ DIF
- DIF
Average amplitude (mm)
Col-0
phyB9
D
phyB9
Col-0
12 13 14 15 16 17 18 19 20 21 22 23 24
Phase shift (h)
E
+DIF
phyB9
Col-0
Ethylene Emission (nL g-1 FW h-1)
9 13 17 21 25 29 33 37 41 45 49 53
Time (h)
F
-DIF
phyB9
Col-0
Ethylene Emission (nL g-1 FW h-1)
9 13 17 21 25 29 33 37 41 45 49 53
Time (h)
G
10 mm
-DIF
+DIF
H
Hypocotyl length
Percentage of 0 µM Control
+DIF Col-0
-DIF Col-0
+DIF phyB9
-DIF phyB9
0 0.1 1 10 100
[ACC] (µM)

Figure 3.6 (see previous page) PHYB represses ethylene-dependent leaf movement. (**A-B**) Projected oscillations for phyB-9 (solid blue line) and Col-0 (dotted black line) leaves developing under (**A**) +DIF or (**B**) -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Average amplitudes of Col-0 and phyB-9 leaves developing under +DIF (black bars) or -DIF (grey bars) conditions, calculated from days 2 to 6 of the projected oscillations (in A and B). Error bars represent SE; n=8. (**D**) Phase shifts of leaf movement between +DIF and -DIF conditions for phyB-9 and Col-0 wild-type leaves. Significant phase shifts ($p<0.05$) are indicated with arrows. Each arrow depicts direction and strength of the shift in phase: the start of the arrow indicates average phase during days 2 to 6 under +DIF conditions, and the arrowhead indicates the average phase for -DIF (days 2 to 6). Error bars represent SE; n=8. (**E-F**) Ethylene emissions of phyB-9 mutants (blue diamonds, solid lines) compared with Col-0 wild-type emissions (white circles, dashed lines) under +DIF (**E**) and -DIF (**F**) conditions. Error bars represent SE; n≥10. Grey areas indicate the dark period. FW, fresh weight. (**G**) Promoter activity analysis of ACS2 using histochemical GUS staining in phyB-9 leaves developed during 10 days of -DIF (top) or +DIF (bottom) conditions on 4 week old plants. (**H**) Relative hypocotyl length of 5 day-old phyB-9 (solid blue line) or Col-0 wild-type (dotted black line) seedlings in response to various concentrations of ACC. After 48 h germination under +DIF (triangles) or -DIF (circles) conditions, seedlings were kept in the dark with temperature cycles for 72 h on various ACC concentrations. Absolute lengths (as depicted in Fig. S3.5D) were normalised for the length at 0 µM ACC (100%). Error bars represent SE; n=60.

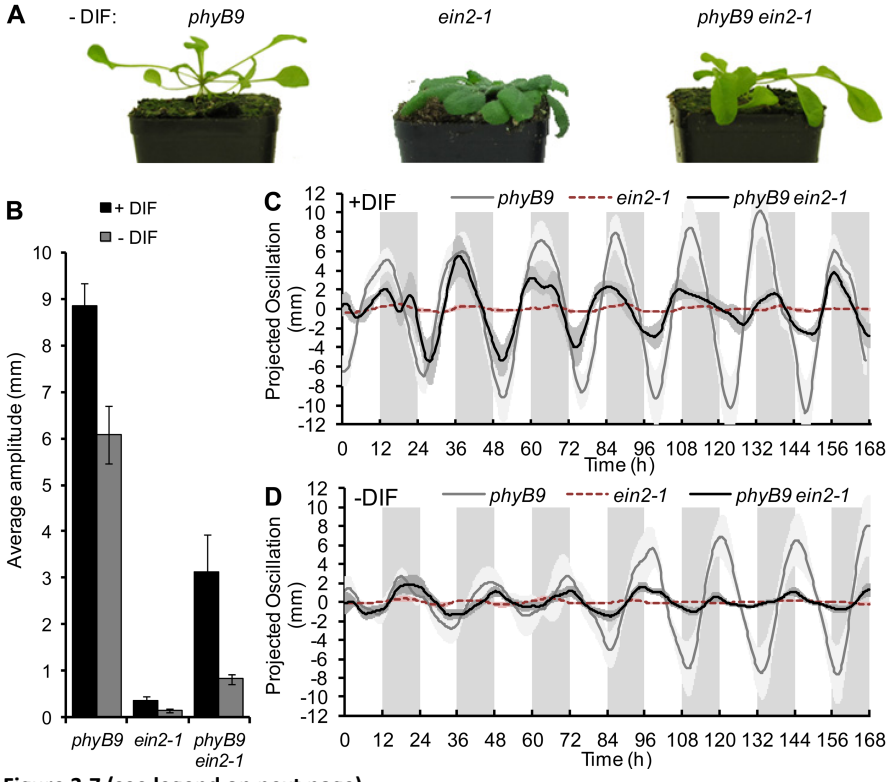


Figure 3.7 (see legend on next page)

Figure 3.7 Dissection of leaf movement in the *phyB-9 ein2-1* double mutant. (**A**) Representative 36 day old *phyB-9*, *ein2-1*, and *phyB-9 ein2-1* rosette plants developing under -DIF conditions at the end of the fourth photoperiod. (**B**) Average amplitudes of *phyB-9, ein2-1,* and *phyB-9 ein2-1* leaves developing under +DIF (black bars) or -DIF (grey bars) conditions, calculated from days 2 to 6 of the projected oscillations (C,D). Error bars represent SE; n=8. (**C-D**) Projected leaf oscillations of *phyB-9*, *ein2-1,* and *phyB-9 ein2-1* double mutant developing under (C) +DIF or (D) -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period.

DISCUSSION

-DIF LIMITS LEAF GROWTH AND MOVEMENT DURING THE DAY

Previous studies have provided a detailed understanding of how hormone and light signalling pathways are integrated and synchronised with daylength to fine-tune plant growth in response to diurnal or seasonal changes [75, 197, 198]. However, in the natural environment, light is accompanied by temperature cycles. Here we performed detailed analysis of leaf growth and movement in response to both natural (+DIF) and artificially (-DIF) imposed light/temperature conditions. Whereas +DIF diurnal conditions result in continuous leaf elongation, accompanied by continuous diurnal leaf movement, the -DIF condition results in cessation of leaf movement and reduced elongation during the photoperiod (Fig. 3.1; Supplemental Fig. S3.1). We found that the effect of -DIF on leaf movement can be traced back to an effect on ethylene levels and ethylene signalling, which become limiting under -DIF, but can be complemented by application of ethephon or ACC (Figs. 3.3A and 3.4H).

Ethylene levels and signalling affect the phase and amplitude of thermoperiodic leaf movement, and the characterisations of phase and amplitude after different chemical treatments and in various mutants are summarised in Fig. 3.8.

In general, high ethylene levels or increased signalling correlate with an early phase and high amplitude of leaf movement, while low ethylene levels or reduced signalling correlate with a late phase and low amplitude of leaf movement (Fig. 3.8A-B). Combined, our results demonstrate that leaf movement amplitude and leaf movement phase do not correlate to total ethylene emissions, but rather are a function of local ACC production by ACS2 (Fig. 3.4). In contrast to lower activity of *ACS2:GUS* under -DIF, *ACS8:GUS* activity was increased under -DIF conditions, and GUS presence increased mostly in the transverse edges of the blade (Supplemental Fig. S3.4A). Previously, rhythmic expression of *ACS8* was reported to correspond with rhythmic ethylene emissions, which are controlled by the circadian clock [195]. Interestingly, *ACS8* transcription is negatively regulated by ethylene signalling [195]. In -DIF, not only local ethylene production decreased (Fig. 3.4G), but also ethylene sensitivity was reduced (Fig. 3.5A-B). A reduced negative feedback on *ACS8* expression is in agreement with the increased *ACS8* activity under -DIF and could explain why overall ethylene emissions are not reduced in -DIF.

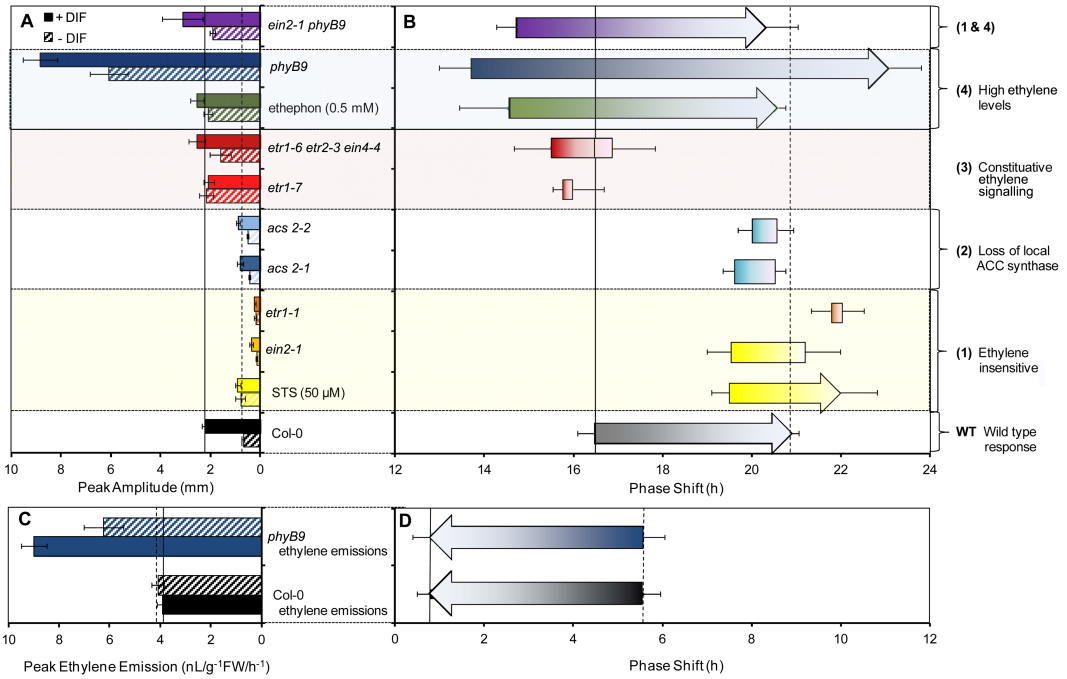


Figure 3.8 Amplitudes and phase shifts of leaf movement and ethylene emission between +DIF and -DIF conditions in mutants and treatments used in this study. (**A**) Averaged amplitudes (measured during days 2 to 6) of leaf movements. Solid bars mark the wild-type response under +DIF conditions and the dashed bars mark the -DIF wild-type response. Error bars represent SE; n=8. (**B**) Phase shifts of leaf movements between +DIF and -DIF conditions compared to wild-type response. Significant phase shifts ($p<0.05$) are indicated with arrows, and non-significant shifts are indicated with bars. Each arrow depicts direction and strength of the shift in phase: the start of the arrow indicates average phase during days 2 to 6 under +DIF conditions and the arrowhead indicates the average phase for -DIF (days 2 to 6). Error bars represent SE; n=8. (**C-D**) Average peak amplitude of ethylene emission (C) and phase shifts of ethylene emission (D) between +DIF and -DIF conditions for *phyB-9* mutants and Col-0 wild-type. Error bars represent SE; n ≥ 10.

PHYB CONTROL OF ETHYLENE PRODUCTION AND SENSITIVITY

Our results confirm previous observations that *PHYB* affects ethylene production [41, 199]. In our conditions, the *phyB-9* mutant showed increased ethylene production compared to wild-type (Fig. 3.6E-F). *PHYB* also repressed ethylene sensitivity, as the *phyB-9* mutant showed increased sensitivity to ethylene in the triple response (Fig. 3.6H). The increased ethylene emission and sensitivity of the *phyB-9* mutant tentatively result in endogenous ethylene signalling levels that are no longer limiting during -DIF. The ethylene signalling towards the leaf movement response is likely mediated by *EIN2*, as mutations in this gene strongly reduce the leaf movement amplitude of the *phyB-9* mutant (Fig. 3.7B). It has been

reported that the role of ethylene in growth is dependent on light [200]. For example, in dark grown seedlings, ethylene inhibits hypocotyl elongation as part of the triple response [196] but in continuous light, ethylene stimulates hypocotyl elongation [201]. This phenomenon was recently explained by the activation of two contrasting pathways by the ethylene signalling transcription factor ETHYLENE-INSENSITIVE 3 (EIN3). In the light, EIN3 activates the growth stimulating transcription factor PHYTOCHROME-INTERACTING FACTOR3 (PIF3). In contrast, in the dark, EIN3 activates the ETHYLENE RESPONSE FACTOR1 (ERF1)-mediated growth-inhibiting pathway [198]. The role of ethylene in the control of elongation is thus condition dependent, which could explain why, in a previous study, performed under continuous light and temperature, ethylene appeared to have no role in controlling the phase of circadian leaf movement [195]. This illustrates that the characterisation of circadian processes under constant conditions are not always easily translated to natural (or economically relevant) diurnal light and temperature conditions.

PHYB CONSTRAINS THE PHASE SHIFTS OF CLOCK REGULATED PROCESSES IN RESPONSE TO -DIF

Previously, it was shown that multiple genes involved in cell elongation are regulated by the circadian clock and the sequential transcription of key steps in cell elongation remained orchestrated under constant conditions [72]. Our results show that for two principally circadian clock regulated processes, leaf movement [71] and ethylene production [195], the phase of activity is shifted under the two diurnal conditions -DIF and +DIF, but the phase shift for each of these processes is different (e.g. 5 h forward for leaf movement, 4 h backward for ethylene production) (Fig. 3.8B, D). Rhythmic ethylene evolution is regulated by the circadian clock, resulting in a specific phase of emission under diurnal entrainment by both light and temperature cycles [195, 202]. In sorghum (*Sorghum bicolor*), temperature was the dominant entrainment signal, setting the phase of ethylene production independently of light cycles [202]. In our experiments with Arabidopsis, the phase of ethylene emission remained within the photoperiod in response to -DIF (Fig. 3.4A). Under -DIF conditions, the phase of leaf movement of wild-type is shifted 5 h forward compared with +DIF conditions, but the phase remained within the dark (warm) period. In contrast to wild-type, the phase of leaf movement of the *phyB-9* null mutant largely adapted to the temperature cycles: *phyB-9* leaves reached the highest position at the start of the cold period independently of the light cycles (Fig. 3.5D). This indicates that the -DIF-induced phase shift of leaf movement in wild-type is partly compensated by PHYB signalling. However, the effect of *PHYB* on the phase is not the same for all clock-controlled processes: loss of *PHYB* function had no effect on the phase shift of ethylene emissions (Fig. 3.8D). It has been reported that -DIF differentially entrains the phase of expression (measured under constant conditions) of two other clock controlled genes, *CHLOROPHYL A/B BINDING2* and *CATALASE3* [203]. A differential effect of -DIF on the rhythmic expression (phase/amplitude) of all clock regulated genes would

reduce coordination of growth and elongation related processes and thus could explain the compactness of plants exposed to -DIF.

FACTORS DOWNSTREAM OF ETHYLENE THAT LIMIT GROWTH DURING THE COLD PHOTOPERIOD

Previously, the average expansion rate of rosette leaves under diurnal conditions was dissected by Pantin et al. [174] It was shown that the expansion rate during the photoperiod is approximately 50% of that during the dark period. If -DIF would completely abolish the expansion rate during the day, those results would predict an approximately 33% reduction in leaf size under -DIF. However, -DIF results in an approximately 40% reduction in leaf size (Supplemental Fig. S3.1B), suggesting that under -DIF also the expansion rate during the night is slightly reduced compared with +DIF conditions. Two factors were identified to be of importance for leaf expansion rate: for emerging leaves the expansion rate of rosette leaves may be limited by starch availability in the early night, while for bigger leaves the expansion during the day was shown to be limited by reduced hydraulic pressure [174]. The leaf movement analysis of our OSCILLATOR-dependent experiments started with leaves 2 days after emergence, in which the expansion rate is mainly limited by hydraulic pressure [174]. However, it is possible that the -DIF treatment also alters clock regulated carbohydrate metabolism such that, for the later stages of leaf development, the required carbohydrates become limiting for cell elongation and leaf movement. It would be interesting to assess the effects of -DIF on the phase and amplitude of expression of core clock components and examine whether carbohydrate availability relates to the altered leaf movement patterns.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis (*Arabidopsis thaliana*) mutants were either from the Nottingham Arabidopsis Stock Centre (www.arabidopsis.info), with accession numbers in parentheses, or from the authors who described the mutant. All transgenic lines and mutants are in the Col-0 (N1092) background: *ACS2::GUS*/GFP (N31380), *ACS4::GUS* (N31381), *ACS5::GUS* (N31382), *ACS6::GUS*/GFP (N31383), *ACS8::GUS*/GFP (N31385), *ACS9::GUS*/GFP (N31386), *ACS11::GUS*/GFP (N31387), *acs2-1* (N16564), *acs2-2* (N16565), *ein2-1* (N3071) [196], *etr1-1* (N237) [204], *etr1-7*, *etr1-6 ein4-4 etr2-3* [190], and *phyB-9* [22]. The *phyB-9 ein2-1* mutant and the *phybB9 ACS2::GUS*/GFP and *phyB-9 ACS8::GUS*/GFP line were generated by crossing the *phyB-9* mutant with *ein2-1* or *ACS2::GUS* and *ACS8::GUS* (Col-0), respectively. Plants with *phyB-9* phenotype were selected in the F2 and selfed (F3). The *phyB-9 ein2-1* double mutant was detected in the F3 by selecting for ethylene insensitivity (lack of triple response phenotype in the presence of 10 µM ACC). The *phyB-9 ACS::GUS*/GFP line were selected based on GUS presence. The presence of the *phyB* allele was confirmed by testing hypocotyl elongation under continuous red (630 nm) light-emitting diode light. The selected lines were selfed, and the F4 progeny displayed no phenotypic segregation and were used in this study.

Plant growth under +DIF (control) conditions was performed as previously described [186]. All experiments were performed in automated climate controlled WEISS (http://www.wkt.com) cabinets (12 h/12 h light/dark cycle). Relative humidity was kept constant at 60% (v/v) and photosynthetic active radiation was 150 µmol m^2 s^{-1} from white fluorescents tubes (PHILIPS, type T5, colour code: 840). Ambient temperature cycles for growth under +DIF conditions were 22°C (photoperiod) and 12°C (dark period) with a temperature ramp of 0.33°Cmin^{-1}. Measurements showed that soil temperature lagged approximately 20 minutes behind ambient air temperature. Plants were grown in fertilised peat/perlite based soil in square (5 cm x 5 cm x 5 cm) plastic pots with different genotypes placed at random positions in the growth cabinet on an irrigation mat which was watered automatically to saturation through porous tubing from a basin containing tap-water every 3 days at the start of the photoperiod. After 20 days, plants were irrigated once with half strength Hoagland-nutrient solution [205] instead of water to provide extra nutrients. Five days later, plants were transferred to a second climate cabinet for imaging with similar conditions and an infrared camera system (OSCILLATOR; see below) with infrared lights and allowed to acclimate for 7 days before the onset of imaging. For -DIF treatment plants were grown under +DIF conditions and at the start of the photoperiod, the temperature cycles were reversed to -DIF 12°C (photoperiod) and 22°C (dark period) with a temperature ramp of 0.33°C min^{-1} in the same growth cabinet. All other growth parameters were kept equal to +DIF conditions.

PLANT GROWTH ANALYSIS BASED ON THE OSCILLATOR GROWTH MONITORING SYSTEM

Plant growth imaging, image data analysis, and extraction of parameters of leaf growth oscillations using the OSCILLATOR setup were performed as described previously [186]. OSCILLATOR uses a continuous top-down imaging of mature Arabidopsis rosette plants with an infrared camera system, and enables extraction and wavelet analysis of oscillating leaf movements. The distance between the leaf tip and the plant apex for each image is calculated and plotted as projected length. Subsequently, a best-fit 2° degree polynomial trend line is automatically calculated for each individual projected leaf length curve, and the residual values are subtracted from this line. The resulting residual is inverted to allow maximum upright leaf position to correspond to maximum peak height. These raw projected oscillations are then smoothed using WAVECLOCK script [176], imbedded in the OSCILLATOR script providing the (smoothed) projected oscillations from which the amplitude and phase for each day for single leaves are automatically calculated. Calculation of phase and amplitude is based on the selection of the data point with the highest value (peak), which has to be flanked by two lower value data points for each individual day (24 h). For this 'peak' the *x* coordinate represents the phase, and the *y* coordinate represents the amplitude. Averages of phase and amplitude were calculated from the values for individual leaves for independent days. All plants analysed were in the same developmental stage, and for each plant, rosette leaf

number 12 and 13 (in order of emergence) were used for leaf movement analysis. Two leaves per plant were analysed, with a minimum of four plants (total n=8-10, referring to the replicate number of leaves included in the analysis). Statistical comparisons between treatments were performed by two-tailed Student's t-test (Excel). All experiments were conducted at least twice using independent trials with similar set-ups and similar outcomes.

ANALYSIS OF LEAF MORPHOLOGY

All leaves present at t=0 h (less than 7 mm) were minimally marked with red paint (Vingerverf CREAL). Unmarked leaves which developed during the treatment were harvested and subsequently scanned using a CANON flatbed scanner (CanoScan 5600F). Morphological parameters were calculated using ImageJ (rsb.info.nih.gov/ij/).

HISTOCHEMICAL GUS STAINING

Histochemical GUS staining of *ACS::GUS* leaves was performed as previously described [206]. All leaves present at t=0 h (more than7 mm) were minimally marked with red paint (Vingerverf CREAL), and plants were kept under +DIF or -DIF conditions. After 10 days, whole rosettes were harvested and marked leaves that developed before the treatment were removed. After staining, fixation, and ethanol clearing of the intact rosette plants, the individual leaves were separated and scanned with a Canon flatbed scanner (CanoScan 5600F). Backgrounds of the scanned images were removed using Adobe Photoshop version 3.1.

PHARMACOLOGICAL EXPERIMENTS

All plant growth regulators used were dissolved in deionised water with 0.005% surfactant Tween-20 (Sigma-Aldrich) and applied to aerial parts of rosette plants by spraying (airbrush) at the start of the experiment (t=0 h). Mock plants were treated with identical solutions that lacked the active components. For ethephon treatments of rosette plants, Ethrel-A (Luxan) which contains 2-chloroethylphosphonic acid (480 g L^{-1}) stabilised in a potassium phosphate buffer was diluted to desired concentration (0, 0.25, 0.5, or 1 mM) before application. STS was prepared by mixing 80% (v/v) 0.1 M STS (Sigma-Aldrich) with 20% (v/v) 0.1 M silver nitrate (Sigma-Aldrich) as described in Sigma protocols and subsequently diluted to 50 µM STS.

HYPOCOTYL ELONGATION RESPONSE TO ACC

Seeds were sterilised in 2.5% sodium hypochlorite solution for 5 min, washed three times with water, sown on petri dishes containing Murashige and Skoog (MS) enriched plant agar (8 g L^{-1} plant agar (Duchefa), 2.2 g L^{-1} MS (Duchefa)) and different concentrations (0, 0.1,

1, 10, and 100 µM) of ACC (Sigma-Aldrich), and stratified in the dark for 4 days (4 °C) to synchronise germination. Seeds were then exposed to either +DIF or -DIF conditions for 48 h (two photoperiods) until radicle protrusion. The timing of radical protrusion was equal between +DIF and -DIF conditions. The seedling plates were then transferred to darkness (covered with aluminium foil) at the start of the third photoperiod or left uncovered under the diurnal experimental conditions (+DIF/-DIF) for two cycles (72 h). Seedlings were placed in a horizontal position, photographed, and hypocotyl lengths were measured using ImageJ software.

ETHYLENE EMISSION ANALYSIS

Ethylene release was measured real-time as described previously [187] using a laser-driven photo acoustic ethylene detector (Sensor Sense, Nijmegen, the Netherlands) for 4 to 5 week old Col-0 or *phyB-9* plants growing under experimental conditions with the following modifications. Single plants including the pot were placed in 350 mL gastight translucent tissue culture pots equipped with tubes to accommodate inflow and outflow under +DIF or -DIF conditions 1 day before the start of the measurement. Pots were flushed continuously with ethylene-free air (1 L h^{-1} flow rate). From day 2 onward, emissions were measured with 2 h intervals for a period of at least 48 h. Pots containing only soil without plants were included in the cultivation procedure and included in the analysis as background emissions.

ACC EXTRACTION, DETECTION, AND QUANTIFICATION BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

ACC extraction was performed as previously described [207]. Frozen leaf material (about 200 mg fresh weight) was ground in liquid nitrogen with a ball mill (MM400; Retsch) in a 2 mL Eppendorf tube, and then extracted with 1 mL of extraction solvent (water:ethanol, 20:80 (v/v)) and sonicated for 5 min at 22°C in a Branson 3510 ultrasonic bath (Branson Ultrasonics). After centrifugation (10 000 rpm for 15 min at 4°C), the supernatant was collected and the pellet was reextracted with 1 mL of extraction solvent. [^{2}H2]ACC (OlChemim) was added to the second extraction solvent as an internal standard. After centrifugation (10 000 rpm for 15 min at 4°C), the supernatant was collected and evaporated to dryness in vacuo at 55°C (SpeedVacuum Savant SPD121P, Thermo Scientific). The residue was dissolved in 2 mL of distilled water, 2 mL of dichloromethane was added, and after vortexing (10 s), the mixture was centrifuged at 500 to 1000 g for 5 min. The upper phase was collected and evaporated to dryness in vacuo at 55°C. The residue was suspended in 50 µL methanol. After centrifugation (10 000 rpm for 15 min at 4°C), 10 µL of the supernatant was used for derivatisation of the ACC for liquid chromatography-tandem mass spectrometry analysis as described previously [208], with the modification that the Waters AccQ•Fluor Reagent Kit (for amino acid derivatisation) was used (Waters). For ACC derivitisation, 10 µL of ACC extract supernatant

were mixed with 20 µL reconstituted AccQ•Fluor reagent in 70 µL of borate buffer and heated at 55°C for 10 min.

Analysis of ACC in Arabidopsis leaf extracts was performed by comparing retention times and mass transitions with those of standard ACC (Sigma) as described previously [209] with the following modifications. A Waters Xevo tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionisation (ESI) source and coupled to an Acquity ultra-performance liquid chromatography system (Waters, USA) was used. Chromatographic separation was achieved on an Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.8 µm) (Waters, USA) by applying a methanol-water gradient to the column, starting from 0.1% MeOH for 0.1 min and rising to 9.1% MeOH at 5.74 min, followed by a 2 min gradient to 21.2% MeOH, followed by a 0.3 min gradient to 25% MeOH, which was maintained for 0.56 min before going back to 0.1% MeOH using a 0.15 min gradient. The column was equilibrated for 2.25 min, using this solvent composition prior to the next run. The run time was 11 min. The column was operated at 55°C with a flow-rate of 0.7 mL min^{-1}. Sample injection volume was 5 µL. The mass spectrometer was operated in positive ESI mode. Cone and desolvation gas flows were set to 50 L h^{-1} and 1000 L h^{-1}, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C, and the desolvation temperature at 650°C. The cone voltage was optimised for derivatised ACC and [$^2H_2$]ACC standard using the IntelliStart MS Console (Waters). Argon was used for fragmentation by collision induced dissociation. Multiple reaction monitoring (MRM) was used for ACC identification and quantification. Parent-daughter transitions were optimised for derivatised ACC and [$^2H_2$]ACC standards using the IntelliStart MS Console. For identification, the following MRM transitions mass-to-charge ratio *m/z* 272.20 > 170.98 at a collision energy of 24 eV and *m/z* 272.20 > 115.97 at 46 eV; and for [$^2H_2$]ACC, the transitions mass-to-charge ratio *m/z* 276.20 > 170.96 at a collision energy of 22 eV and *m/z* 276.20 > 115.97 at 46 eV. Cone voltage was set to 28 eV. ACC was quantified using a calibration curve with known amount of standards and based on the ratio of the area of the MRM transition *m/z* 272.20 > 170.96 for ACC to the MRM transition *m/z* 276.20 > 170.96 for [^{2}H2]ACC. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters). The summed area of all the corresponding MRM transitions was used for statistical analysis.

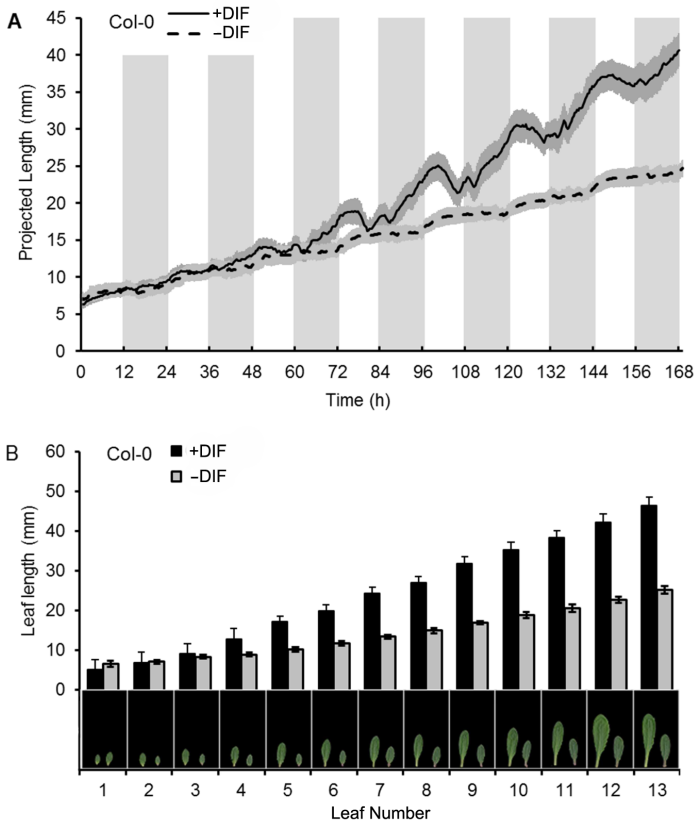
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers.

ACKNOWLEDGMENTS

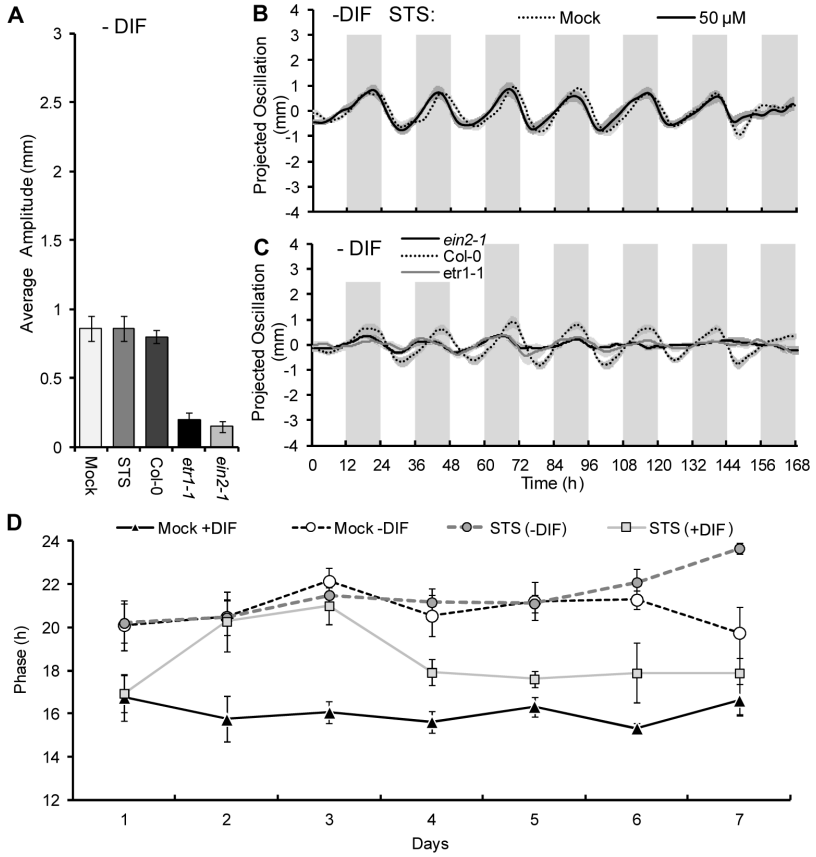
We are very grateful to T. Charnikova for setting up and supervising the ACC analysis. We thank B. Binder for his advice and for providing seeds of the ethylene mutants and R. Welschen, F. Verstappen, M. Schreuder, C. MengFoong, and W. Kohlen for advice and technical assistance.

SUPPLEMENTAL DATA

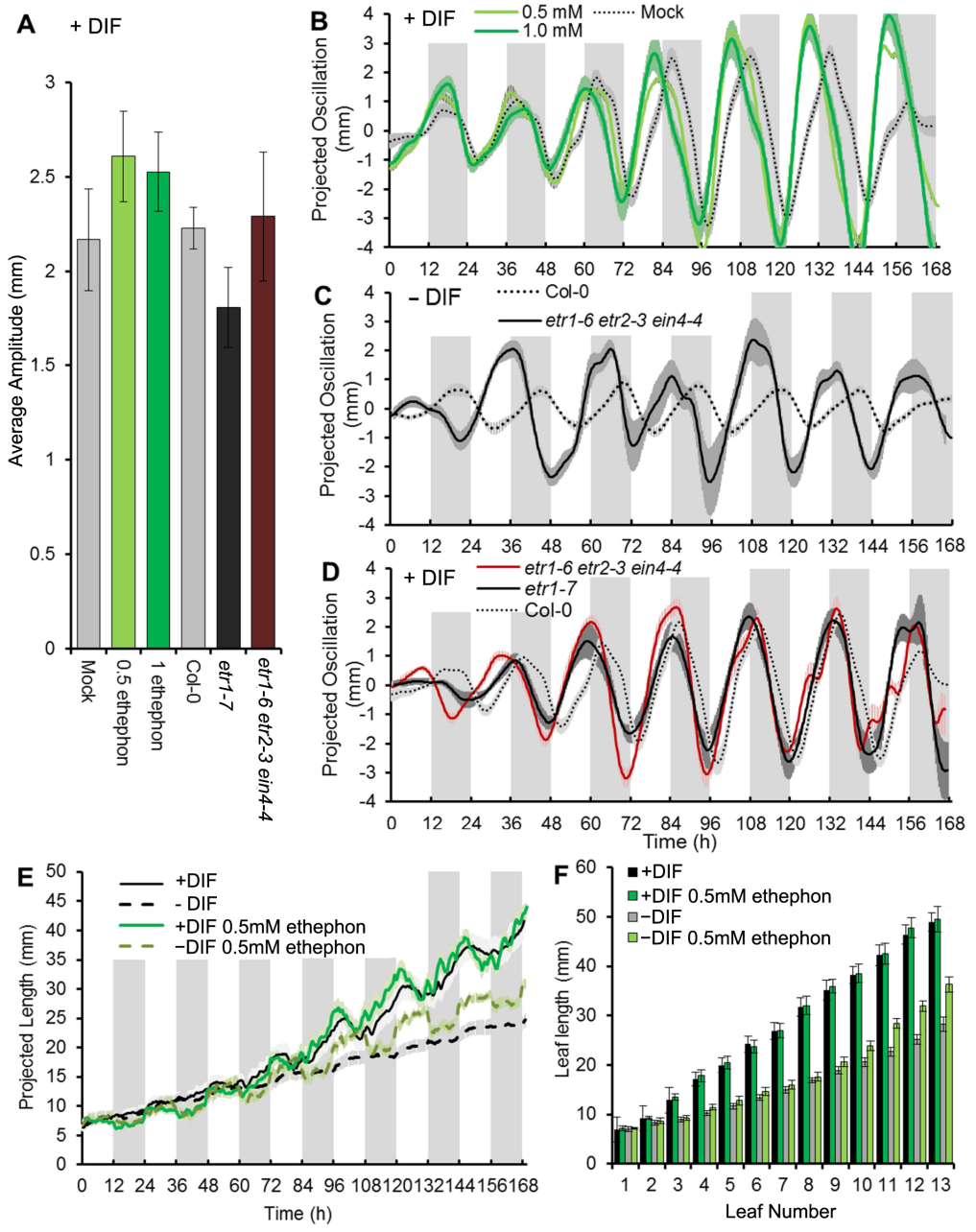
Supplemental File S3.1 Infrared time-lapse recording of Col-0 rosette plants developing under +DIF or -DIF conditions. Infrared images of representative Arabidopsis plants growing under +DIF (left) or -DIF (right) conditions were captured every 20 minutes for 1 week. Image stacks were compressed into a video of 20 frames per s. Labels below the plants indicate the light and temperature conditions.



Supplemental Figure S3.1 Projected and absolute leaf lengths decrease under -DIF conditions. (**A**) Projected leaf length of Col-0 leaves, as measured by the OSCILLATOR system every 20 minutes for 7 days of development under +DIF (solid) or -DIF (dashed) conditions. Projected leaf length represents the projected distance between the leaf tip and the rosette centre, measured for each subsequent image. SE are depicted per time point as shading, n=8 leaves. Grey areas indicate the dark period. (**B**) Absolute leaf lengths (mm) (as measured from rosette centre to leaf tip) and representative images of Col-0 leaves emerged and developed during 10 days of +DIF (black) or -DIF (grey) conditions. Leaf age increases from left to right. Error bars represent SE; n=10.

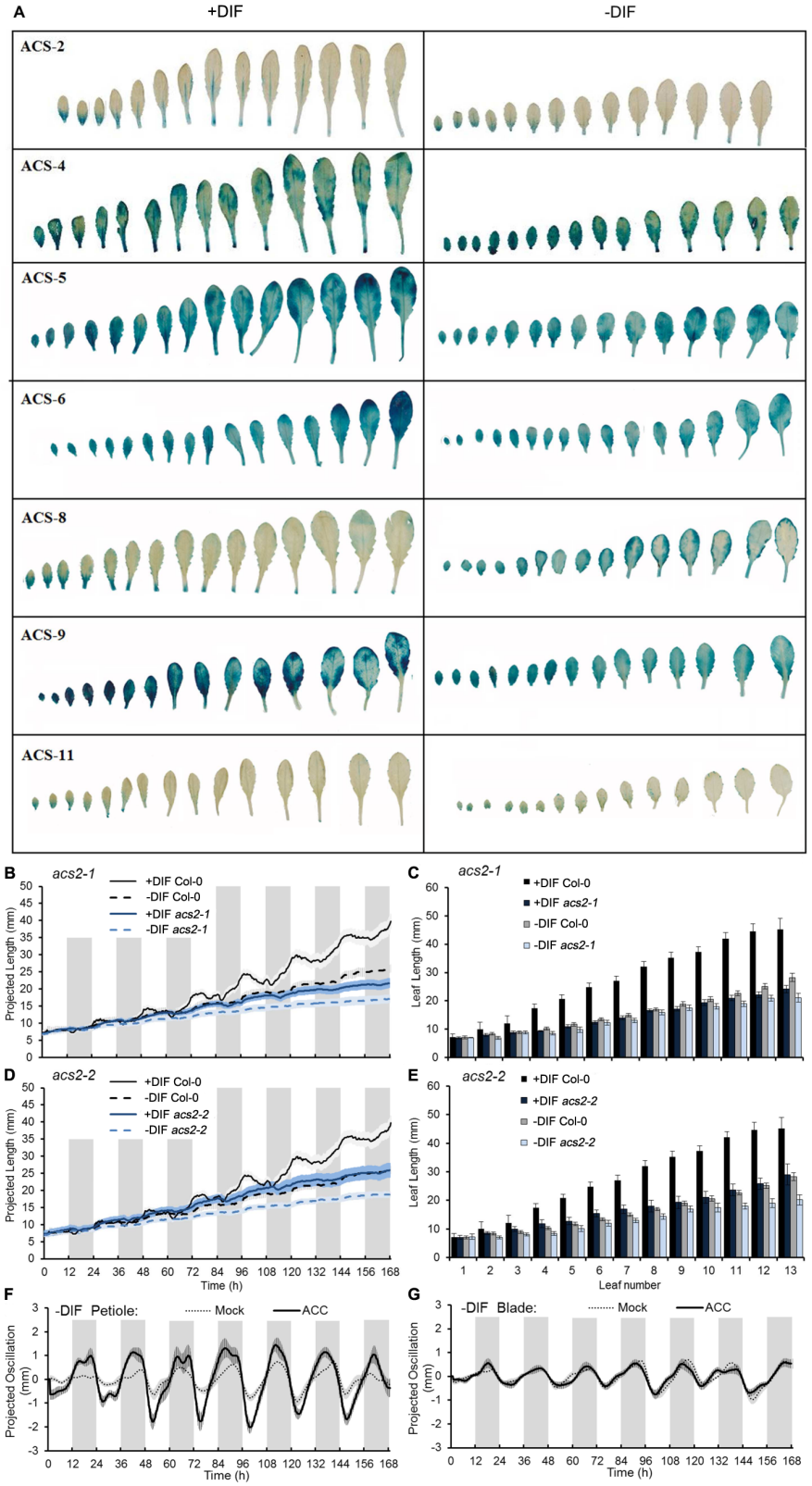


Supplemental Figure S3.2 Ethylene signalling controls amplitude and phase of diurnal leaf movement. (**A**) Average amplitudes (days 2 to 6) calculated from the projected oscillations (B-C) for leaves treated with mock (white), 50 µM STS (grey), Col-0 (dark grey), *etr1-1* (black), and *ein2-1* (grey) developing under -DIF conditions. Error bars represent SE; n=8. (**B**) Projected oscillations of Col-0 leaves treated with 50 µM STS (solid) compared to mock (dashed) under -DIF conditions. Error bars represent SE; n=8.Grey areas indicate the dark period. (**C**) Projected leaf oscillations of *etr1-1* (solid black) and *ein2-1* (solid grey) compared to Col-0 (wild-type) (black, dashed) under -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**D**) The phase of oscillations for leaves developing under -DIF conditions treated with mock (dashed, white circles), with 50 µM STS (dashed, grey circles), and leaves developing under +DIF conditions treated with mock (solid, black triangles) or with 50 µM STS (solid, grey squares) for 7 days of development. Error bars represent SE; n=8.

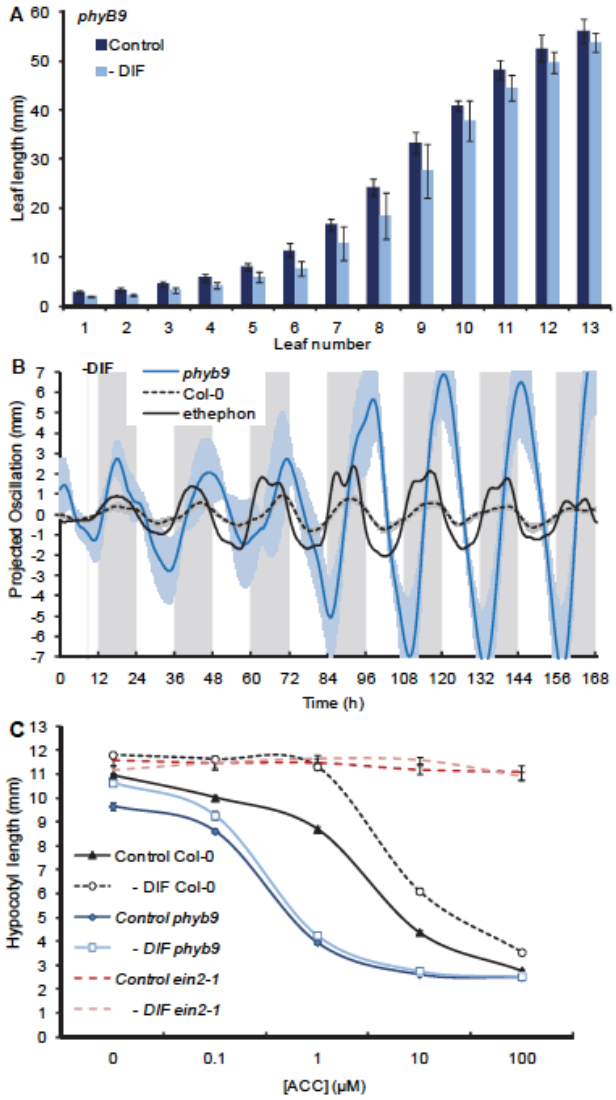


Supplemental Figure S3.3 Effect of ethylene on leaf growth and movement. (**A**) Average amplitudes of days 2 to 6 calculated from the projected oscillations for leaves treated with mock (grey), 0.5 mM (light green), and 1 mM (green) ethephon, and for Col-0 (grey), *etr1-7* (black), and *etr1-6 etr2-3 ein4-4* (dark red) developing under +DIF conditions. Error bars represent SE; n=8. (**B**) Ethephon dose-response of projected oscillations for Col-0 leaves developing on plants sprayed at t=0 h, with two ethephon

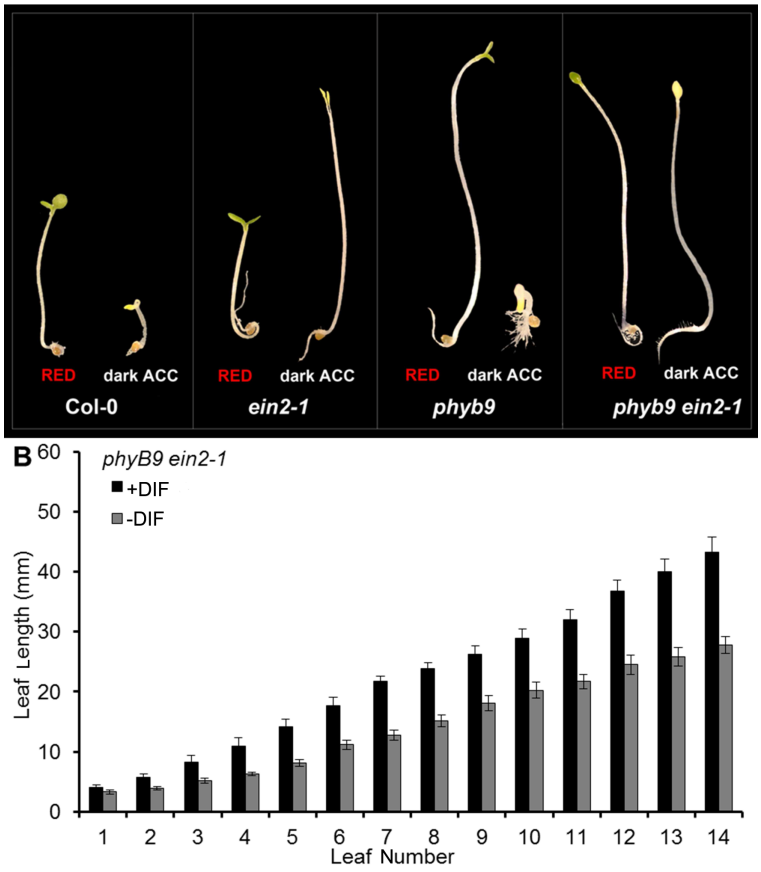
concentrations, 0.5 mM (light green) and 1 mM (dark green) compared to mock, 0.0 mM (dashed). Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Projected oscillations of constitutive signalling mutant *etr1-6 etr2-3 ein4-4* (solid) compared to wild-type (dashed) under -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**D**) Projected oscillations of constitutive signalling mutants *etr1-7* (grey) and *etr1-6 etr2-3 ein4-4* (red) compared to wild-type (dashed) under +DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**E**) Projected leaf length for 7 days of development under +DIF (solid) and under -DIF (dashed) conditions treated at t=0 h with mock (black) or 0.5 mM ethephon (green). SE are depicted per time-point as shading, n=8 leaves. Grey areas indicate the dark period. (**F**) Absolute leaf lengths (mm) of leaves emerged and developed during 10 days of +DIF (solid) or -DIF (dashed) conditions and with addition of 0.5 mM ethephon at t=0 h (green). Leaf age increases from left to right. Error bars represent SE; n=10.


A
+DIF
-DIF
ACS-2
ACS-4
ACS-5
ACS-6
ACS-8
ACS-9
ACS-11
B
acs2-1
+DIF Col-0
-DIF Col-0
+DIF acs2-1
-DIF acs2-1
Projected Length (mm)
C
acs2-1
Leaf Length (mm)
D
acs2-2
+DIF acs2-2
-DIF acs2-2
Time (h)
E
Leaf number
F
-DIF Petiole:
Mock
ACC
Projected Oscillation (mm)
G
-DIF Blade:

Supplemental Figure S3.4 (see previous page) ACS::*GUS* activities in Arabidopsis leaves and leaf lengths and oscillations of acs2-1 and acs2-2 mutants. (**A**) Promoter activity analysis of ACS using histochemical GUS staining in leaves developed during 10 days of +DIF and -DIF (antiphase) conditions on 4 week old plants were stained for promoter *ACS::GUS* activity. All images represent typical results of at least 10 plants and 2 independent experiments. (**B**) Projected leaf length for Col-0 (black) or *acs2-1* for 7 days of development under +DIF (solid) or under -DIF (dashed) conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Absolute leaf lengths (mm) of Col-0 or *acs2-1* leaves emerged and developed during 10 days of +DIF (solid) or -DIF (dashed) conditions. Leaf age increases from left to right. Error bars represent SE; n=10. (**D**) Projected leaf length for Col-0 (black) or *acs2-2* for 7 days of development under +DIF (solid) or under -DIF (dashed) conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**E**) Absolute leaf lengths (mm) of Col-0 or *acs2-2* leaves emerged and developed during 10 days of +DIF (solid) or -DIF (dashed) conditions. Leaf age increases from left to right. Error bars represent SE; n=10. (**F-G**) Projected oscillations of Col-0 leaves locally treated with ACC (solid) or mock (dashed) lanolin paste on the petiole (F) or the blade (G) under -DIF conditions. Error bars represent SE; n=8.



Supplemental Figure S3.5 PHYB affects ethylene sensitivity and leaf growth and movement under diurnal light and temperature cycles. (**A**) Absolute leaf lengths (mm) of *phyB-9* leaves emerged and developed during 10 days of +DIF (dark blue) or -DIF (light blue) conditions. Error bars represent SE; n=10. (**B**) Projected leaf oscillations of *phyB-9* (solid, blue) Col-0 (dashed, black) and Col-0 treated with 0.5 mM ethephon (solid, black) at t=0, developing under -DIF conditions. Error bars represent SE; n=8. Grey areas indicate the dark period. (**C**) Absolute hypocotyl length (mm) of 5 day old Col-0, *phyB-9,* and *ein2-1* seedlings germinated until radicle protrusion (48 h) on 0.5 MS-medium containing various concentrations of ACC at either +DIF or -DIF conditions and then kept in the dark with thermo-cycles for an additional 72 h. Ethylene-insensitive *ein2-1* mutants were used as +DIF. Error bars represent SE; n=60-70.



Supplemental Figure S3.6 Hypocotyl and leaf elongation of the phyB-9 ein2-1 double mutant. (**A**) Phenotypic validation of the *phyB-9 ein2-1* double mutant. Five day old seedlings of the *phyB-9 ein2-1* double mutant do not display the triple response in response to 10 µM ACC in the dark similar to the *ein2-1* parent and in addition show elongated hypocotyls similar to the *phyB-9* parent under continuous red (630 nm) LED light. (**B**) Absolute leaf lengths (mm) (as measured from rosette centre to leaf tip) of *phyB-9 ein2-1* leaves emerged and developed during 10 days of +DIF (dark purple) or -DIF (light purple) conditions. Leaf age increases from left to right. Error bars represent SE; n=10.

On crosstalk…

“Contraries harmonise with each other; the finest harmonies springs from things that are unalike…”

- Hercules

4

THERMOPERIODIC CONTROL OF HYPOCOTYL ELONGATION DEPENDS ON AUXIN-ETHYLENE INDUCED PIF3 EXPRESSION

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IN REVIEW:

Plant Physiology

ABSTRACT

Antiphase light/temperature cycles (-DIF) inhibit hypocotyl growth in Arabidopsis (*Arabidopsis thaliana*). Here we show that this is caused by reduced cell elongation due to decreased ethylene and auxin signalling. Cell elongation in the basal part of the hypocotyl under -DIF was restored by both ACC (ethylene precursor) and auxin. Expression of several *ACC Synthases* (*ACS*) was reduced under -DIF but could be restored by auxin application. The reduced hypocotyl elongation under -DIF in ethylene biosynthesis and signalling mutants could not be complemented by auxin, indicating that auxin functions upstream of ethylene. The PHYTOCHROME INTERACTING FACTORS (PIF), PIF3, PIF4, and PIF5 were previously shown to be important regulators of hypocotyl elongation. We now demonstrate that, in contrast to *pif4* and *pif5* mutants, the reduced hypocotyl length in *pif3* cannot be rescued by either ACC or auxin. In line with this, treatment with ethylene or auxin inhibitors did not reduce hypocotyl elongation in *PIF3ox* (over-expressor) plants whereas *PIF4ox* and *PIF5ox* hypocotyl length decreased significantly. PIF3 promoter activity was strongly reduced under -DIF but could be restored by auxin application in an ACS dependent manner. Combined, these results show that PIF3 is a regulator of hypocotyl length, downstream of an auxin and ethylene cascade, while PIF5 regulates hypocotyl length upstream of it. Under -DIF, reduced auxin biosynthesis and sequential downstream ethylene signalling become limiting for *PIF3* expression, resulting in seedlings with shorter hypocotyls.

INTRODUCTION

To ensure optimal growth, plants need to adapt their physiological and developmental programs to a constantly changing environment. Light and temperature are two of the strongest environmental signals affecting plant development [9]. Both signals vary in diurnal cycles, which usually oscillate in phase. The natural cycle of warm days and cool nights is referred to as positive day/night temperature difference (+DIF). If the light and temperature cycles are provided in antiphase (cold days and warm nights), this is referred to as negative day/night temperature difference (-DIF). The difference between day and night temperatures strongly affects plant development and the responses of plants to diurnally fluctuating temperatures are collectively referred to as thermoperiodism [76]. For many plant species, elongation is stimulated when the positive difference between day and night temperature increases [79]. However, in horticulture excessive elongation growth decreases crop quality and especially during the seedling stage, excessive elongation of the fragile hypocotyl is unwanted [135, 210] and -DIF is frequently used to reduce elongation [79]. In Arabidopsis (*Arabidopsis thaliana*), -DIF inhibits inflorescence and leaf elongation and a 10°C -DIF resulted in a 40% decrease of final leaf length compared to warm days and cold nights (10°C +DIF) [80, 81]. Although economically important, a full mechanistic understanding of how -DIF specifically affects plant cell elongation is still lacking.

Environmental signals like light and temperature are perceived by plants and are translated into different hormonal signals, which frequently operate in crosstalk with each other and regulate specific plant developmental programs in response to changes in environmental conditions [211]. One of the best studied phytohormones in relation to elongation is auxin [212]. Auxin induced cell elongation is under strict control of environmental conditions. For instance, the rapid elongation of hypocotyls in response to high temperature under continuous light conditions [213] depends on increased upregulation of auxin biosynthesis genes [49]. Significant crosstalk exists between auxin and the gaseous hormone ethylene as ethylene affects auxin transport [214] and auxin can induce ethylene biosynthesis [215]. The effect of ethylene on plant growth is highly dependent on environmental conditions; in darkness ethylene inhibits, while in the light it stimulates hypocotyl elongation [200, 201].

Recently, in a study on the photoperiodic control of plant growth it was demonstrated that light negatively influences elongation by activating phytochrome light receptors [14]. Once activated, these proteins stimulate the degradation of growth stimulating basic helix-loop-helix (bHLH) transcription factors called PHYTOCHROME INTERACTING FACTOR (PIF) [216]. PIF proteins have also been reported to feedback on phytohormone pathways. PIF4 and PIF5, for example, activate auxin biosynthesis genes and/or auxin signalling [49, 75, 217, 218]. PIF4 and PIF5 were shown to be positive regulators of hypocotyl elongation [75]. In addition to PIF4 and PIF5, also PIF3 promotes elongation in seedlings grown under diurnal light/dark conditions. The stimulatory effect of ethylene on hypocotyl elongation under continuous light has been shown to be mediated by PIF3 [198]. It was demonstrated that PIF3 directly binds to a G-box domain in the promoter region of know growth marker genes such as the transcription factors *PIL1 (PHYTOCHROME-INTERACTING FACTOR 3-LIKE 1), HFR1 (LONG HYPOCOTYL IN FAR-RED 1)* and the xyloglucan endotransglycosylase-related *XTR7 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7),* potentially involved in cell-wall growth [21, 219, 220]. However, in the studies mentioned above, the positions of the PIF3, PIF4, and PIF5 transcription factors within the signal pathway that control cell elongation were not fully established. Also, studies on PIF3 were performed under constant temperature (22°C) and the effect of (diurnal) temperature cycles on PIF3 action on cell elongation were not investigated.

In this work we expand these studies on the control of hypocotyl elongation by environmental factors (light and temperature) and endogenous phytohormones (auxin and ethylene). We compare the effect of diurnal light cycles on cell elongation in Arabidopsis hypocotyls under contrasting diurnal temperature cycles (+DIF and -DIF). Using different mutants we link the PIF functions in elongation to auxin and ethylene signalling and we demonstrate that under -DIF, both auxin and ethylene become limiting for cell elongation. Auxin acts upstream of ethylene and links to the induction of ethylene biosynthesis via a localised transcriptional activation of several *ACC synthase* genes. In addition, our genetic interaction studies demonstrate that PIF3, PIF4, and PIF5 act at different positions in this signalling interaction. Auxin activates transcription of *PIF3* in an ethylene dependent fashion,

which positions PIF3 downstream of the signalling cascade. Both PIF4 and PIF5 seem to function upstream and control elongation by regulating the auxin and ethylene signal input. While earlier studies indicate an essential role for PIF4 in plant elongation under constant temperature (22°C or 28°C) and diurnal light [49, 75], PIF4 does not seem to be required for the growth inhibition response to -DIF.

RESULTS

REDUCED ARABIDOPSIS HYPOCOTYL CELL ELONGATION UNDER -DIF CAN BE COMPLEMENTED WITH ACC

The growth response of seedlings to -DIF was characterised by comparing Arabidopsis seedling growth under +DIF and -DIF diurnal cycles. Seedlings grown for seven days under either condition are in a similar developmental stage [81]. However, -DIF treated plants showed a 40% reduction in hypocotyl length compared to control grown (+DIF) seedlings (Fig. 4.1A-C). Closer examination of the epidermal cells of seedlings showed that the reduction in hypocotyl length under -DIF can be attributed to reduced cell elongation rather than to reduced cell divisions (Fig. 4.1D-F).

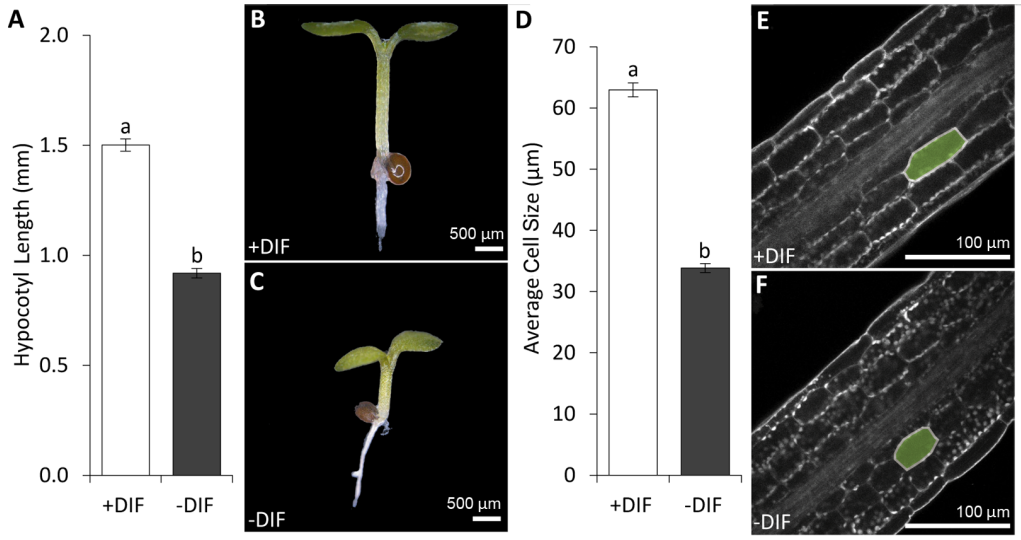


Figure 4.1 Decreased hypocotyl length under -DIF is due to reduced cell elongation. (**A**) Average Arabidopsis hypocotyl length after seven days of growth under +DIF and -DIF. (**B**-**C**) Bright field image of representative Arabidopsis seedlings grown for seven days under +DIF (B) or -DIF (C), scale bars represent 500 µm. (**D**) Average hypocotyl cell sizes at basal site hypocotyl scored at seven days after germination. (**E**-**F**) Confocal microscopy image of +DIF hypocotyl cells (E) and -DIF hypocotyl cells (F) scale bars represent 100 µm. Bars represent mean±SE. Bars with a different letter differ significantly (p-value<0.05).

Because -DIF reduces ethylene sensitivity in Arabidopsis [81] and because ethylene stimulates hypocotyl elongation in the light [201] we assessed whether ethylene was limiting hypocotyl elongation under -DIF. Indeed, application of the ethylene precursor (ACC) increased hypocotyl length of the Arabidopsis seedlings in a dose dependent manner (Fig. 4.2A-C). Analysis of the hypocotyl epidermal cells showed that ACC rescues the hypocotyl length phenotype under -DIF by enhancing cell elongation (Fig. 4.2D). Because the action of ethylene is tightly linked to that of auxin [221] and auxin is involved in the regulation of cell elongation [222, 223] we subsequently investigated the role of auxin in the seedling growth response to -DIF.

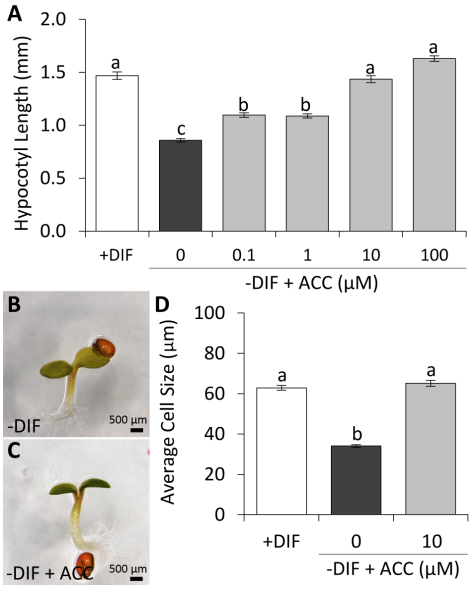


Figure 4.2 ACC complements hypocotyl elongation under -DIF conditions. (**A**) Average hypocotyl length of seven day old Col-0 grown under +DIF or -DIF with and without increasing concentrations ACC. (**B-C**) Bright field image of representative seven day old Arabidopsis seedlings grown under -DIF (B), or -DIF treated with 10 µM ACC (C), scale bars represent 500 µm. (**D**) Average cell size at the basal part of the hypocotyl for seedlings grown for seven days under +DIF and -DIF with the addition of 0 or 10 µM ACC. Bars represent mean±SE. Bars with a different letter differ significantly (p-value<0.05).

ENDOGENOUS AUXIN LEVELS AND AUXIN SIGNALLING ARE REDUCED IN -DIF GROWN SEEDLINGS

Similar to the application of ACC, also application of 1-naphthaleneacetic acid (NAA) or Indole-3-acetic acid (IAA) to seedlings growing under -DIF resulted in enhanced hypocotyl elongation in a dose dependent manner (Fig. 4.3A-C, Supplemental Fig. S4.1). Moreover, examination of the seedlings treated with NAA revealed that this effect on hypocotyl length under -DIF is also due to increased elongation of the hypocotyl cells rather than increased cell division (Fig. 4.3D). This indicates that like ethylene, also auxin is limiting for cell elongation under -DIF. To verify whether limiting auxin under -DIF results from reduced auxin biosynthesis or a reduction in auxin signalling, free auxin levels of whole seedlings were analysed at mid-day (t=6 h after lights on) for both +DIF and -DIF conditions. Under -DIF, the levels of free auxin were 40% lower than in seedlings grown under +DIF (Fig. 4.4A). The activity of the eDR5-luciferase reporter (eDR5::*LUC*) [224] is often used as a proxy for auxin signalling in plants. To study the effect of -DIF on auxin signalling capacity under different diurnal light and temperature cycles we analysed eDR5-luciferase reporter activity in seedlings grown under +DIF or -DIF. Results show that in the

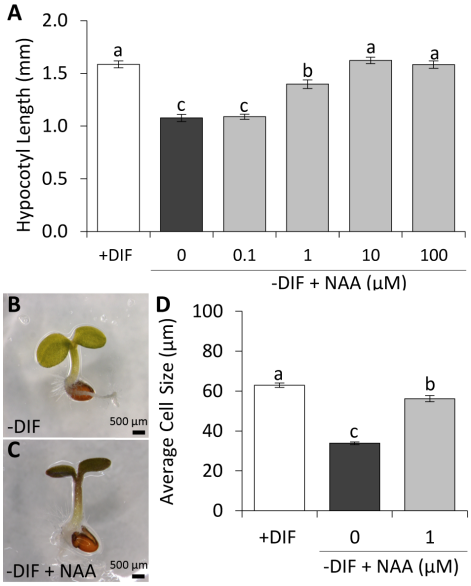


Figure 4.3 NAA complements hypocotyl elongation under -DIF conditions. (**A**) Average hypocotyl length of seven day old Col-0 grown under +DIF or -DIF with and without increasing concentrations NAA. (**B-C**) Bright field image of representative seven day old Arabidopsis seedlings grown under -DIF (B), or -DIF treated with 1 µM NAA (C), scale bars represent 500 µm. (**D**), Average cell size at the basal part of the hypocotyl for seedlings grown for seven days under +DIF and -DIF with the addition of 0 or 1 µM NAA. Bars represent mean±SE. Bars with a different letter differ significantly (p-value<0.05).

control plants (+DIF), luciferase activity was primarily localised in the apical meristem, the cotyledon vasculature, and in the cotyledon tips. In contrast, under -DIF, the eDR5 activity at all these sites was severely reduced (Fig 4.4B).

The DII Venus auxin output reporter produces a constitutive YFP signal in plant cells, which is rapidly degraded in the presence of auxin signalling activity [225]. The DII Venus reporter therefore reveals sites of no or reduced auxin signalling. In line with the eDR5 reporter plant results (Fig. 4.4), no YFP signal was observed in the apex or hypocotyl of DII-Venus reporter plants grown under +DIF (Fig 4.5A-D), indicative of active auxin signalling in these tissues. In contrast, -DIF grown DII-Venus reporter seedlings showed signal accumulation in the apex (Fig. 4.5E-F) and predominantly in the basal part of the hypocotyl (Fig 4.5E, G, H), indicating no or reduced auxin signalling at these sites compared to seedlings grown under +DIF. After application of 1 µM NAA to the growing medium, the YFP signal in -DIF grown DII-Venus reporter plants disappeared (Fig. 4.5I-L), indicating that endogenous auxin levels rather than auxin sensitivity is limiting auxin-signalling capacity under -DIF conditions.

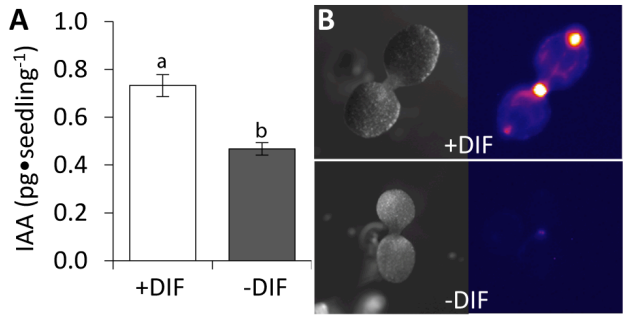


Figure 4.4 Auxin levels are reduced under antiphase temperature and light cycles (-DIF). (**A**) Free IAA levels measured in whole seedlings grown for seven days under +DIF and -DIF. Seedlings were sampled during midday (6 hours after the start of the photoperiod). (**B**) eDR5::*LUC* activity in seven day old Arabidopsis seedlings, as quantified under +DIF and -DIF conditions at midday (6 hours after the start of the photoperiod). Bars represent mean±SE. Bars with a different letter differ significantly (p-value<0.05).

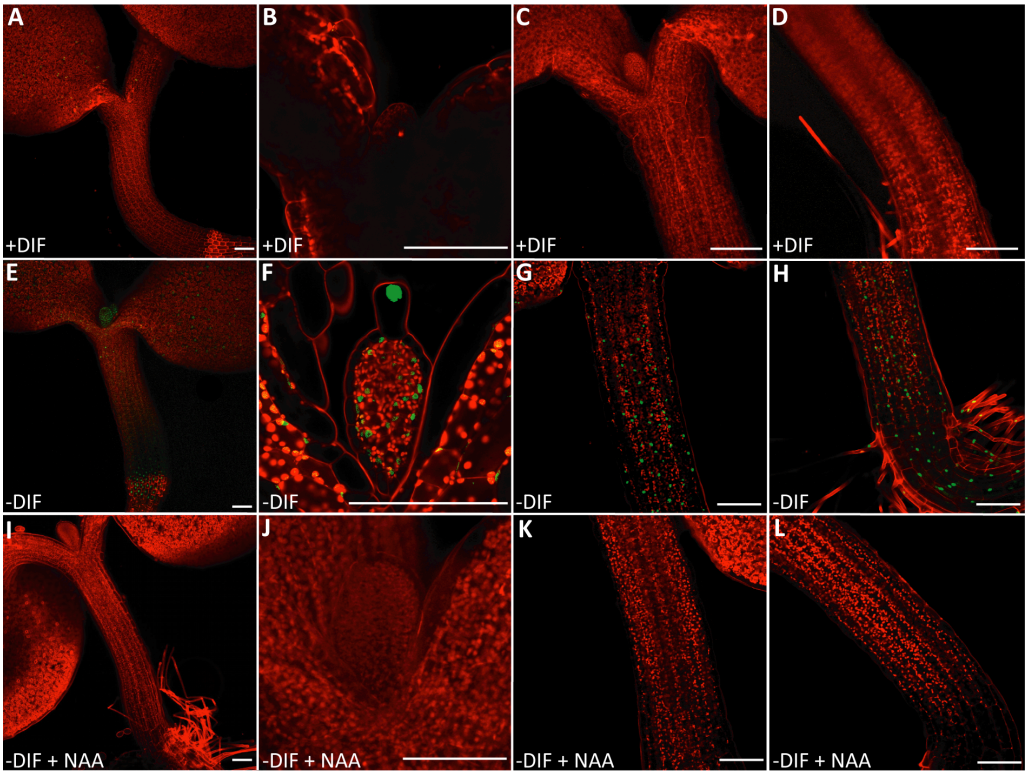


Figure 4.5 Local auxin signalling is reduced under -DIF due to reduced auxin levels. Confocal image of seven day old seedlings carrying the *DII::VENUS YFP* reporter, (**A**-**D**) +DIF conditions, overview (A), leaf primordium (B), apical hypocotyl (C) and basal hypocotyl (D). (**E**-**H**) -DIF conditions, overview (E), leaf primordium (F), apical hypocotyl (G) and basal hypocotyl (H). (**I**-**L**) -DIF conditions treated with 1 µM NAA, overview (I), leaf primordium (J), apical hypocotyl (K) and basal hypocotyl (L). Scale bars (A, E, I) 200 µm and (B-D, F-H, J-L) 100 µm.

-DIF DISRUPTS AUXIN INDUCED ETHYLENE BIOSYNTHESIS

As both application of auxin and ACC restore hypocotyl growth under -DIF, we used the ethylene signalling mutant *ein2-1* [196] and the ethylene biosynthesis *acc synthases* (*acs*)-*octuple* KO mutant [193] to investigate how auxin and ethylene are linked in this response. The hypocotyl length of the ethylene insensitive mutant *ein2-1* is approximately 40% shorter compared to wild-type when grown under control (+DIF) conditions. When grown under -DIF its length was not reduced further, in contrast to wild-type (Fig. 4.6A). Moreover, NAA application to *ein2-1* seedlings did not result in increased hypocotyl length under -DIF, again, in contrast to wild-type seedlings (Fig. 4.6A). This indicates that the auxin regulation of hypocotyl elongation (Fig. 4.3) occurs upstream of ethylene signalling. To narrow down the regulatory relationship between auxin and ethylene, we assessed whether auxin affects

ethylene biosynthesis under -DIF conditions. NAA application increased ethylene emission from -DIF grown seedlings by 40% compared with mock treated plants (Fig. 4.6B).

One of the rate limiting steps in ethylene biosynthesis is the production of ACC by *ACS* [191], which are encoded by a multi-gene family [193]. To determine whether -DIF limits ethylene production through auxin-regulated changes in *ACS* activity, the *acs-octuple* (*acs2 acs4 acs5 acs6 acs7 acs9 amiRacs8 amiRacs11*) loss-of-function mutant [193] was grown under control and -DIF conditions. This strong ACC biosynthesis mutant has highly reduced ethylene production [193] and behaved similar to the ethylene insensitive *ein2-1* mutant (Fig. 4.6A, C). Also, just as in *ein2-1,* NAA application to -DIF grown *acs-octuple* mutant did not rescue hypocotyl growth (Fig. 4.6A, C). However, when these plants were treated with ACC, hypocotyl length was restored to wild-type levels under +DIF conditions (Fig. 4.6A, C).

In a complementary assay we tested the effect of chemical inhibition of ACS activity in wild-type plants by aminoethoxyvinylglycine (AVG) [226]. Application of AVG did not result in a further reduction of wild-type seedling hypocotyl length under -DIF (Fig. 4.6A, D). Addition of ACC bypassed the reduced endogenous ACS activity caused by the AVG treatment and hypocotyl length was restored close to wild-type +DIF length (Figure 6A, D). NAA application did not increase hypocotyl length in AVG treated seedlings under -DIF (Fig. 4.6C) just as in the *acs-octuple* mutant (Figure 6D), in contrast to wild-type seedlings grown without AVG (Fig. 4.6A). Combined, these experiments indicate that under -DIF ethylene induced cell elongation is limited by reduced auxin-regulated ACC synthase activity.

To further determine how ACS activity is regulated by -DIF, all known functional Arabidopsis *ACS* family members were analysed using promoter *GUS* reporter lines [194]. Under +DIF the promoters of all *ACS* genes were active in the basal part of the hypocotyl and in the leaf primordia, with the exception of *ACS9* (Fig. 4.6E, Supplemental Fig. S4.2). -DIF reduced the activity of *ACS2*, *4*, *5*, *6*, *7* and *11* in the basal part of the hypocotyl, but increased activity of *ACS8* (Fig. 4.6E). Application of NAA restored promoter activity of *ACS2, 5, 6, 7, 8* and *11* in the basal part of the hypocotyl (Fig. 4.6E) and *ACS2, 4, 5, 6 and 7* in the leaf primordia (Supplemental Fig. S4.2). Combined, the results suggest that the reduced auxin levels under -DIF disturb the transcriptional activation of a subset of *ACS* genes, which correlated with reduced cell elongation under -DIF.

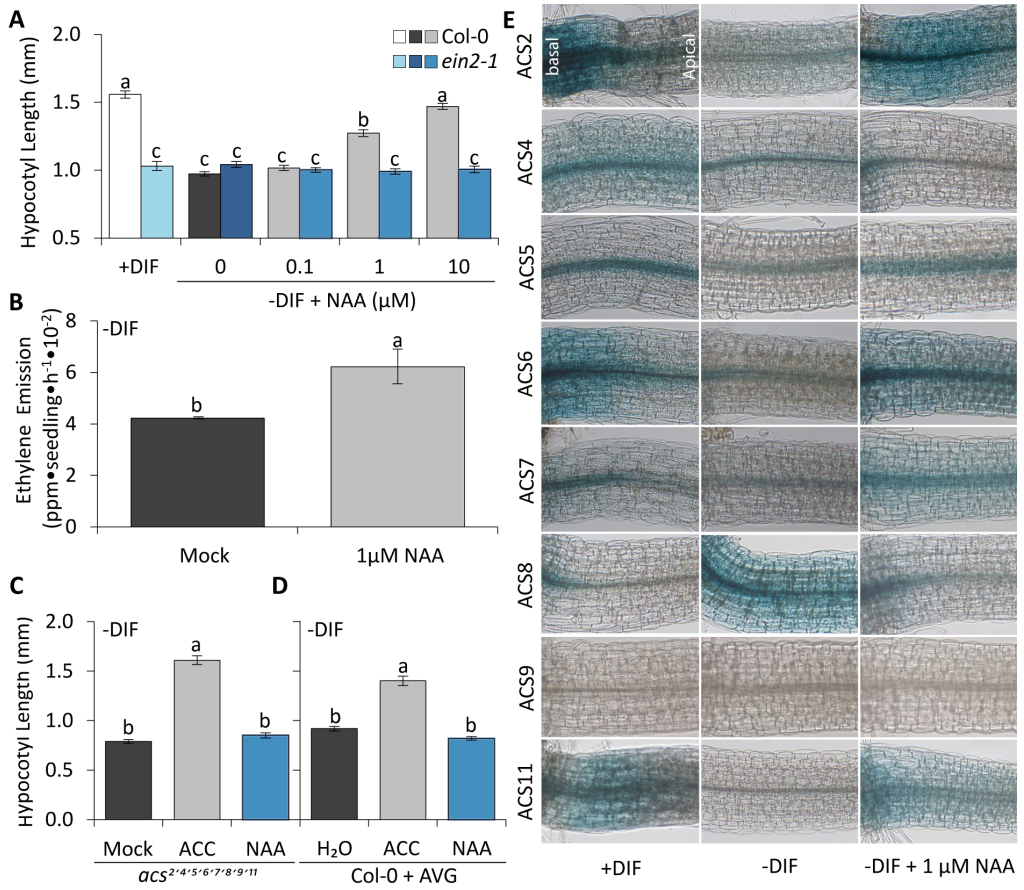


Figure 4.6 Auxin acts upstream of ethylene biosynthesis. (**A**) Average hypocotyl length of seven day old Arabidopsis seedlings Col-0 and *ein2-1* with and without NAA. Control represents untreated seedlings developed under +DIF. (**B**) Total ethylene emitted per hour of Arabidopsis seedlings grown under -DIF with and without 1 µM NAA. (**C**-**D**) The effect of 10 µM ACC and 1 µM NAA application on hypocotyl elongation in *acs*2,4,5,6,7,8,9,11 (*acs2-1acs4-1acs5-2acs6-1acs7-1acs9-1amiRacs8acs11*) seedlings grown for seven days under -DIF and wild-type seedlings grown in the presence of AVG. (**E**) $ACS^{(2,4,5,6,7,8,9,11)}$*::GUS* staining in seven day old seedlings in the basal part of the hypocotyl under +DIF, -DIF and -DIF in the presence of 1 µM NAA. Bars represent mean±SE. Bars with a different letter differ significantly (p-value<0.05).

PIF3 ACTS DOWNSTREAM OF AUXIN INDUCED ETHYLENE BIOSYNTHESIS

Recently, the transcription factors PIF3, PIF4 and PIF5 were shown to be involved in the regulation of hypocotyl elongation [75, 220, 227]. Also, it was demonstrated that ACC-induced hypocotyl elongation in the light depends on transcriptional activation of *PIF3* [198]. To examine the involvement of PIFs in the -DIF phenotype, *pif* single, double and triple mutants (*pif3*, *pif4*, *pif5*, *pif4 pif5* and *pif3 pif4 pif5*) were grown under +DIF and -DIF with or

without ACC or NAA. Under +DIF, all *pif* mutants had shorter hypocotyls than wild-type seedlings (Fig. 4.7A), while under -DIF the hypocotyl length of wild-type and mutant seedlings was the same (Fig. 4.7B). Under +DIF, ACC and NAA restored the hypocotyl length of *pif4*, *pif5* and *pif4 pif5* to wild-type length. Under -DIF, hypocotyl length of *pif4*, *pif5* and *pif4pif5* did not reach full wild-type length but increased significantly upon either application. However, *pif3* and *pif3 pif4 pif5* hypocotyl length was unresponsive to either treatment, both for seedlings grown under +DIF (Fig. 4.7A) and seedlings grown under -DIF (Figure. 4.7B). Combined these results indicate that the effect of auxin and ethylene signalling on cell elongation are located downstream of -or parallel to- PIF4 and PIF5, but upstream of PIF3.

To further investigate the role of these different PIFs in thermoperiodic hypocotyl elongation, we examined the -DIF response of *PIF3*, *PIF4* and *PIF5* over-expressor lines (*PIFox*) as well as a *phyB* mutant. PIF proteins are degraded by PHYB signalling in the light [228, 229] and as a consequents the level of endogenous PIF is elevated in a *phyB* mutant [230]. Under +DIF, the hypocotyl length of *PIF3ox* and *PIF4ox* was only marginally increased compared with wild-type, while *phyB-9* and particularly *PIF5ox* displayed a strong increase in hypocotyl length (Fig. 4.7C). When grown under -DIF, the hypocotyl length of wild-type seedlings strongly decreased, as expected (Fig. 4.7C-D). However, *PIF3ox, PIF5ox* and *phyB-9* hypocotyl length was not affected by -DIF. The *PIF4ox* line did however respond to -DIF and compared to +DIF showed a 40% reduction similar to wild-type (*t-test p-value*<0.05). Thus the *PIF4ox* line is sensitive to the effects of -DIF on growth, indicating that PIF4 is not involved in the -DIF response (Fig. 4.7C-D). Application of AVG reduced *PIF4ox*, *PIF5ox* and *phyB-9* hypocotyl length, but did not affect *PIF3ox* under either +DIF or -DIF (Fig. 4.7C-D). Combined these results indicate that PIF5 and PHYB function upstream of ethylene while PIF3 acts downstream of ethylene.

We also tested the effect of the polar auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA) in these lines as previously it was shown that NPA reduces hypocotyl length of light grown seedlings in an auxin dependent manner [231]. The application of NPA resulted in reduced hypocotyl length in wild-type seedlings under +DIF but did not further reduce hypocotyl length of seedlings grown under -DIF, indicating that auxin is important for growth under +DIF, and that hypocotyl length under -DIF cannot be further reduced (Fig. 4.7C-D). In contrast, NPA did not affect hypocotyl length of *PIF3ox* when grown under either +DIF or -DIF, indicating that in relation to this phenotype these lines are insensitive to NPA (Fig. 4.7C-D). In contrast, in *PIF4ox, PIF5ox* and *phyB-9* auxin is required for the elongation response under +DIF, and in these lines NPA treatment reduced hypocotyl elongation. Under -DIF, the *PIF4ox* line was only minimally affected by NPA compared with the strong reduction observed in *PIF5ox* and *phyB-9* (Fig. 4.7C-D). These results confirm that under +DIF, PIF4 and PIF5 depend on auxin and that under -DIF in wild-type plants auxin becomes limiting for the elongation response. However, for the *PIF5ox* and *phyB-9* mutant the increased hypocotyl elongation under -DIF is dependent on auxin. The *PIF3ox* insensitivity to NPA, further indicates that PIF3 acts downstream of the auxin signal responsible for elongation.

Because of the downstream role of PIF3 in the elongation response we tested whether PIF3 expression is reduced under -DIF. For this purpose a *pPIF3::GUS* reporter line was examined under control and -DIF conditions. Indeed, less *pPIF3::GUS* activity was detectable under -DIF compared with +DIF (Fig. 4.7E, H). Moreover, this higher *PIF3* promoter activity under +DIF was also reduced in response to exogenously applied AVG or NPA (Figure 7F, G). In contrast, *PIF3* promoter activity under -DIF conditions was upregulated by ACC and NAA/IAA treatment (Fig. 4.7I, J; Supplemental Fig. S4.3). Combined, the results indicate that *PIF3* is an essential downstream target of an auxin-ethylene signal interaction towards elongation growth and that the interaction between diurnal light and temperature cycles regulates the input and signalling capacity of this cascade, possibly through the interaction of other PIFs (e.g. PIF5) and PHYB.

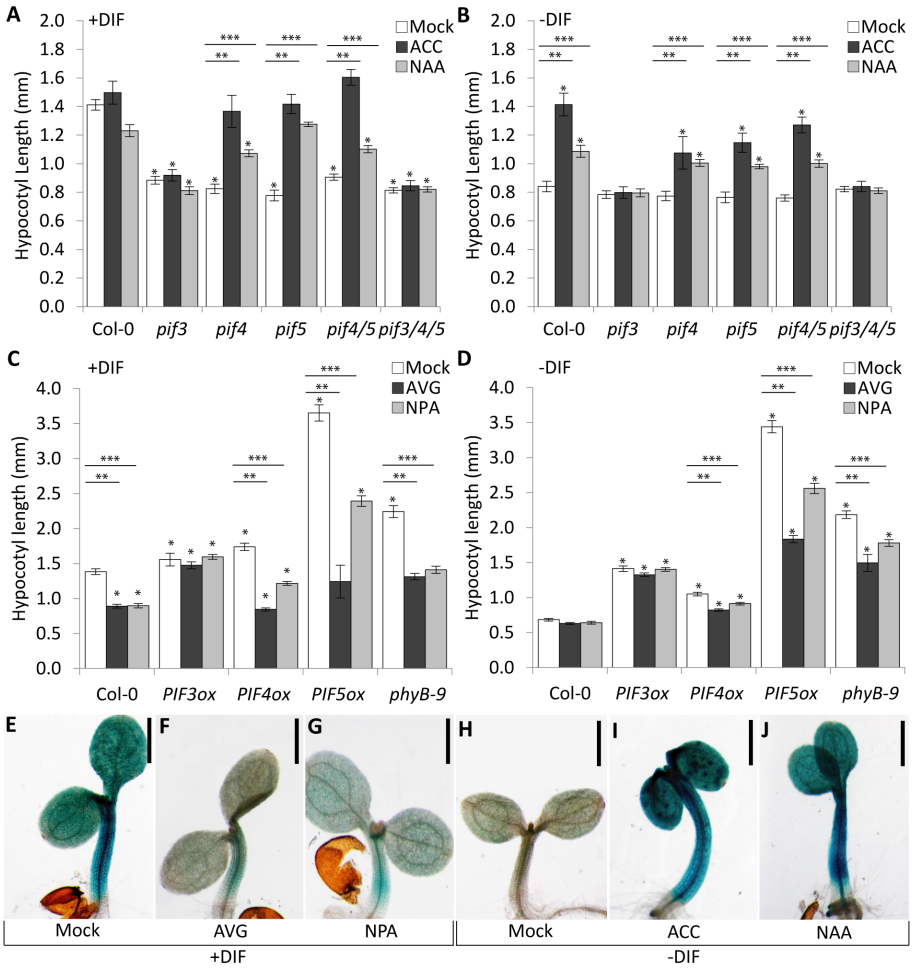


Figure 4.7 The role of *PIF3, 4* and *5* in auxin or ethylene mediated regulation of hypocotyl elongation. (**A**) The effect of 10 µM ACC and1 µM NAA application on hypocotyl elongation in Col-0, *pif3*, *pif4*, *pif5*, *pif4 pif5* and *pif3 pif4 pif5* under +DIF conditions. (**B**) The effect of 10 µM ACC and1 µM NAA

application on hypocotyl elongation in Col-0, *pif3*, *pif4*, *pif5*, *pif4 pif5* and *pif3 pif4 pif5* under -DIF conditions. (**C**) The effect of 2.5 µM AVG and 1 µM NPA on hypocotyl elongation in Col-0, *PIF3ox*, *PIF4ox*, *PIF5ox* and *phyB-9* under +DIF conditions. (**D**) The effect of 2.5 µM AVG and 1 µM NPA on hypocotyl elongation in Col-0, *PIF3ox*, *PIF4ox*, *PIF5ox* and *phyB-9* under -DIF conditions. (**E**-**J**) PIF3::*GUS* promoter activity in seven day old seedlings grown under +DIF (**E**), +DIF in the presence of 2.5 µM AVG (**F**), +DIF in the presence of 1 µM NPA (**G**), -DIF (**H**), -DIF in the presence of 10 µM ACC (**I**), and -DIF in the presence of 1 µM NAA (**J**). Single asterisk indicates significant difference compared to mock treated wild type, double asterisks indicate significant difference between white bar and black bar per genotype and triple asterisks indicate significant difference between white bar and gray bar per genotype (p-value<0.05).

DISCUSSION

Here we show the effects of an antiphase diurnal temperature and light cycle on hypocotyl elongation. While under constant light conditions there is a positive relationship between temperature and growth [49, 213], under diurnal light/temperature cycles it is mostly the day (photoperiod) temperature that limits growth [81]. In the work presented here we analysed key components of the signalling cascade involved in the growth response of plants (auxin, ethylene, PIFs, PHYB) and determined the position and the role of these different components under natural and antiphase light and temperature cycles. Previously we identified that in mature plants ethylene becomes limiting for leaf elongation growth under -DIF conditions [81]. We now show that this also holds true for hypocotyl elongation and is a direct consequence of reduced auxin signalling under -DIF. Furthermore, we show that the reduced growth under -DIF can be attributed to limited activity of the growth promoting transcription factors PIF for which we have identified distinct positions in the ethylene-auxin signalling interaction towards cell elongation.

AUXIN-ETHYLENE INTERACTION TO ELONGATION IS MODULATED BY LIGHT/TEMPERATURE CYCLES.

The results indicate that cell elongation in the basal part of the hypocotyl is a function of ethylene signalling. Moreover, the results indicate that auxin signalling acts upstream of ethylene biosynthesis and signalling in the thermoperiodic elongation response. First, NAA application failed to restore hypocotyl elongation in *ein2-1* and in AVG treated plants. In addition, auxin induced expression of most *ACS* genes (Fig. 4.6), thus restoring ethylene emission and consequently restored elongation under -DIF. The reduced ethylene production under -DIF was linked to reduced activity of different members of the *ACS* gene family at the site of reduced cell elongation. Only the *ACS8* promoter activity in seedlings responded opposite to the other *ACS* genes to -DIF. This upregulation of *ACS8* expression under -DIF has also been observed in rosette leaves [81] and indeed, it has been shown that *ACS8* expression is negatively regulated by ethylene [195]. While on the one hand the elevated *ACS8* expression thus confirms reduced ethylene signalling under -DIF, on the other hand the increased expression of this single gene is apparently not able to compensate for the low

endogenous ethylene levels. In contrast, application of ACC to seedlings under -DIF was able to restore the hypocotyl elongation response (Fig. 4.2).

PIF5 SEEMS TO CONTROL CELL ELONGATION BY REGULATING INPUT INTO THE PIF3 DEPENDENT SIGNALLING CASCADE

The changes in auxin and ethylene signalling under +DIF and -DIF are linked to the activity of different PIFs. The PIFs are frequently considered to be highly redundant transcription factors with overlapping roles in the control of elongation [232]. However, here we demonstrate that these transcription factors act at different positions in the auxin-ethylene-elongation cascade. We demonstrate that the elongation response in *pif4*, *pif5* and *pif4 pif5* mutants can be complemented by both auxin and ethylene, indicating that auxin and ethylene act either downstream or independent from PIF4 and PIF5. In contrast, the elongation response of the *pif3* and *pif3 pif4 pif5* mutants was not complemented by either auxin or ethylene. This indicates that PIF3 functions downstream and dependently of auxin and ethylene in the signal transduction chain that regulates thermoperiodic hypocotyl elongation (Fig. 4.8).

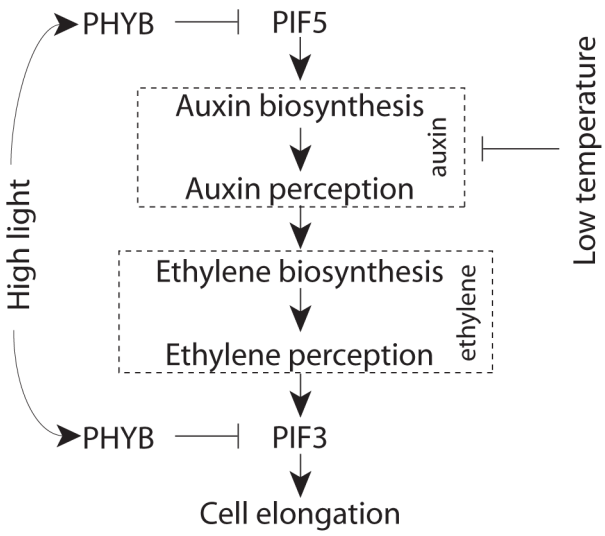


Figure 4.8 Proposed model for the PIF mediated auxin-ethylene crosstalk in relation to hypocotyl elongation.

The observation that the *PIF5ox* line is insensitive to -DIF indicates that PIF5 is located upstream of the auxin-ethylene signalling cascade. The *PIF4ox* line responded to -DIF similar as wild-type, which suggests that PIF4 is not involved in the -DIF response. A growth promoting role of PIF4 independent of ethylene fits with the observation that *ein2-1* still displays hypocotyl elongation in response to high temperature (29°C) under continuous light [213], a response which was later shown to depend specifically on PIF4 [49].

PIF protein stability is highly dependent on PHYB mediated degradation [14] and a *phyB* mutant is therefore expected to have increased levels of PIF protein. We previously demonstrated that *phyB-9* has elevated ethylene emissions and increased *ACS* expression [81] which is suggestive for increased PIF5 activity in *phyB-9*. The loss of PHYB function results in increased hypocotyl elongation, which is insensitive to -DIF [81, 86]. Because the hypocotyl elongation in *pif5* is sensitive to NPA this places PIF5

upstream of auxin. However, under -DIF in wild-type plants, auxin becomes limited for the elongation response. When PIF5 activity is elevated (either by overexpression or by reduced turnover) the increased hypocotyl elongation under -DIF is sensitive to NPA, suggesting that the elevated PIF5 activity results in higher endogenous auxin levels.

It was previously observed that the *PIF5ox* has increased expression of several *ACS* genes and consequently overproduces ethylene [233], while PIF5 has also been shown to modulate auxin signalling [218]. This could be interpreted as PIF5 increasing auxin synthesis and auxin inducing *ACC synthase* genes which results in increased ethylene production. Furthermore, the increased ethylene production seems to be a unique feature of the *PIF5ox*, as this was not observed in *PIF3ox* and *PIF4ox* Lines [233]. This would be in agreement with a position of PIF5 upstream of ethylene, as we propose (Fig. 4.8).

DIURNAL LIGHT AND TEMPERATURE CYCLES AFFECT PIF3

We show that the reduced elongation response of wild-type seedlings under -DIF is the consequence of a reduced activity of the downstream acting factor PIF3 (Fig. 4.8). We demonstrate that exogenous application of the upstream components ethylene (ACC) and auxin can enhance *PIF3* transcription under -DIF. Similarly, the inhibition of the normal hypocotyl elongation response under +DIF by exogenous application of chemicals which either inhibit ethylene biosynthesis or auxin transport correlates with a reduction in *PIF3* transcription levels (Fig. 4.7E).

When ACC application under -DIF conditions was combined with AVG, *PIF3* expression could be fully restored, whereas NAA application combined with AVG only modestly increased *PIF*3 expression (Fig. 4.7; Supplemental Fig. S4.3). This indicates that the effect of auxin on *PIF3* expression is indirect and requires ethylene production. Furthermore, the *PIF3ox* line is insensitive to NPA, which again places PIF3 downstream of the auxin signal towards elongation (Fig. 4.8). The regulation by (indirect) auxin and (direct) ethylene signalling of *PIF3* expression fits well with the finding that the ethylene signalling component EIN3 acts a regulator of *PIF3* expression. EIN3 was shown to bind to the *PIF3* promoter [198], providing a direct link between ethylene signalling and PIF3 activity. Combined, the results indicate that in wild-type seedlings the auxin-ethylene induced *PIF3* expression is the main determinant of thermoperiodic hypocotyl elongation.

THE CIRCADIAN CONTROL OF RHYTHMIC GROWTH IS DEPENDENT ON BOTH LIGHT AND TEMPERATURE

As both light and temperature are strong entrainment signals for the clock, the altered growth response under -DIF could be the result of an altered clock function under this condition. Both ethylene and auxin biosynthesis and signalling are regulated by the circadian clock [195, 224]. In addition, the circadian clock regulates *PIF4* and *PIF5* expression.

Transcriptional analysis of *PIF4* and *PIF5* under diurnal light conditions (constant temperature) shows that there are strong oscillations in their expression, with highest expression occurring during the light period [75]. However, in combination with the light-induced degradation of PIF proteins by PHYB, the PIF4 and PIF5 proteins reach peak levels during a limited period at dusk, at which time they stimulate maximum elongation [75]. Targets of PIF4 and PIF5 are enriched for genes from the auxin biosynthesis pathway and indeed auxin responses are severely affected in the *pif4 pif5* double mutant [218]. This corresponds well with our hypothesis that the reduced auxin signalling under -DIF is indicative of limited PIF5 activity under this condition. In this work we provide the framework by which auxin and ethylene control thermoperiodic hypocotyl elongation. Future challenge will be to unravel how these components are rhythmically coordinated under diurnal light and temperature conditions.

METHODS

PLANT MATERIAL AND GROWING CONDITIONS

Arabidopsis (*Arabidopsis thaliana*) seeds were either obtained from the Nottingham Arabidopsis Stock Centre (NASC) or from the authors who first described the line. All transgenic lines and mutants are in Col-0 (N1092) background. Lines used in this research are: pACS2::*GUS/GFP* (N31380), pACS4::*GUS* (N31381), pACS5::*GUS* (N31382), pACS6::*GUS/GFP* (N31383), pACS7::*GUS/GFP* (N31384), pACS8::*GUS/GFP* (N31385), pACS9::*GUS/GFP* (N31386), pACS11::*GUS/GFP* (N31387), *ein2-1* (N3071), *eDR5::*LUC [224], *DII::VENUS* [225], *acs2 acs4 acs5 acs6 acs7 acs9 amiRacs8 amiRacs11* [193], *phyB-9* [22], *pif3* (N66042), *pif4* (N66043), *pif5* (N66044), *pif4 pif5* [217], *pif3 pif4 pif5* (N66048), *PIF3ox* [234], *PIF4ox* [75], *PIF5ox* [233], *pPIF3*::*GUS* [198]. All seedlings were surface sterilised and stratified in the dark for 2-3 days (4°C) to synchronise germination and then exposed to treatment conditions for 7 days. All seedlings were grown on petri-dishes (Ø 6 cm) containing 10 ml ½ Murashige-Skoog (MS) enriched plant agar, (0.22 $g \cdot l^{-1}$ MS (Duchefa), 8 $g \cdot l^{-1}$ plant agar (Duchefa) under either +DIF or -DIF conditions as previously described [186].

HYPOCOTYL ELONGATION RESPONSE TO GROWTH REGULATORS

Surface sterilised seeds were grown for 7 days under +DIF or -DIF conditions with different concentrations (0, 0.1, 1, 10, 100 µM) of 1-naphthaleneacetic acid (NAA, Sigma-Aldrich), Indole-3-acetic acid (IAA, Duchefa), 1-N-Naphthylphthalamic acid (NPA, Sigma-Aldrich) Aminoethoxyvinylglycine (AVG, Sigma-Aldrich), 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma-Aldrich).

ANALYSIS OF HYPOCOTYL LENGTH

Seedlings were placed in a horizontal position, photographed (mounted Nikon D90 camera) and hypocotyl lengths were assessed using ImageJ software [235] All seedlings were harvested for further use.

HISTOCHEMICAL BETA-GLUCURONIDASE (GUS) STAINING

Histochemical GUS staining of *pACS2/4/5/6/7/8/9/11*::*GUS* and pPIF3*::GUS* reporter hypocotyls grown under +DIF or -DIF was performed as previously described [206]. Incubation times varied between 10 and 60 minutes but were always identical within experiments. After staining, fixation and clearing individual hypocotyls were imaged.

LUCIFERASE IMAGING AND ANALYSIS

Luciferase imaging and analysis was performed as previously described [236] with the following modifications. Seedlings containing the eDR5::*LUC* reporter construct [224] were grown on 0.5 ml medium in 12 well black plates (Promega). Each well contained 50 seedlings evenly spread out. Seedlings developed in 12 h white light (~150 µmol m^{-2} sec^{-1}), 12 h dark cycles for 4 d with +DIF or -DIF temperature cycles. 24 hours prior to the start of imaging a thin layer of 0.3 ml of D-luciferin (Duchefa) solution (1 mM firefly D-luciferin, sodium-salt, Duchefa, 0.01% Tween 80) was added to the media. Seedlings were assayed for bioluminescence by acquiring images with an exposure time of 15 min using a Pixis 1024B (1024x1024) camera system (Princeton Instruments) equipped with a 35 mm, 1:1.4 Nikkor SLR camera lens (Nikon) fitted with a DT Green filter ring (Image Optics Components Ltd.) to block delayed auto-fluorescence chlorophyll emissions. Images of luciferase activity are depicted with false color scales (blue indicating low activity, red indicating high activity).

MICROSCOPY

For confocal microscopy hypocotyls of 7-day-old seedlings were incubated for 10 min in 1 mM propidium iodide (PI, SIGMA) and imaged on a Zeiss 700 Axio Imager confocal laser scanning microscope (Carl Zeiss) using either EC Plan-Neofluar 40x (Numerical Aperture 1.30) Oil DIC M27 or EC Plan-Neofluar 10x (Numerical Aperture 0.3) Ph1 objective. Samples were excited with 2% of a 488-nm laser (emission from a 30-mW argon tube) for YFP excitation and 2% of a 555-nm laser (emission from a 1-mW helium-neon tube) for PI excitation. Transmission images were simultaneously collected. Single mid-plane optical sections were selected from z series and compared. Image analysis was done using ZEN 2011 and Adobe Photoshop CS2 (Adobe Systems).

For bright field 7-day-old seedlings were imaged on a Zeiss stereo Discovery (A12) with a Plan S 1.0× FWD 81 mm (1-100×) objective. Images were taken with an AxioCam MRc5 (5 MPix camera; Zeiss) and analysed using AxioVision 4.6 software.

AUXIN ANALYSIS

Arabidopsis seedlings grown under either +DIF or -DIF were harvested at mid-day (6 hours, lights on t=0 h) frozen in liquid nitrogen, ground, extracted and analysed for auxin content as previously described [237].

ETHYLENE ANALYSIS

Thirty seedlings were grown at either +DIF or -DIF for 5 days on 2.5 mL MS medium in 10 mL glass vials sealed with micropore tape to allow gas exchange. At the start of the fifth photoperiod, the vials were capped airtight and returned to the experimental conditions. Ethylene was allowed to accumulate in the experimental conditions for 3 days and ethylene concentrations were quantified in 0.5 ml of headspace using Gas Chromatography (HS-GC) with Flame Ionisation Detection (FID).

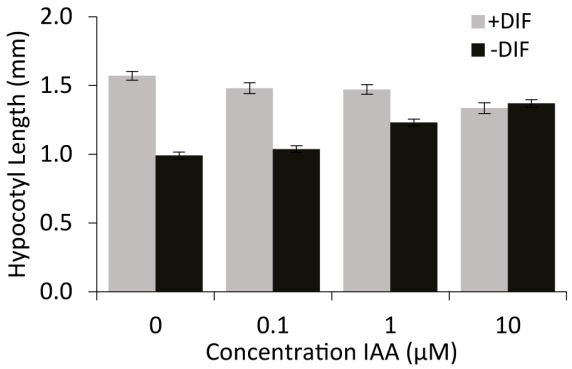
STATISTICAL ANALYSIS

When appropriate, data were subjected to the Student's *t*-test (Microsoft excel). All other data were subjected to one-way analysis of variance (ANOVA). Individual differences were then identified using a post-hoc Tukey test ($p < 0.05$). All analyses were performed using SAS_9.20 (http://www.sas.com/).

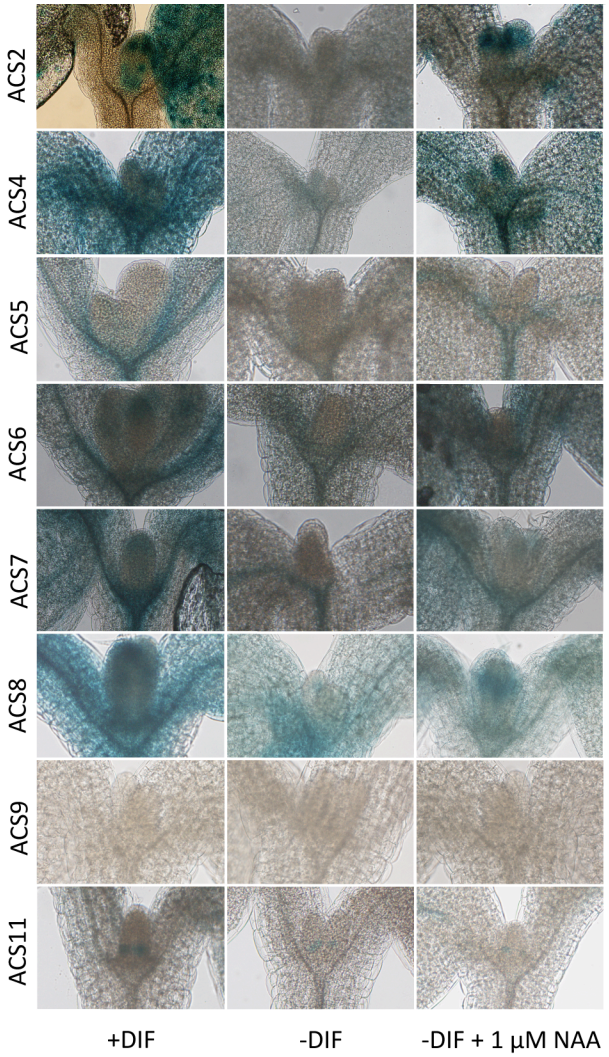
ACKNOWLEDGEMENTS

We thank J. Rienks (Bonn University, Germany), F. Verstappen and T. Charnikhova (Wageningen UR, the Netherlands) for technical support. We are grateful to A. de Montaigu (MPIPZ, Germany) for critical reading of the manuscript.

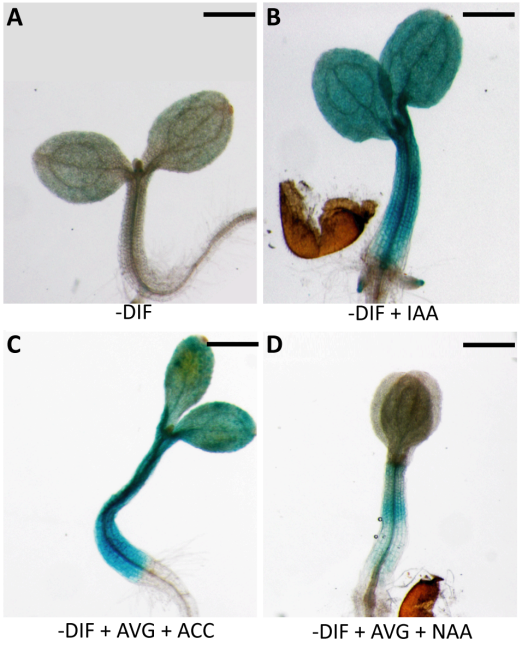
SUPPLEMENTAL DATA



Supplemental Figure S4.1 IAA complements hypocotyl elongation under -DIF conditions. Average hypocotyl length of Col-0 with and without IAA.



Supplemental Figure S4.2 Auxin acts upstream of ethylene signaling. ACS(2,4,5,6,7,8,9,11)::*GUS* promoter activity in the apex of seven day old seedlings under +DIF, -DIF, and -DIF + 1 µM NAA.



Supplemental Figure S4.3 PIF3::*GUS* promoter activity under -DIF. (**A**) -DIF, (**B**) -DIF + 1 µM IAA, (**C**) 2.5 µM AVG + 10 µM AAC, (**D**) 2.5 µM AVG + 1 µM NAA.

On having a jetlag…

“Time is an illusion.”

- Albert Einstein

5

ANTIPHASE LIGHT AND TEMPERATURE CYCLES ALTER THE CIRCADIAN CLOCK, CAUSING CARBOHYDRATE STARVATION AND REDUCED GROWTH IN ARABIDOPSIS: THE ARABIDOPSIS 'JETLAG'

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IN PREPARATION

ABSTRACT

Diurnal light cycles entrain the circadian clock, allowing plants to orchestrate growth processes with the light environment. In addition, coinciding diurnal temperature cycles also affect plant growth. Plant elongation is stimulated by increasing positive differences between day and night temperatures (+DIF). In contrast, a negative temperature difference (-DIF) reduces elongation and leaf movement. The objective of this study is to understand the role of the circadian oscillator in the response of plants to (antiphase) light and temperature cycles. Using luciferase reporters we analysed clock gene transcription in Arabidopsis under +DIF and -DIF. Under +DIF, robust circadian rhythms were observed, while under -DIF the amplitude and phase of *CCA1*, *LHY*, *PRR9*, *GI,* and *TOC1* expression changed. The altered clock gene expression under -DIF affects circadian regulated growth processes, such as starch metabolism. Under +DIF, nocturnal starch degradation rates were adjusted to the anticipated dawn. Under -DIF, however, this coordination was lost. Starch was prematurely mobilised during the photoperiod, resulting in carbohydrate starvation. In accordance with this, the phase-shift in leaf movement of wild-type plants in response to -DIF was phenocopied by starch mutants (*pgm*, *sex1*) grown under +DIF. Previously, GI was shown to be involved in temperature compensation of the clock. We show that under -DIF, g*i* mutants retain optimal growth and maintain robust clock activity, suggesting that misregulation of GI is responsible for the growth reduction under -DIF. Combined, this indicates that reduced circadian robustness under -DIF results in inefficient coordination of diurnal growth processes, resulting in reduced elongation growth.

INTRODUCTION

Optimal plant growth requires the coordination of growth processes with diurnal fluctuations in the environment [74]. Previously, it was shown that the sequential transcription of genes involved in key steps of cell elongation and other growth processes are orchestrated in time and coordinated with the diurnal light cycle [72, 75]. In addition, growth related rhythmic leaf movement under a natural combination of both light and temperature cycles (in phase: warm day, cold night: +DIF) correlates with rhythmic ethylene production, while antiphase light and temperature cycles (cold day, warm night: -DIF) alter the robustness and phase of leaf growth and amplitude of ethylene emission [81]. Thus, the rhythmic activity of growth and growth related processes is determined by a complex interaction of endogenous signals and environmental cues. Likely, the plant circadian clock plays a central role in orchestrating the phase of growth processes to allow optimal growth under natural environmental conditions.

To study the circadian clock, mostly constant conditions are used (e.g. continuous light and temperature), after appropriate entrainment to light or temperature cycles [63, 238]. In Arabidopsis these studies have resulted in the identification of core clock components and a

detailed understanding of the 'mechanics' of the Arabidopsis circadian clock [239]. The intrinsic rhythmic output of the clock, with a period of about 24-hours, is generated by multiple interlocking feedback loops of oscillating expression of core clock genes [240]. The central loop contains the morning-expressed *MYB* transcription factors *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1)* and *LATE HYPOCOTYL ELONGATION* (*LHY*) that negatively regulate the evening-expressed *TIMING OF CAB EXPRESSION 1* (*TOC1)*, which in turn represses *CCA1* and *LHY*. This central loop interlocks with the so called 'morning loop' which includes the *PSEUDO-RESPONSE REGULATORS PRR5*, *PRR7* and *PRR9* that repress the expression of *CCA1* and *LHY*. In addition, the central loop also interacts with an 'evening' loop, containing *GIGANTEA* (*GI*) and *ZEITLUPE* (*ZTL*), which interact on a protein level to suppress TOC1 [58, 59] (for recent reviews see [62, 239]). The circadian clock controls various important aspects of plant growth such as hormone signalling [195, 224], photosynthesis [73], carbohydrate metabolism [241], and leaf movement [160, 161]. However, to ensure optimal coordination between these processes and the environment, the clock requires environmental input signals, that allow the synchronisation of the circadian oscillator with the 24-hour cycle of the earth's rotation. Therefore, clock controlled processes are continuously adjusted to remain synchronous with the environment, a process referred to as entrainment [63].

Light is a principal entrainment signal (*zeitgeber*) for plants, and is perceived through the phytochrome and cryptochrome families of photoreceptors [13]. For most clock controlled growth processes only the response to light entrainment has been studied [160, 161, 165, 242, 243].

Nevertheless, in addition to light, temperature transitions are also strong "*zeitgebers",* and temperature cycles with a minimum difference of 4°C are sufficient to entrain circadian processes in plants [244]. It was previously reported that *prr7* and *prr9* are redundantly functioning in temperature entrainment. In *prr7 prr9* double mutants, circadian processes cannot be entrained to thermo cycles when grown in constant light but they respond similar to wild-type when entrained with light cycles under continuous temperature [65].

In addition to entrainment by light and temperature, another characteristic of the circadian clock is that its rhythmic output remains relatively constant across a range of ambient temperatures. This requires an active temperature compensation mechanism, as temperature intrinsically affects the speed of (bio)chemical processes [245].

A quantitative genetic approach showed that the evening loop clock components GI [69] and ZTL play a role in temperature compensation in Arabidopsis [68, 69]. Recently also a specific role for the 'morning' feedback loop of the circadian clock in temperature compensation in Arabidopsis was demonstrated by studying entrainment of the clock at 12°C and 30°C. It was shown that *prr7 prr9* double mutants overcompensate for a change in temperature, possibly by hyper-activation of CCA1 and LHY [246]. Despite growing knowledge on the architecture of the Arabidopsis circadian clock, the genetic and molecular basis of temperature entrainment and temperature compensation remain poorly understood [67]. Moreover, how the plant deals with temperature effects on clock entrainment, which at the

same time may conflict with effects of temperature on the compensation mechanism, remains an enigma [247].

Plant growth adapts to the light environment and under diurnal light cycles, the clock ensures optimal coordination of a plethora of rhythmic growth related processes, varying from gene expression of transcription factors to phytohormone signalling [75, 195, 224], both of which are involved in the regulation of hypocotyl elongation [165] and leaf movement [248]. In addition, light entrainment was shown to determine dawn anticipation and regulates starch degradation rates [241]. The regulation of starch metabolism is important for maintaining growth at different times of the diurnal day. During the photoperiod, sugars from photosynthesis are used for growth but also partly stored as starch. This starch is subsequently degraded during the night to provide sugars for nocturnal growth. The rate of nocturnal starch degradation is controlled by the circadian clock, which is essential for an accurate anticipation of dawn [241].

Remarkably, only few studies have examined the activity of the circadian clock itself under more natural combinations of both diurnal light and temperature cycles. Studies in Drosophila show that combined light and temperature cycles generate more robust circadian rhythms than either light or temperature entrainment alone [249]. Similarly, Arabidopsis growth under phased light and temperature cycles (+DIF) increased, compared to growth under diurnal light without temperatures cycling (0 DIF) [80].

Moreover, when light and temperature are artificially presented in antiphase, i.e. cold days in combination with warm nights (-DIF), the elongation growth of various plant species is strongly reduced [77, 78, 80, 81]. Arabidopsis plants grown under -DIF (DT/NT 12°C/22°C), display a reduction in leaf elongation from 20% to 40% (depending on the study) compared with the control (DT/NT 22°C/12°C) [80, 81].

As both light and temperature entrain the clock [63], we postulate that antiphase light and temperature cycles alter the coordination of circadian clock controlled processes by differently affecting individual clock components. Some clock components, or circadian output processes, may be more susceptible to light entrainment while others may be more responsive to temperature entrainment. For instance, under -DIF the phase of diurnal ethylene emission in sorghum (*Sorghum bicolor*) is preferentially determined by temperature rather than light, as ethylene emissions always peaked during the warm period, regardless of the photoperiod [202]. Previous research showed that the phase of circadian output gene *CAB2* expression is preferentially entrained by light, whereas the phase of output gene *CAT3* expression is more sensitive to entrainment by temperature [203]. Such preferential response of circadian output genes to temperature over light was also demonstrated for the cold responsive gene *CCR2* and was shown to vary within natural Arabidopsis accessions [247].

While most of these studies recorded the response upon transfer to continuous conditions after diurnal light or temperature entrainment, here we specifically focused on quantifying the activity of the clock and growth related output processes in plants during diurnal light and temperature conditions.

To this aim we semi-constantly quantified the expression of different clock components and output genes under diurnal light and temperature cycles using luciferase (LUC) reporters [250]. For this a custom-build set-up that enabled LUC measurements under +DIF or -DIF conditions was developed. Results showed that compared with robust rhythmic promoter activities under +DIF, all circadian genes tested were differentially altered by -DIF. In addition, *GI* was identified as an essential component for the response of plants to -DIF. We propose that the altered expression of clock genes alters the 'timekeeping ability' of the clock under -DIF, resulting in a less efficient coordination of clock regulated output processes essential for optimal growth.

RESULTS

-DIF CONDITIONS ALTER CIRCADIAN LEAF MOVEMENT

Previously we demonstrated that the phase of diurnal leaf movement for plants grown under -DIF is delayed compared with plants grown under +DIF. The upward diurnal leaf movement is determined by the temperature rather than the light cycle, a phenotype which is easily visible at the end of the photoperiod of both conditions (Supplemental Fig. S5.1A-B) [81]. Arabidopsis cotyledon or seedling primary leaf oscillations under constant light and temperature are an easy to measure, yet reliable output of the circadian clock [71]. Therefore, circadian cotyledon movement has been used extensively to identify clock mutants e.g. [160] and characterise physiological effects on the clock [160, 243]. To demonstrate that leaf movement of mature Arabidopsis leaves is also under control of the clock, we imaged rosette leaf movement during the switch from +DIF or -DIF to constant conditions. Hereto, three week-old plants were imaged under +DIF or -DIF conditions for three days and the conditions were subsequently switched to constant light and constant temperature (22°C) at dawn (t=0 h) while imaging continued. Under +DIF and -DIF the leaf movement adapted to the new conditions and rhythmicity persisted after the switch to constant conditions (Fig. 5.1A). Leaf movement was analysed using OSCILLATOR [186], which provides projected leaf movement (Supplemental Fig. S5.1B), from which amplitude (Supplemental Fig. S5.1C-D) and phase (peak amplitude relative to t=0 h) (Supplemental Fig. S5.1C, E) are quantified [81, 186]. Upon the switch to constant conditions the amplitude of leaf movement of +DIF entrained plants was instantly reduced, while for plants entrained to -DIF the amplitude initially remained similar (Fig. 5.1B). Moreover, the phase of leaf oscillations for +DIF entrained plants shifted from t=16-18 h under +DIF to t=19-22 h under free running conditions (Fig. 5.1C). In contrast, for plants entrained to -DIF the phase decreased, and shifted from t=21 h to about t=4-6 h under free running conditions, but only after a 32 h period of no peak, (effectively 'skipping' one 24-hour cycle) (Fig. 5.1A, C). Remarkably, when observed diurnally, leaf movement peaks during the warm period (night) under -DIF, and during the cold period (night) under +DIF (Fig. 5.1A). But when released into constant conditions, leaf movement of

both +DIF and -DIF entrained leaves peaks during the subjective cold period (Fig. 5.1A, C). Combined, these results indicate that for the circadian phase of leaf movement of rosette plants, temperature entrainment is dominant over light entrainment.

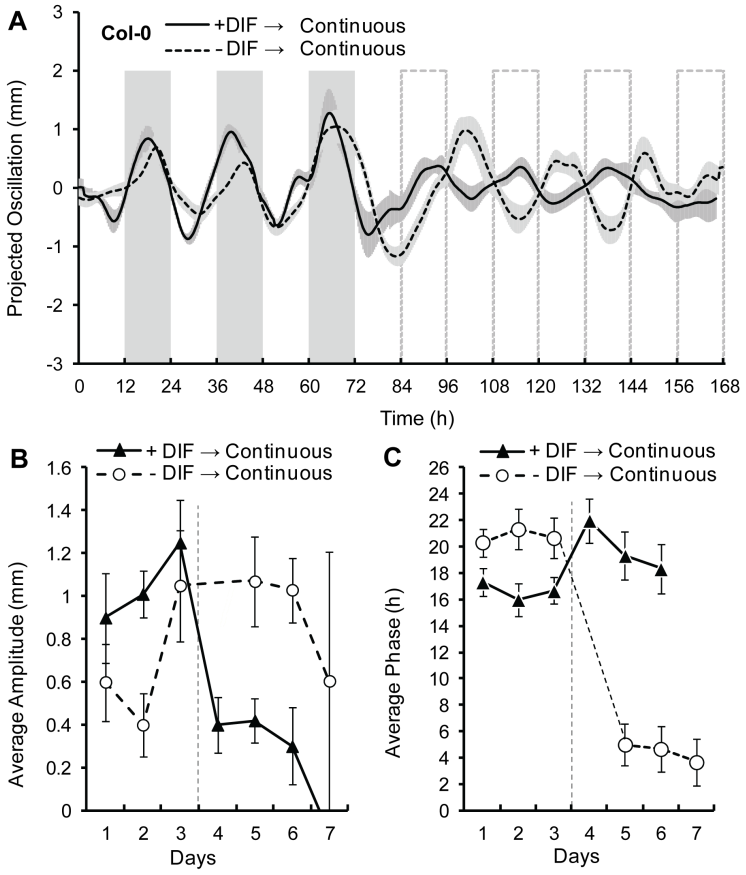


Figure 5.1 -DIF alters circadian leaf movement parameters. (**A**) Projected oscillations of developing leaves switched to continuous conditions after 72 h of control (+DIF, solid line) or -DIF (dashed line) entrainment of 4 weeks old Arabidopsis (Col-0) rosette plants pregrown under +DIF. Solid grey boxes indicate night period, dotted boxes indicate subjective night. Error bars represent SE, n=8. (**B**) Average amplitudes calculated from the projected oscillations of leaves switched to continuous conditions after 72 h of control (+DIF, solid) or -DIF (dashed) entrainment. Horizontal dotted line indicates this switch to constant conditions. Error bars represent SE; n=8. (**C**) The average phase of leaves switched to continuous conditions after 72 h of control (+DIF, solid line) or -DIF (dashed line) entrainment. Horizontal dotted lines indicates the switch to constant conditions. Error bars represent SE; n=8. (B-C): note that from (A) the seventh phase for +DIF could not be detected and that in -DIF no phase occurs at day 4, which is replaced by a connecting dotted line in both (B) and (C).

-DIF CONDITIONS DIFFERENTIALLY ALTER THE DIURNAL EXPRESSION PATTERNS OF CIRCADIAN CLOCK GENES

The altered phase output of clock regulated leaf movement and other clock regulated processes such as ethylene emission under -DIF [81, 202], suggests that the expression of clock genes themselves may be altered under -DIF compared with +DIF. We therefore set out

to compare clock gene expression under +DIF and -DIF conditions using clock luciferase reporter plants [240, 251, 252]. Previously, the LUC reporter system has been successfully employed to analyse clock gene activity in Arabidopsis seedlings under temperature cycles in constant light or in light cycles under constant temperature [65]. In contrast, we monitored LUC luminescence driven by different clock promoters in mature rosette plants under combined light and temperature cycles (+DIF and -DIF). For this a novel custom-build set-up was developed that allowed monitoring of luminescence under different diurnal light and temperature conditions (see methods). Because diurnal fluctuations in light and temperature may affect *in vivo* luminescence, independent of promoter activity, we first tested how +DIF and -DIF conditions affected *in vivo* LUC activity when driven by the constitutive 35S promoter (35S::*LUC+*) [251] (Supplemental Fig. S5.2). Bioluminescence was imaged every hour (10 min exposure) on three week old plants transferred to the luminometer setup after three days of adjustment to +DIF or -DIF conditions in growth cabinets. Bioluminescence under +DIF and -DIF was measured for four subsequent days in the rosette plants. The *in vivo* LUC activity was affected by both temperature and light, and displayed a minor but distinguishable rhythmic trend under both +DIF and -DIF (Supplemental Fig. S5.2A). Similar effects of light cycles on LUC activity have been reported for Arabidopsis 35S::*LUC+* seedlings [251]. The average bioluminescence in response to the different light and temperature combinations was compared and the overall effects on LUC activity were minor (Supplemental Fig. S5.2B).

Subsequently, LUC activity in Arabidopsis CCA1::*LUC+,* CCR2::*LUC+* [253], TOC1::*LUC+* [240], and LHY::*LUC+*, GI::*LUC+*, PRR9::*LUC+* [251] reporter plants was monitored under +DIF and -DIF conditions. Remarkably, the activity of each of the tested clock genes was differently affected by -DIF compared with +DIF conditions (Fig. 5.2A-F). For all reporters measured (except *TOC1*), the peak amplitude and thus rhythmicity of bioluminescence was strongly reduced under -DIF. The TOC1::*LUC+* activity initially showed higher amplitudes at day two and three under -DIF, but at day four of imaging under -DIF, the amplitude of TOC1::*LUC+* activity collapsed (Fig. 5.2B). The phase of the rhythmic clock gene activity under -DIF is difficult to determine as the unsmoothed pattern of LUC activity we (purposely) present shows transient increases at the light and temperature transitions, which was reported before in response to light [68, 251] and is also observable in 35S::*LUC+* activity under +DIF or -DIF conditions (Supplemental Fig. S5.2B). These small transition peaks are thus likely a promoter, independent of light or temperature response artefact. However, we note that the changes at the light/temperature transitions are much more pronounced for *TOC1* and *GI* promoter activity, indicating that these genes may have a direct response to changing light/temperature conditions (Fig. 5.2B-C). Although complicated to determine, when compared to +DIF the phase of CCA1::*LUC+* appears to show a small delay under -DIF (Fig. 5.2A). In contrast, compared with +DIF the phase of GI::*LUC+* (Fig. 5.2C) and PRR9::*LUC+* (Fig. 5.2D) seemed slightly advanced under -DIF, hinting that in addition to the strong effects on amplitude, there could be a different phase shift for individual clock genes. Remarkably, the CCR2::*LUC+* activity displays two peaks under +DIF, one during the day and one at night (Fig.

5.2E). Since both of these peaks occur in the middle of the day or the night period, where no changes in light or temperature occur, it can be assumed that these peaks relate to genuine *CCR2* promoter activity. Combined, the strongly reduced amplitudes of *CCA1*, *GI*, and *PRR9* expression, possibly in combination with small but differential phase shifts of the clock genes under -DIF conditions likely results in reduced clock robustness. This may explain the altered coordination of circadian output processes such as leaf movement [81], ethylene emission [81, 202], and flowering time [80] previously reported to occur under -DIF.

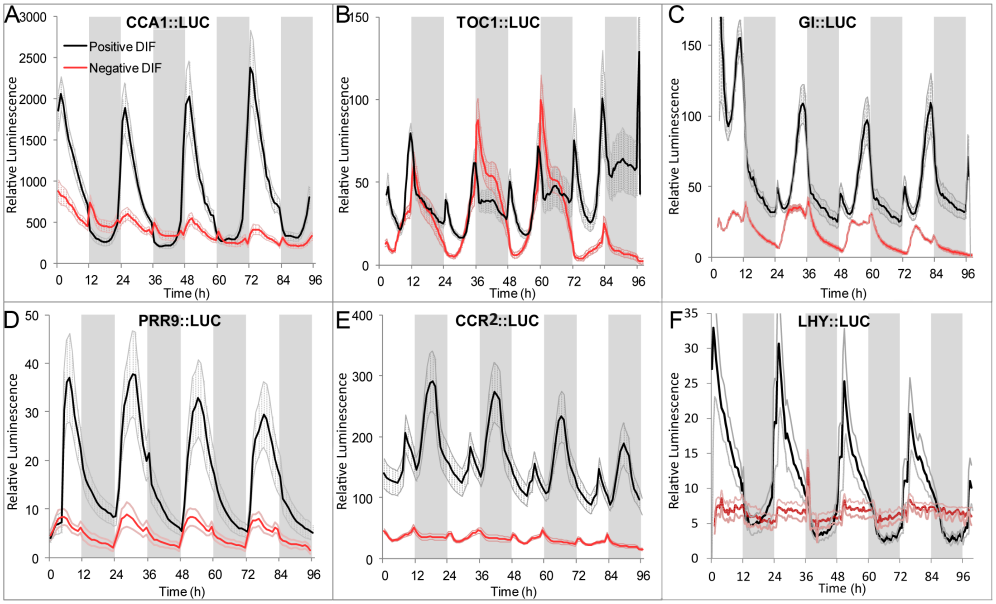


Figure 5.2 -DIF alters rhythmic activities of circadian clock genes. (**A-F**) Average Luciferase bioluminescence of *CCA1* (**A**), *TOC1* (**B**), *GI* (**C**), *PRR9* (**D**), *CCR2* (**E**), and *LHY* (**F**) *LUC+* reporters under -DIF (red line) and +DIF (black line). Plants were pregrown for 3 weeks under +DIF, and then pretreated with 3 days of +DIF or -DIF conditions after which bioluminescence was imaged for 4 subsequent days under either +DIF or -DIF diurnal conditions (depicted here). Grey boxes indicate the dark period. Error bars represent SE; n=8.

GIGANTEA MEDIATES THE SENSITIVITY TO THE GROWTH INHIBITING EFFECTS OF -DIF

In previous work, the analysis of a series of mutants under -DIF conditions identified genes that are involved in the growth response of plants to -DIF. In one such screen the photoreceptor PHYTOCHROME B (PHYB) was identified and the elongation of *phyB-1* mutants was only minimally affected by -DIF compared with wild-type [80]. Recently we showed that PHYB affects the phase of the growth related leaf movement and compensates for the partial phase shift in leaf movement induced by -DIF, which resulted in fully temperature entrained leaf movement in *phyB-9* [81]. To determine whether some of the clock components play a role in either robustness or phase shift in the clock output under -DIF, we screened selected clock mutants for insensitivity to -DIF. Previously it was shown that *PRR7* and *PRR9* are redundantly involved in temperature entrainment of the circadian oscillator [65]. Similarly, *GI* was shown to be involved in temperature compensation of the Arabidopsis clock [68]. Therefore, leaf growth parameters of specifically *ppr7 prr9 (prr7/9)* double mutants [65] and two independent *GI* loss-of-function mutants, *gi-100* [254] and *gi-11* [68], were analysed under +DIF and -DIF conditions. Under -DIF, *prr7/9* displayed the same leaf growth movement as the Col-2 wild-type (Supplemental Fig. S5.3). However, both *gi-100* and *gi-11* were insensitive to the effects of -DIF. Both *gi* loss-of-function mutants maintained strong elongation growth (Supplemental Fig. S5.4A-B) and displayed stronger leaf movement amplitudes

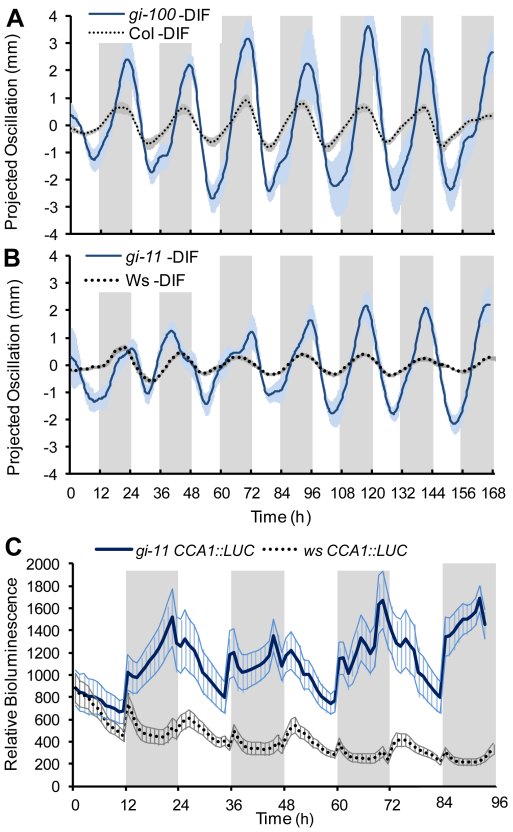


Figure 5.3 Loss of GIGANTEA results in insensitivity to the effects of -DIF on rhythmic growth and circadian rhythms. (**A**) Projected oscillations for *gi-100* (solid blue) and Col-0 (dotted black) leaves developing under -DIF conditions. Error bars represent SE; n=8. Grey boxes indicate the dark period. (**B**) Projected oscillations for *gi-11* (solid blue) and Ws (dotted black) leaves developing under -DIF conditions. Error bars represent SE; n=8. Grey boxes indicate the dark period. (**C**) Average CCA1::*LUC+* Bioluminescence in *gi-11* (blue) or Col-0 (black) plants. Plants were pregrown for 3 weeks under +DIF, and then pretreated with 4 days of +DIF or -DIF conditions after which bioluminescence was imaged for four subsequent days under either +DIF or -DIF conditions. Error bars represent SE; n=8. Grey boxes indicate the dark period.

under -DIF compared with their respective wild-types, Col and Ws (Fig. 5.3A-B). This demonstrates that *GI* loss-of-function results in insensitivity to the growth inhibiting effects of -DIF.

To determine the effect of *GI* under -DIF on clock function, the activity of the CCA1::*LUC+* promoter was examined in the *gi-11* background. Indeed, the rhythmic *CCA1* expression was less reduced in the *gi-11* mutant background compared with the wild-type, and maintained strong diurnal oscillations under -DIF (Fig. 5.3C). Combined, this indicates that GI is essential for changes in the robustness of clock gene expression and the maintenance of diurnal growth in response to -DIF.

DIURNAL STARCH DEGRADATION RATES ARE AFFECTED BY -DIF

We next set out to further investigate the link between altered clock function and the reduced leaf growth movement under -DIF. Diurnal growth is sustained by carbohydrates produced during the day by photosynthesis, and during the night from starch degradation [255]. The role of starch metabolism in growth has been extensively studied [256], and it was demonstrated that the clock is important for the coordination of starch degradation in relation to the anticipated photoperiod [241]. Because -DIF alters the regulation of clock controlled processes, it was of interest to investigate the effect of -DIF on starch metabolism. Altered clock function could result in less efficient coordination of starch degradation rates with the anticipated dawn, resulting in carbohydrate starvation. In order to monitor the carbohydrate starvation state of plants, a carbohydrate starvation inducible promoter coupled to luciferase (carbohydrate starvation reporter) was used [241]. In plants grown under control conditions, the carbohydrate starvation reporter does not show strong *LUC* bioluminescence, but when grown under -DIF, activity of the starvation reporter was induced with a pronounced peak at dawn (Fig. 5.4A). Bioluminescence was most pronounced in the shoot meristem region, the petiole, and the leaf midvein (Fig 5.4B). To confirm that activation of the starvation induced reporter gene indeed results from altered starch metabolism, starch levels were quantified in plants growing under control and -DIF conditions as previously described [257]. Even though this quantification method proved sensitive to saturation and therefore may lead to underestimation of the starch levels at the end of the day, a clear trend in the starch content in leaves was detected. In plants exposed to -DIF, starch levels already started to decline eight hours after the start of the photoperiod, while under +DIF the relative starch levels started to decline after dusk, reaching the lowest levels just before dawn (Fig. 5.4C) as previously described for optimal conditions [256]. Combined, the results indicate that circadian controlled starch degradation [241] is altered under -DIF, and less well coordinated with the photoperiod, resulting in carbohydrate starvation peaking at dawn.

STARCH MOBILISATION AFFECTS GROWTH RELATED DIURNAL LEAF MOVEMENT

So far we showed that -DIF alters the phase of leaf movement [81] and induces carbohydrate starvation (Fig. 5.4A-B). To determine whether the carbohydrate starvation relates to the altered phase of leaf movement under -DIF conditions we analysed leaf growth movement parameters in two starch degradation mutants; *starch excess 1* (*sex1*), which is unable to degrade starch [258] and *phosphoglucomutase* (*pgm*), which is considered to contain severely reduced starch levels [259]. Both the *sex1* and *pgm* mutant leaves display a delayed phase compared to wild-type leaves under +DIF (Fig. 5.5A). This results in a leaf movement phase that is similar to the phase of wild-type plants under -DIF (Fig. 5.5B-C). In wild-type the late phase under -DIF results from a 5 h phase shift compared with +DIF [81]. This indicates that starch degradation is indeed important for a correct timing of growth related leaf movement. Changes in the circadian coordination of starch mobilisation, as induced by -DIF, are likely responsible for the phase shifts in leaf movement in -DIF compared to +DIF.

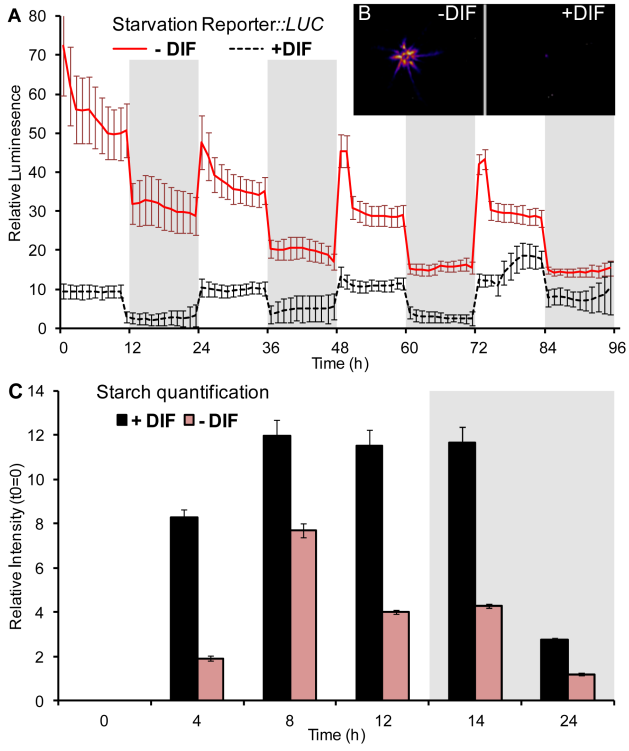


Figure 5.4 Starch metabolism is altered under -DIF. (**A**) Plants transformed with the carbohydrate starvation reporter (pAt1g10070::*LUC*) construct were grown under +DIF for 3 weeks. At this point plants were either kept 3 more days in +DIF, or transferred to -DIF for 3 days. After this period the plants were imaged for *LUC* bioluminescence under either +DIF or -DIF conditions, form the start the photoperiod (t=0 h) on, for 4 subsequent days. Values are means of measurements on 8 individual rosettes. Error bars are SE. (**B**) Representative images of pAt1g10070::*LUC* starvation reporter bioluminescence in rosette plants after 3 days of -DIF (left) or +DIF (right) conditions at the start of the photoperiod (t=0 in A). (**C**) Rosette plants were grown under +DIF for 3 weeks. After this period half of the plants were transferred to -DIF conditions and the other half were kept +DIF conditions. Aboveground rosettes tissue for individual plants were harvested at t=0 h (before transfer), 4, 6, 12, 14, and 24 hours after the start of the photoperiod. Starch was extracted and quantified using lugol staining and spectrophotometry. Values are expressed as relative intensities by pairwise normalising individual measurements with the individual colour density recorded at t=0 h (untreated control). Grey box indicates night. SE are depicted as error bars; n=16.

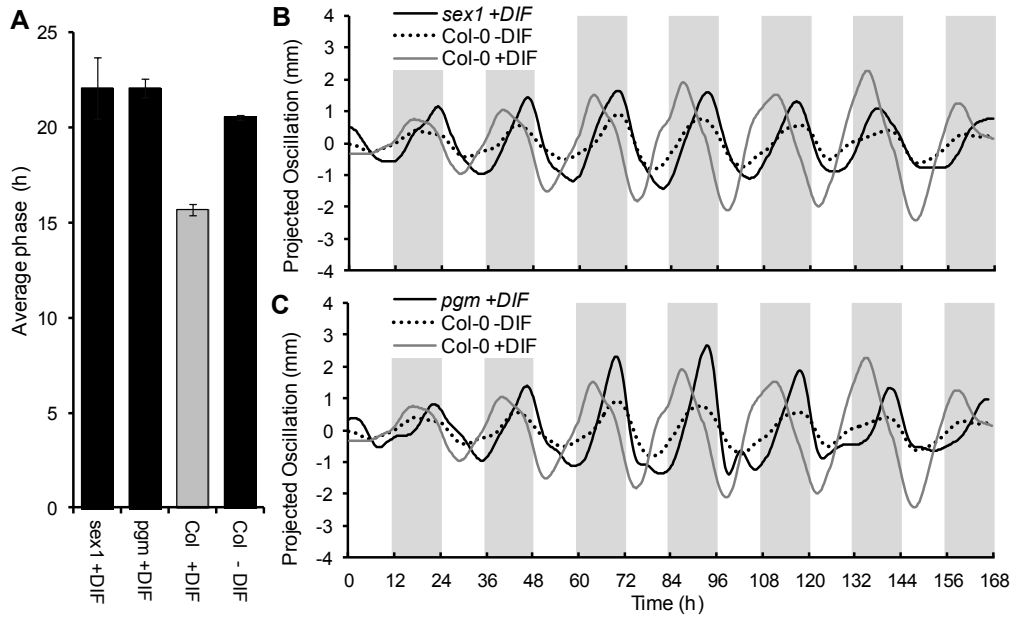


Figure 5.5 *sex1* and *pgm* starch mutants phenocopy the late phase of -DIF treated plants. (**A**) Averaged phase of leaf movement oscillations of starch mutants *sex1* and *pgm* grown under +DIF compared to Col-0 plants grown either under +DIF or -DIF. Error bars represent SE; n=8. (**B**-**C**) Projected leaf oscillations of *sex1* (B) and *pgm* (C) under +DIF compared to Col-0 plants grown under +DIF or -DIF. SE are omitted for clarity but vary between -1.38 and 2.65; n=8. Grey boxes indicate the dark period.

DISCUSSION

Remarkably few studies have examined the functioning of the Arabidopsis circadian clock under combined light and temperature cycles. In this study, we demonstrate that the phase coordination of light and temperature cycles (+DIF or -DIF) directly affects the endogenous circadian clock, and thereby alters the phase and amplitude of a number of clock regulated growth processes.

NATURAL (+DIF) LIGHT AND TEMPERATURE CYCLES ENHANCE CIRCADIAN ROBUSTNESS

The coordination of growth processes by the circadian clock, which allows anticipation of the day (and night) length, provides an adaptive advantage: plants of which the circadian period was matched with the period of the light cycles in which they grew, displayed increased growth, photosynthesis, and survival compared to plants with circadian periods differing from their environment [73]. In addition to light, temperature is another common environmental signal, and both signals are *zeitgebers*, entraining the circadian clock of many organisms [56]. Interestingly, light entrained Arabidopsis plants maintained a different period length of the circadian output gene *CCR2* than temperature entrained plants [247]. In

addition, the magnitude of this response depended on natural variation, illustrating that the temperature sensitivity of the Arabidopsis circadian clock may adapt to the local environment [247]. In our experiments, the period of both clock genes and output processes under -DIF always remained 24 hours. The effects of -DIF on growth are therefore not related to an altered period length. Leaf movement amplitudes can be considered as a proxy of the robustness of circadian oscillations. When leaf movement amplitude is compared between +DIF and continuous light and temperature conditions (compare Supplemental Fig. S5.1B with Fig. 5.1A day 4-7), it is clearly observable that the amplitudes under +DIF are much higher, indicating increased circadian robustness under this condition. We propose that the presence of synchronous light and temperature cycles results in increased clock robustness. This correlates with optimal growth, as in Arabidopsis elongation increases with increasing positive day temperature difference compared with constant temperature [80]. In Drosophila (*Drosophila melanogaster*), the transcript levels of multiple clock components (e.g. *period*, *timeless*, *clock,* and *vrille*) in neurons of adult flies were quantified under either light or temperature cycles or a combination of both cycles. Indeed, when light and temperature were synergistically combined (+DIF), the RNA oscillations became much more robust compared with separate light or temperature cycles, as indicated by increased amplitudes [260]. Combined, these and our results indicate that in both Arabidopsis and Drosophila the natural combination of both light and temperature cycles synergistically enhances the robustness of circadian rhythms and diurnal output processes, providing a clear adaptive advantage.

THE EXCEPTIONAL BEHAVIOUR OF TOC1 UNDER -DIF

The most pronounced effect of -DIF on circadian clock activity was the strong reduction in the amplitude of all tested genes (except *TOC1*) compared with +DIF (Fig. 5.2). The reduced activity of *CCA1* early in the morning under -DIF does not coincide with a simultaneous altered activity of *TOC1.* This is unexpected as a mutual negative feedback loop exists between *CCA1/LHY* and TOC1 protein, which is the core of current clock models [58]. The decreased *CCA1/LHY* expression under -DIF could be considered to mimic *cca1 lhy* mutants. Interestingly, red light is able to induce a biphasic pattern in *TOC1* expression, similar to what we observe under +DIF, and the clock mutant *cca1-1 lhy12* maintains wild-type red light induced expression of *TOC1* [261]. In our assays a direct light response of TOC1::*LUC+* is observed, both under +DIF and -DIF (Fig. 5.2). A potential combination of circadian regulation and acute (red)light induction could explain why *TOC1* amplitudes remain high under -DIF. In addition, there could be other factors that specifically stabilise TOC1 protein under -DIF. Recently ABA levels were shown to positively and specifically regulate TOC1 [58, 262]. Under -DIF, ethylene signalling was reduced which resulted in reduced leaf movement amplitudes [81]. ABA was shown to antagonise ethylene induced upward leaf movement [164]. Therefore, it is possible that ABA levels are enhanced by -DIF. It will be a future

challenge to determine whether direct light induction, increased ABA levels or another factor is responsible for the relative robustness of TOC1 under -DIF. The other way around, the stable expression of TOC1 explain the reduced expression of the other clock genes, as the TOC1 protein acts as a transcriptional repressor and antagonises *LHY*, *CCA1*, *PRR9*, and an evening complex of which GI is a part [58, 263].

TEMPERATURE ENTRAINMENT VERSUS TEMPERATURE COMPENSATION

In Arabidopsis, *PRR7* and *PRR9* were shown to be important for temperature entrainment of the clock [65]. However, under -DIF the *prr7/9* double mutant showed a similar phase shift and amplitude-reduction of leaf movement as the wild-type (Supplemental Fig. S5.3A-B). We thus conclude that, while *PPR7* and *PPR9* are redundantly required for temperature entrainment of the clock, they are dispensable for the growth response to -DIF.

In Arabidopsis the clock component *GI* has been shown to play an important role in temperature compensation. A mechanisms in which *GI* maintains circadian rhythmicity and accuracy at higher temperatures by regulating TOC1 was proposed [68]. Our results show that GI expression under diurnal light and temperature conditions is sensitive to how light and temperature are combined as *GI* expression is reduced under -DIF compared to +DIF (Fig. 5.2C). It is striking that *gi-11* and *gi-100* are insensitive to the growth retarding effects of -DIF (Fig. 5.3A, B). *gi-11* surprisingly has higher amplitudes of CCA1::*LUC+* compared to wild-type under -DIF (Fig. 5.3C). This indicates that despite decreased expression under -DIF, GI protein is still fulfilling an active role in the -DIF response.

Moreover, GI was shown to be involved in PHYB dependent light signalling [254], and GI is required for maintaining robust amplitudes of clock genes through its effect on light entrainment [264]. While previously we have shown that *PHYB* is important for compensating for phase shifts occurring upon the change from +DIF to -DIF, *GI* seems to be responsible for the compensation of circadian amplitudes upon transfer from +DIF to -DIF. As previous effects of GI in temperature compensation were measured under continuous light [68], it remains to be examined if GI dependent temperature compensation is determined by the temperature in combination with the light cycle. Perhaps such a light and temperature depended interaction could result from its potential interaction with activated PHYB in the nucleus [254].

ALTERED CLOCK FUNCTION UNDER -DIF AFFECTS THE CIRCADIAN COORDINATION OF GROWTH

Our hypothesis was that the reduced growth under -DIF is caused by an altered coordination of growth processes with the environment. This should be most pronounced in the effect of -DIF on the phase of clock regulated processes. Although the phase of clock gene expression under -DIF is sometimes difficult to determine due to low amplitudes and sometimes shows dual peak activity, it remains possible that the phase of the examined clock

genes shifts differentially in response to -DIF (Fig. 5.2). This could suggest that the effect of -DIF on the phase is not the same for all individual clock components. This trend is much clearer in clock regulated output processes. The phase of leaf movement shifted backwards under -DIF but this shift was absent in constitutive ethylene signalling mutants. In addition ethylene emission shifted forward under -DIF, hinting at a disturbance in the temporal coordination between these two output processes [81]. Clearly, different clock controlled processes are not affected the same by light and temperature cycles. After entrainment with -DIF, *CAT3* (evening gene) and *CAB2* (morning gene) shifted differently when measured under constant conditions. The phase of the CAB3 was more responsive to the temperature entrainment, while the phase of CAB2 responded preferentially to light entrainment [203]. Similarly, the start of starch degradation, if swiftly followed by maximal leaf angles in the early night under +DIF, again hints at coordination between these processes, which becomes altered in response to -DIF.

Similar to Arabidopsis, zebrafish are also sensitive to both light and temperature entrainment of their circadian activity, under subsequent constant conditions. However, in contrast to Arabidopsis growth, when zebrafish are exposed to -DIF their circadian activity remains preferentially synchronised to the light cycles [265]. This indicates that in this species, for this process, light is a dominant signal that is able to overwrite temperature input.

We conclude that selective sensitivity of output processes to light and temperature cycles is key to the differential shifts of output processes under -DIF. This is a different conclusion then the existence of two different circadian oscillators which differ in their sensitivity to light and temperature as previously proposed [203]. Combined, our results show that the synchronous integration of both light and temperature signals by the circadian clock is essential for coordinating different growth processes. Under -DIF, this coordination is lost, explaining the reduced growth under -DIF, which was first observed by Went in 1944 [76].

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS

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 are in this research are: Col-0 (N1092), Col-2 (N28170), CCA1::*LUC+* (N9965), TOC1::*LUC+* (N9960), GI::*LUC+* N9961 [266], PPR9::*LUC+* (N9962) [266], CCR2::*LUC+* (N9808), 35S::*LUC+* (N9966), pAt1g10070::*LUC* [241], *pgm1-1* (N210), *sex1-1*, (N3093), *gi-100* [254], *gi-11* [68], *prr7 prr9* [65]. *gi-11* mutants carrying the CCA1::*LUC+* reporter and the Ws wild-type were a gift from Andrew Millar. The growth conditions were as previously described [186].

LUCIFERASE IMAGING IN ROSETTE PLANTS AND LUC ACTIVITY ANALYSIS

Luciferase imaging and analysis was performed as previously described [236] with the

following modifications. Wild-type and plants containing LUC reporter constructs were allowed to develop in +DIF conditions in climate cabinets for 3 weeks at +DIF as described [81] with the exception that the plant were grown on (pre-washed) 5 cm x 5 cm x 5 cm Rockwool blocks (Grodan), watered weekly with 0.25 Hoagland solution [205] and the irrigation mats after 1.5 weeks before watering. Plants were randomised after 10 days at the start of the photoperiod in the growth cabinet. For comparing +DIF and -DIF the plants were imaged separately but were always of the same age, which required two batches with 4 days difference between germination. The separate batches were labelled and grown randomly distributed but in parallel in the same climate room. Three weeks after germination, 8 reporter plants and 1 wild-type without LUC construct (for background correction) were placed in a custom made grassed surface black plastic box containing 9 (3x3) smaller individual square boxes (8 cm x 8 cm x 8 cm each). This block, containing the 9 plants in the individual smaller blocks, was placed in either +DIF conditions or -DIF conditions for 3 days and the individual Rockwool blocks were soaked in 4 ml of D-luciferin (Duchefa) solution (0.5 mM firefly D-luciferin, sodium-salt, Duchefa, 0.01% Tween20 dissolved in 0.5 times Hoagland solution). Twenty-four hours prior to the start of imaging the plants were again supplied with 4 mL of 0.5 mM Luciferin (prepared as above, except the Hoagland solution was replaced with dH_2O). Before the next dawn the box containing the 9 plants was covered with aluminium foil and was quickly placed into a custom build climate box for bioluminescence imaging under +DIF or -DIF. This box was constructed from Styrofoam glued to an aluminium frame fitted with the exhausts of two coolers (Mobicool) and one incubator heater (Stuart scientific). All devices were controlled by timer thermostats (Conrad), which allowed maintaining a stable ambient temperature of 12°C or 22°C (± 1°C) in the box. The temperature ramp for heating and cooling the box, 10°C was 60 minutes (0.33°C min^{-1}) and divided into 30 min before and 30 min after lights on/off. This climate controlled box was equipped with a transparent lid fitted with a insulated opening to accommodate the luminometer camera lens. Approximately 70 µmol m^{-2} sec^{-1} of light at the leaf surface was provided from 2 multicolour LED arrays (15W E27) emitting solely in the red, blue and orange (6:3:1) light spectrum (Kweekotheek.nl) which were suspended above the transparent lid. During imaging, the relative humidity was kept constant at an approximate average of 60% (v/v) throughout the cycles. The lights and camera were synchronised with a custom made macro using Metamorph 6.2 software. Lights switched off two min before dark imaging to eliminate background LED afterglow. At the start of the photoperiod the plants were assayed for bioluminescence by acquiring images every 1 or 2 h with exposure times of 10 min. Imaging was done with a air-cooled CCD chip (-70°C) Pixis 1024B camera system (Princeston Instruments) equipped with a 35 mm, 1:1.4 Nikkor SLR camera lens (Nikon) fitted with a DT Green filter ring (Image Optics Components Ltd.) optimised to block any delayed chlorophyll fluorescence. Of the whole camera set up, only the optical end of the lens was present inside the climate box. The constant cooling of the cameras CCD chip resulted in heat exchange and maintained the camera body at a steady 27°C which was conducted to the lens, preventing

lens condensation. This novel setup allowed the maintenance of 12 h light periods in combination with 12°C/22°C or 22°C/12°C temperature cycles throughout the four days of imaging, identical to the cycles present in the commercial growth cabinets (Weiss) used in all other experiments [81]. Images of luciferase activity in plants are depicted with false colour scales (blue indicating low activity, red indicating high activity). Relative luminescence was analysed for each plant in each image as mean gray value using ROI manager with ImageJ software. Image background subtraction was performed for all luciferase-imaging experiments. Each experiment was repeated 3 times with comparable results.

MEASUREMENT OF LEAF MOVEMENT PARAMETERS AND LENGTH

Measurement of leaf movement parameters and leaf lengths under +DIF and -DIF were preformed as previously described [81, 186] with the following modifications: for analysis of leaf movement parameters under continuous conditions 3 week old rosette plants were imaged using OSCILLATOR [186] for 3 days under +DIF or -DIF conditions starting at the start of the photoperiod (t=0 h) in parallel in two separate growth chambers (Weiss). At the start of the 4^{th} photoperiod (t=72 h) the imaging growth chambers were switched to continuous light (same quality and intensity) and continuous temperature (22°C), while parallel imaging continued. All image data was further processed and quantified as previously described [186].

STARCH QUANTIFICATION

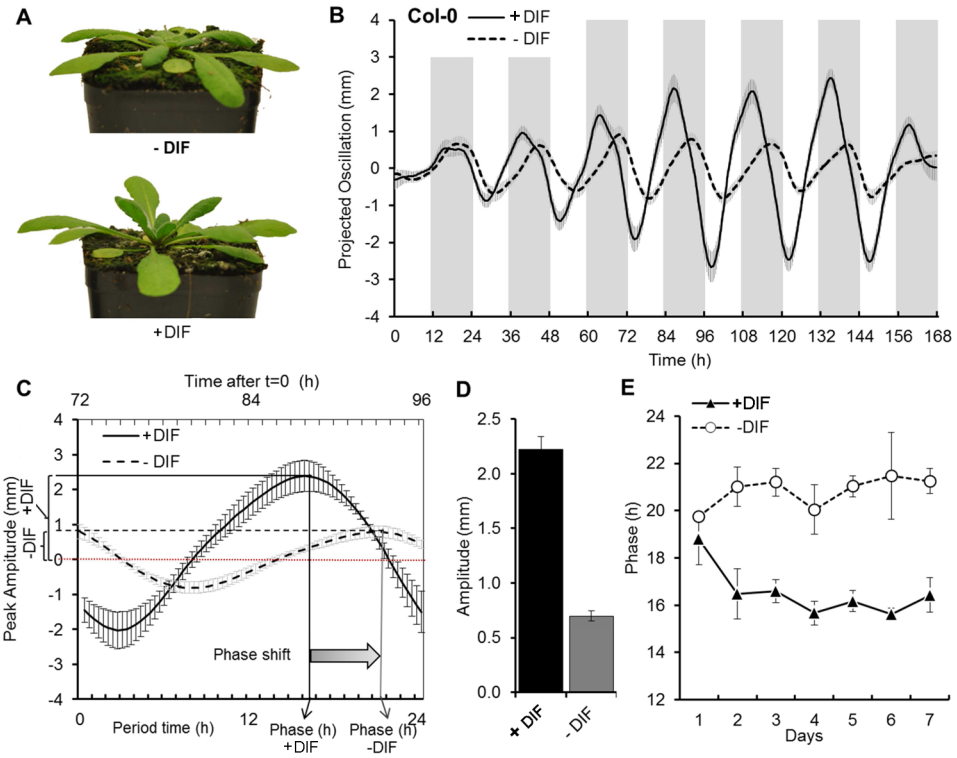
Starch content was quantified as previously described [257], with the following modifications: three week old +DIF grown plants were randomised in +DIF conditions and then the next morning randomly transferred to either +DIF or -DIF conditions. Subsequently whole rosette (green)tissue was harvested in a time course and immediately frozen in liquid nitrogen. The material was ground in liquid nitrogen with a ball mill (MM 400; Retch) and 100 mg of each homogenised sample was exhaustively extracted (5x) with 1 ml 80% aq. ethanol Extraction Solvent (ES) in 2 ml Eppendorf tubes in an ultrasonic bath (Branson Ultrasonic) at 50°C for 15 min. The ES insoluble residue was spun down at 10.000 RPM and the ES carefully decanted. Next the ES insoluble residue was vacuum-dried in a Speed Vac (Speed Vacuum Savant, Thermo scientific). The water starch in the resulting pellets was solubilised in boiling water for 15 min in 2.5 mL Eppendorf tubes with punctured lids to release pressure. The tubes were weighted before and after boiling, the volumes were readjusted to the start weight with dH_2O to account for water evaporation during solubilisation. The resulting solution was vortexted and centrifuged at 10.000 RPM at 22°C for 10 min. The supernatant was carefully removed with a pipet (Eppendorf) and reacted with lugol solution (Sigma-Aldrich) in 0.1% (v/v) ratio by mixing (frequent pipetting) in a plastic cuvette (Bio-Rad) to a final volume of 1 mL. The resulting (blue) colour density for each individual plant was recorded after 1 min incubation at A_{620} using a spectrophotometer (Thermo Scientific) for

each individual plant. This experiment was repeated twice with similar outcomes.

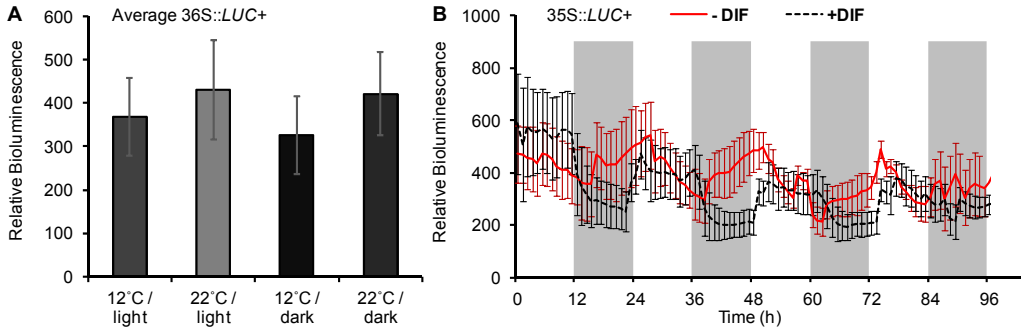
ACKNOWLEDGEMENTS

We are grateful to A. Pokhilko and A. Millar for advice on experimental procedures and for kindly donating seeds of the *gi-11* CCA1::*LUC* luciferase reporter. We acknowledge funding by the Top Technological Institute Green Genetics (TTI-GG, grand 2CFL009RP to AvdK) and the Dutch science foundation NWO (ALW to AvdK) for the development of the diurnal luminometer system.

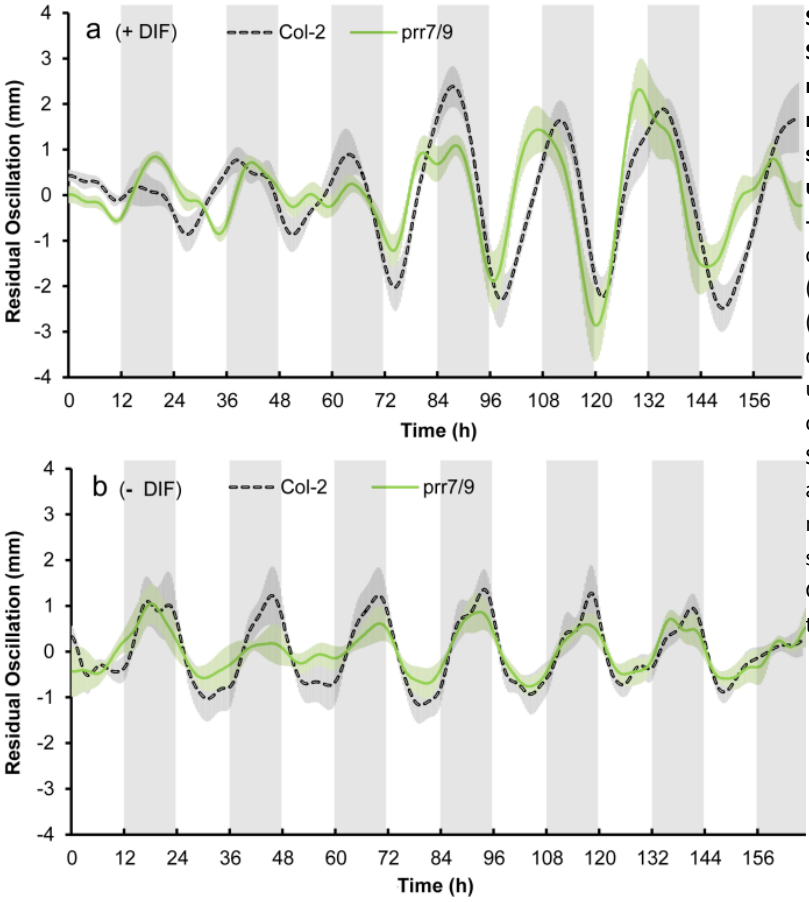
SUPPLEMENTAL DATA



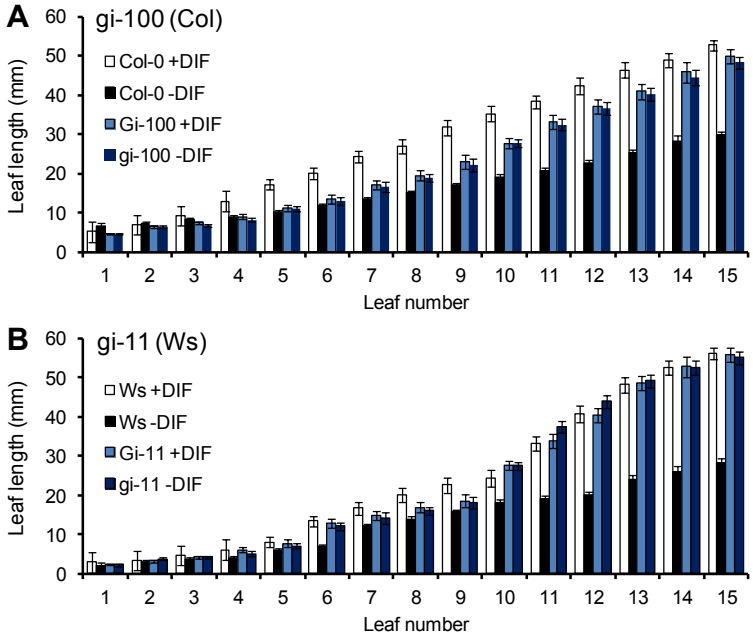
Supplemental Figure S5.1 Rhythmic growth and movement of leaves under control and -DIF conditions. (**A**) Thirty-six day old Arabidopsis (Col-0) rosette plants exposed to -DIF (top) or control (+DIF) (bottom) conditions as photographed at the end of the fourth photoperiod. (**B**) Projected oscillations for leaves developing 7 days under control (solid) or -DIF (dashed) conditions. Standard errors (SE) are depicted per 20 min time-point as shading, n=8 leaves. Grey boxes indicate the dark period. (**C**) Close-up of (B) for day 4, depicting an example of amplitude, phase and phase shift for control leaves compared to -DIF. Error bars represent SE; n=8. (**D**) Average amplitudes of day 2-6 calculated from the projected oscillations (B) for leaves developing under control (black) or -DIF (grey) conditions. Error bars represent SE; n=8. (**E**) The phase of oscillations for leaves developing under -DIF conditions (dashed, white circles) shifts compared to control (solid, black triangles). Error bars represent SE; n=8. Adapted with permission from [81].



Supplemental Figure S5.2 Effect of light and temperature on 35S::*LUC+* bioluminescence. (**A**) Bioluminescence of 35S::*LUC+* reporter was measured every hour during different combinations of light and temperature and averaged over 12 hour periods. Error bars represent SE; n=8. (**B**) Plants were pregrown for 3 weeks under +DIF, and then pretreated with 4 days of +DIF or -DIF conditions after which bioluminescence was imaged for four subsequent days under either -DIF (red line) +DIF (dotted black line) diurnal conditions. Error bars represent SE; n=8. Grey boxes indicate the dark period.



Supplemental Figure S5.3 Rhythmic leaf movement of *prr7/9* mutants responds similar to wild-type under both + DIF and -DIF. (**A**-**B**) Projected oscillations for *prr7/9* (Solid green) or col-2 (dotted black) leaves developing 7 days under control (A) or -DIF (B) conditions. Standard Errors (SE) are depicted per 20 min time-point as shading, n=8 leaves. Grey boxes indicate the dark period.



Supplemental Figure S5.4 Leaf elongation growth of *gi* mutants are insensitive to the effects of -DIF compared to their wild-type. (**A**-**B**) Leaf lengths (mm) of leaves emerged and developed during 10 days of +DIF (light) or -DIF (dark) conditions for Col-0 and *gi-00* (A) and *Ws* and *gi-11* (B) plants pregrown at +DIF. Leaf age increases from left to right. Error bars represent SE; n=10

On finishing this thesis…

“Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis, we ourselves are a part of the mystery that we are trying to solve.”

- Max Planck

6

GENERAL DISCUSSION

AS THE EARTH TURNS THINGS HEAT UP

The earth spins on its axis with a period of approximately 24 hours, creating diurnal cycles of sunlight. When sunlight hits an object, it increases the temperature of the object, a process known as thermal radiation [267]. Thus, sunrise is usually accompanied by an increase in temperature and sunset by a decrease in temperature. Even though in nature, diurnal light and temperature cycles (predominantly) occur in phase, there are separate signalling pathways for light and temperature in plants [9], which may seem somewhat redundant. Based on the results obtained in this thesis, it is proposed that one adaptive advantage of these apparently redundant signalling pathways (and thus their conservation through evolution) in part arises from the synergistic effects between light and temperature signalling under daily diurnal conditions. In addition, the adaptive responses to extreme heat [10, 268] and extreme cold [269] are also important factors, undoubtedly contributing to the conservation of both light and temperature signalling pathways in Arabidopsis (*Arabidopsis thaliana*), and in plants in general [270].

In this thesis we investigated plant growth in response to combinations of diurnal light and temperature cycles. When the positive difference between the day and night temperature increases (+DIF), this has a positive effect on plant growth and development. In contrast, when light and temperature cycles are applied in antiphase (-DIF), this leads to an inhibition of elongation in many plant species [76, 79, 80].
To investigate the cause of this difference in plant growth responses between +DIF and -DIF, two main topics were studied:

1) The physiological and genetic regulation of diurnal growth.
2) The role of the circadian clock under the diurnal conditions.

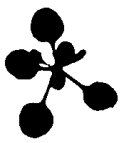
As a general approach, for both processes we quantified how they are affected by -DIF (as experimental treatment), and compared this response to +DIF (the control conditions). The final discussion is therefore centred around the main question: how do these two topics combine: how do the observed changes in the Arabidopsis circadian clock relate to the observed changes in rhythmic growth in response to -DIF, compared to their behaviour under +DIF.

LIGHT AND TEMPERATURE INCREASE CIRCADIAN CLOCK ROBUSTNESS

Circadian clocks are widespread in living organisms and allow for the coordination of physiological and developmental processes with the environment [56]. Many clock components have been identified, and in all organisms that posses circadian oscillators these components interconnect into more or less intricate regulatory feedback loops. Combined, these loops create a steady oscillating output with a period of approximately 24 hours [56]. By combining mathematical modelling with the wealth of experimental data on clock

mutants, the core molecular mechanism underlying the Arabidopsis circadian clock is well understood [59]. However, new clock components and complexes [271] as well as novel entrainment loops and feedback signals [262, 272] are still being discovered. These discoveries are constantly being integrated into improved mathematical models of the clock [58, 59, 263]. The core of the Arabidopsis clock is composed of a set of transcriptional feedback loops. Reduced to its simplest form, the clock consists of a morning, a central, and an evening feedback loop [60]. For the clock to retain its rhythmic activity it needs to be entrained by environmental signals, termed *zeitgeber.* This allows for a continuous synchronisation of the clock with the 24 h cycle of the earth's rotation. In addition it allows development to adjust to the changing photoperiods throughout the seasons. Light and temperature are both capable of entraining the circadian clock of many organisms [63].

By analysing the leaf movement of Arabidopsis rosette leaves, as one of the outputs of the clock, with the OSCILLATOR system (chapter 2), we could demonstrate that leaf movement amplitudes are more robust under +DIF than under constant light and temperature or under -DIF (chapter 4,5). However, based on the data presented in chapter 4, we could not directly claim that combined light and temperature cycles affect the clock itself. To test whether the interaction between light and temperature cycles increases clock robustness we needed to quantify the expression of clock genes under both +DIF and -DIF. To this aim we developed a novel setup that allowed quantification of clock promoter driven LUCIFERASE (LUC) activity in mature rosette plants growing under diurnal conditions of combined light and temperature cycles. This setup enabled the (semi-)continuous monitoring and quantification of LUC clock reporter activity under +DIF and -DIF. Indeed these measurements confirmed that the clock itself was altered, and seemed less robust, under -DIF compared with +DIF. The strongest indications for this claim are the decreased amplitudes of most clock genes (chapter 5) in -DIF exposed plants. What these measurements did not show was the difference between clock gene expression under combined light and temperature (our data) versus continuous conditions, temperature cycles only, or light cycles only (of which the latter is frequently used in growth chambers). CCA1::*LUC* amplitudes measured under light cycles and temperature cycles only are included in the study of Salome and colleagues (2005) [65]. But in this research the bioluminescence was measured in seedlings instead of mature rosette plants and unfortunately bioluminescence data were only shown as normalised values [65], which complicates a direct comparison with our data. An absolute comparison between in phase light and temperature cycles, i.e. +DIF (light dark & hot cold, LDHC), continuous conditions (LLHH), only light cycles (LDHH) or only temperature cycles (LLHC) can be extracted from a collection of Arabidopsis time course microarray experiments (performed with by various groups) using the DIURNAL tool [273]. Data for these different conditions are present in this database, and can be easily compared. One limitation is that these data are only available for seedlings. This can be a limitation because certain processes, such as the regulation of flower induction and starch metabolism are controlled by the circadian clock [241, 274], but do not play a prominent role in seedlings. Nevertheless, as an



example, we compared the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) expression in seedlings under all conditions mentioned above. The results obtained from this DIURNAL analysis do indeed show that amplitudes and thus likely clock robustness dramatically increase with +DIF, indicating that the most common occurring natural environment gives the strongest clock output (Fig. 6.1).

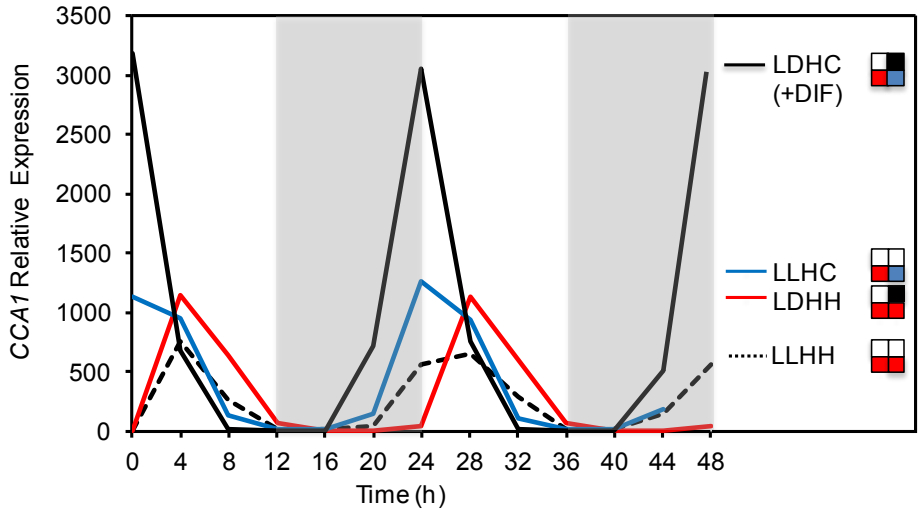


Figure 6.1 CCA1 amplitudes in response to various conditions. Normalised expression levels of *CCA1* measured every 4 hours in seedlings grown in continuous conditions (dashed line, LLHH), only light cycles (red line, LDHH), only temperature cycles (blue line, LLHC) and combined light and temperature cycles (black line, LDHC, i.e. +DIF). Two full diurnal days are depicted. Grey bars indicate the dark period. Square pictograms illustrate the experimental conditions: A full square represents a 24h diurnal day and is divided into four 12h sub-squares. The top sub-square indicates the photo- (white) and dark (black) period. The bottom sub-squares indicate the synchronous temperature conditions; 22°C (red) and 12°C (blue).

Furthermore, there is no difference in amplitude of *CCA1* clock gene expression under light cycles at constant temperature (LDHH, 22°C) compared with the amplitude of *CCA1* clock gene expression under temperature cycles under constant light conditions (LLHC; 12°C-22°C), indicating that in this respect both signals are of equal strength in entraining the clock. However, while individual light and temperature signals provide a similar time cue to the clock, and in this way may be viewed as redundant in circadian entrainment, when combined, they increase the amplitude of clock gene expression. A major contribution to the field would be the complementation of DIURNAL with time course gene expression data obtained from seedlings grown under -DIF as well as acquiring expression data for rosette plants.

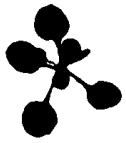
THE EVOLUTIONARY CONSERVATION OF INTEGRATING BOTH LIGHT AND TEMPERATURE CYCLES

Similar to Arabidopsis, also in Drosophila (*Drosophila melanogaster*), combining the two *zeitgebers*, results in more robust transcripts of clock components and clock output processes [249, 260]. In fact, both light and temperature entrainment are evolutionarily conserved in plants [63], insects [249], and fish [265]. However, with the evolution of obligate warm blooded organisms, which maintain a stable body temperature independent of the environment (homeothermy) [275], the importance of temperature entrainment is expected to be lost. Indeed monkeys and marmosets exposed to temperature cycles in continuous light, display random circadian locomoter activity rhythms [276, 277]. The lack of temperature entrainment in these homeotherms indicates that they may have become solely light dependent. However, the body temperature of homeothermic animals still shows minor diurnal fluctuations, and when cultured mammalian cells are exposed to only these minor temperature fluctuation in constant light, they do sustain rhythmic gene expression [278]. Thus, throughout evolution, temperature entrainment seems to be conserved.

WHAT HAPPENS TO THE ARABIDOPSIS CIRCADIAN CLOCK UNDER -DIF?

If the combined effect of light and temperature (+DIF) enhances clock function, then the opposite condition - antiphase light and temperature (-DIF) - may alter the circadian clock of Arabidopsis. Indeed, clock gene amplitudes were drastically altered in response to -DIF (Fig 5.2). The phase of most clock genes shifted slightly but in a gene specific manner, indicating that the effect of -DIF is different for the different genes that constitute the clock. In addition, a strong reduction in the amplitudes of the clock genes - *CCA1*, LATE ELONGATED HYPOCOTYL (LHY), *GIGANTEA* (*GI*) and *PSEUDORESPONSE REGULATOR9* (*PRR9*) - was observed. Only the amplitude of TIMING OF CAB EXPRESSION 1 (TOC1) remained relatively high under -DIF. This demonstrates a strongly altered functioning of the Arabidopsis circadian clock under -DIF conditions compared with +DIF. By extrapolating known feedback loops in the Arabidopsis circadian clock, the changes observed in the components measured under -DIF can be integrated in a simplified graphical model and compared with +DIF (Fig. 6.2). It is important to note that this model only contains the components we either directly measured under +DIF and -DIF or discuss in this chapter. For a complete model of clock interactions see [58].

In this model, TOC1 is proposed to be a strong repressor of CCA1, LHY, PRR5/7/9 (Fig 6.2B), and an evening complex that includes GI [58, 59, 263]. A repressor function for TOC1, combined with increased *TOC1* expression under -DIF, explains the reduced expression of *CCA1*, *LHY*, *PRR9* and *GI* observed under -DIF (Fig. 6.2B). GI also plays a key role in the -DIF response as *gi* mutants maintain strong leaf movement and increased *CCA1* expression amplitudes under -DIF. Remarkably *GI* expression itself was reduced under -DIF (Fig 6.2) but apparently GI is still active in a pathway suppressing clock amplitudes. The role of *GI* in temperature compensation of the amplitudes of other clock genes was proposed to depend on temperature dependent regulation of TOC1 [68]. GI plays a role in the post-transcriptional



regulation of the clock as it stabilises (ZEITLUPE) ZTL. Blue-light-enhanced binding of GI to ZTL results in *TOC1* expression rhythms with high amplitudes [279]. Thus light and temperature interconnect with GI and ZTL to control TOC1 (Fig 6.2). Furthermore, GI is a component of the PHYTOCHROMEB (PHYB) signalling pathway and based on the interaction between GI and PHYB in the nucleus [254] we postulate that GI acts as a light regulated thermo-sensor. Under -DIF, the mismatch between temperature and light (indicated by the grey lightning bolts in Fig. 6.2B) could result in GI dependent stabilisation of TOC1 and subsequent antagonism of other clock genes. The consequences of alterations in such conceptual models are hard to predict without mathematical calculations, and more input parameters may be needed for constructing a predictive mathematical model of +DIF and -DIF. For instance the component ZTL was not analysed in this thesis but judging from the changes that occur during -DIF, it is not unlikely that ZTL is essential for the -DIF response. GI stabilises ZTL in a protein complex and this interaction determines TOC1 stability [279]. To have a better predictive power and allow the generation of testable hypotheses it would be essential to incorporate the altered behaviour of the clock genes that we determined into existing mathematical models.

WHAT HAPPENS TO ARABIDOPSIS GROWTH UNDER -DIF?

There are numerous processes in plants that retain rhythmic activity even in the absence of environmental input (under continuous conditions), which implies that they are (partially) under control of the endogenous circadian clock [75, 195, 224, 241, 280]. These processes are not part of the circadian oscillator itself, but are part of the rhythmic output of the clock. Analysis of clock regulated genes demonstrated, for example, that the obligate sequential steps required for cell elongation [281] are orchestrated by the circadian clock [72]. This study provided the basis for the theory that the coordination between different output processes by the circadian clock regulates plant growth and development in relation to the environment. The next important step was the integration of the effect of diurnal light cycles on clock output. Expression of the growth promoting transcription factors *PHYTOCHROME INTERACTING FACTORs PIF4* and *PIF5* is regulated by the clock in such a way that their transcript levels peak during the day [75, 220]. Yet their degradation by light activated PHYB results in reduced protein levels during the photoperiod [216]. Under short days, the combination of circadian expression and light degradation creates a small window of PIF protein presence at the end of the night/early dusk corresponding with maximal hypocotyl elongation at that time of the day [75]. Although only the interaction of these output processes with light was investigated, it is the first example of how the integration of clock regulated output processes with the environment determines rhythmic plant growth.

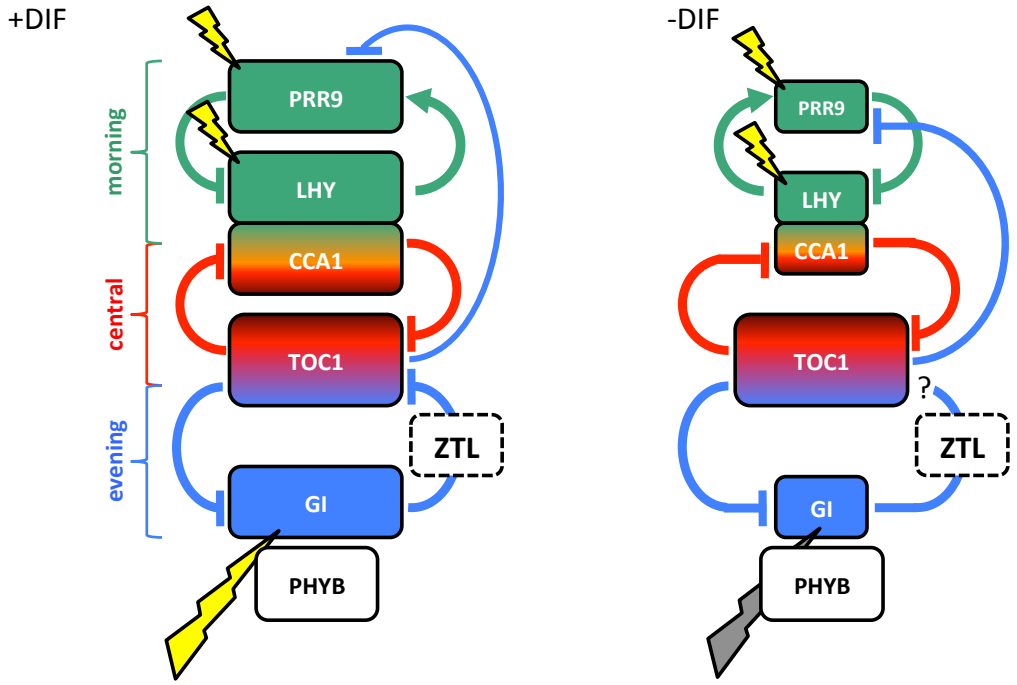


Figure 6.2 Graphical model illustrating the changes observed between -DIF and +DIF for the measured clock genes, including their reported (protein) interactions. These interactions are based on [58]. The amplitudes of the clock components are represented by the size of the boxes. Boxes with solid lines depict components studied here, while boxes with dashed lines represent components not analysed in this thesis. The colours represent the morning loop (green), central loop (red), and evening loop (blue) activity. Known light input is indicated with lightning bolts.

-DIF INHIBITS GROWTH DURING THE PHOTOPERIOD

In this thesis we showed that growth of Arabidopsis leaves under -DIF is mainly inhibited during the day. This reduction in growth is due to decreased ethylene signalling and changes in local ethylene production (chapter 3). Despite the importance of this observation, in chapter 3 we only provide evidence on the inhibiting effect of -DIF on leaf growth during the day. To further underpin these results, and allow for more general conclusions, I have also analysed diurnal inflorescence elongation -using a modified OSCILLATOR setup- under +DIF, -DIF and under -DIF in the presence of ethephon (Fig. 6.3). Indeed inflorescence elongation ceased during the cold day, a response that could be partly overcome by treatment with ethephon (chemical ethylene precursor) (Fig. 6.3). This allows us to conclude that the -DIF inhibition of growth during the day is a general phenomenon and that ethylene is the limiting factor for growth under -DIF.

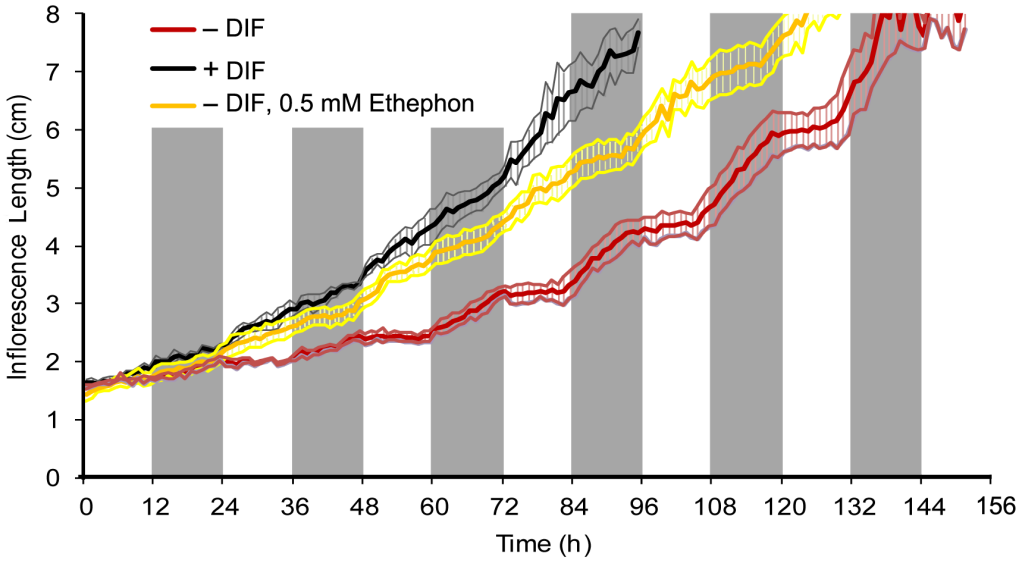


Figure 6.3. Diurnal Arabidopsis inflorescence elongation. Plants bolting under +DIF were transferred to +DIF (black) or -DIF (red) conditions and imaged with OSCILLATOR. Inflorescence elongation under -DIF was inhibited during the photoperiod, while nocturnal elongation rates remained similar to +DIF. The -DIF growth inhibition is partly restored to +DIF levels by spraying the bolting rosette at t=0 with 0.5 mM ethephon (chemical ethylene precursor) (yellow). Grey bars indicate the dark period. Error bars represent SE, n=5.

THE PIFS AS MOLECULAR GLUE BETWEEN THE CLOCK, THE ENVIRONMENT, AND GROWTH

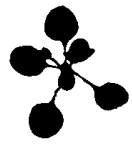
In chapter 4 we demonstrated that in response to -DIF, auxin levels decrease in seedlings during the day. This was previously also shown for inflorescence stems [80]. By using an auxin input eDR5::*LUC* reporter [224] and the auxin output sensor DII-venus [225], we could now pinpoint tissue specific limitations in auxin signalling (chapter 4). In addition we identified auxin to be one of the regulators of ethylene signalling under +DIF and -DIF, confirming previous reports of the interaction between auxin and ethylene in the control of various growth processes [221]. This showed that the limiting effect of ethylene for growth under -DIF, as concluded in chapter 3, is actually caused by limiting auxin production. Moreover, we linked this hormonal signalling cascade with *PIF* genes, as important components for controlling cell elongation in response to day and night temperature differences. Previously PIF4 was shown to be involved in high temperature induced upregulation of auxin biosynthesis to stimulate hypocotyl elongation [49]. Remarkably, PIF4 over-expressors (PIF4OX) responded to -DIF similar as wild-type, excluding an essential role of PIF4 in the -DIF response (Fig. 4.7) Perhaps PIF4 inducible auxin biosynthetic pathway genes, such as *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA1*) [282] and the cytochrome P450 *CYP79B2* that converts tryptophan to indole-3-acetaldoxime [283], are specifically suppressed by -DIF. This would be in line with the strongly reduced auxin levels

and signalling in -DIF grown seedlings (Fig. 4.4). Furthermore both *TAA1* and *CYP79B2* are upregulated during shade avoidance (SA) and this correlates with SA elongation [282]. It is very likely that -DIF suppresses SA as it inhibits elongation also in dense crop canopies. In addition, *phyB-1* and *phyB-9* are described as having a constitutive SA phenotype [284] and are both insensitive to -DIF [81, 86].

In contrast to PIF4 we could place PIF5 upstream of the auxin induced ethylene signalling cascade which becomes limiting under -DIF. PIF5 induces *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), an auxin responsive gene, which is involved in photoperiodic growth [227]. In line with an increased auxin response, PIF5OX plants constitutively overproduce ethylene as a result of activation of *ACC SYNTHASEs* (*ACSs*) [233]. Because mutants are insensitive to auxin inhibitors and PIF5OX plants are insensitive to -DIF (chapter 4) we putatively placed PIF5 upstream of auxin (chapter 4). An alternative explanation could be that PIF5 circumvents auxin biosynthesis and directly activate *ACS* expression. In that case there must also be feedback from ethylene signalling to auxin response, as was indeed shown to take place in Arabidopsis root elongation [215]. It seems PIF5 remains functional under -DIF when sufficient protein levels accumulate (OX, *phyB*). This could be experimentally investigated by measuring auxin levels in *phyB-9* and in PIF5OX plants made ethylene insensitive by introducing *ein2-1.* Either way, the auxin-ethylene interaction ultimately activates *PIF3* transcription, which is limiting cell elongation under -DIF (chapter 4). Of the PIFs mentioned so far, only PIF3 lacks a role in stimulating elongation during the night [198]. In contrast, PIF4 and PIF5 are highly abundant regulators of nocturnal hypocotyl elongation [285, 286]. This supports the conclusion that limiting PIF3 expression results in reduced cell elongation during the -DIF light period (chapter 4).

ETHYLENE AND THE TIMING OF LEAF MOVEMENT

Leaf movement is perhaps the best known clock output process and its persistence under continuous conditions was already demonstrated in 1729 [159]. Because its quantification is non-invasive and relatively easy to do under constant light [160] it is perhaps the most frequently studied clock output process [182, 244, 287]. Upward leaf movement can be triggered by external factors such as heat and low light (hyponastic growth) and is positively regulated by ethylene [12, 181]. Ethylene regulated differential cell elongation in the petiole is the driving force behind this process [181, 288]. Similar to upward leaf movement, ethylene emission is also a clock output process [195]. Thus rhythmic ethylene biosynthesis and upward leaf movement are perhaps examples of two output processes that are interdependent. Our data shows that diurnal ethylene emission and leaf movement are differentially regulated by light and temperature. Under +DIF, wild-type upward leaf movement takes place during the warm day but peaks in the cold night. In response to -DIF the upward movement shifts and now takes place during the warm night (Fig. 3.1). When leaves were entrained with +DIF or -DIF and then deprived of any time cues, the leaf



movement again peaked during the subjective cold period. Thus leaf movement seems preferentially regulated by temperature. In contrast to leaf movement, ethylene is preferentially light regulated: Ethylene emissions display a peak in the middle of the light (high temperature) period during +DIF. However, in response to -DIF, peak ethylene emission shifts, but remains within the light (now cold) period. Under +DIF, leaf movement is initiated slightly before the peak in ethylene emission. Because ethylene is preferentially entrained by light and leaf movement preferentially entrained by temperature, under -DIF, the leaf movement is initiated in the warm (dark period) while ethylene peak emission remains in the light but now cold period, which coincides with a reduced amplitude of leaf movement. This implies loss of a temporal relationship between ethylene biosynthesis and upward leaf movement. It is however important to note that the cumulative ethylene emission measured is not *per se* representative for the local ethylene signalling in the leaf. Analysis of the expression of *ACS2* showed a specific reduction in the petiole under -DIF which correlates with the reduced leaf movement amplitude (Fig 3.4) The same is observed in seedlings, where local *ACS* activity in the basal part of the hypocotyl correlated with cell elongation (chapter 4). It is remarkable that a gaseous compound such as ethylene can have such specific local signalling effect. But when ethylene is scaled up to the level of a fire ant (*Solenopsis spec.*), ethylene's diffusion speed across the cytoplasm of an average plant cell is equivalent to a fire ant crossing a football (soccer) field in 5.35 minutes.

WHAT HAPPENS TO STARCH REGULATION OF GROWTH UNDER -DIF?

Throughout the light period, photosynthesis provides sucrose, which is immediately available for growth. Part of this sucrose is stored as starch in the leaves. These carbohydrate reserves are degraded during the night to support nocturnal growth [289]. Starch degradation during the night is essentially linear and in Arabidopsis starch is almost, but not completely, remobilised by the end of the night [290].

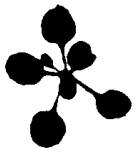
A correct anticipation of dawn is important for preventing premature depletion of carbohydrate reserves for growth. Therefore, the starch degradation rates are tightly controlled by the circadian clock [241]. This coordination between the clock anticipated dawn and starch degradation rates, maximises growth potential while it avoids carbohydrate starvation. Indeed, when the circadian clock is disrupted through mutations (e.g. *cca1/lhy*), starch is degraded too quickly, resulting in reduced growth and a carbohydrate starvation status of the plant at the end of the night [241]. In line with this we found that under -DIF, *CCA1* and *LHY* activity were greatly reduced (Fig. 6.2). The activation of the carbohydrate starvation reporter under -DIF, confirmed that starch was degraded too quickly (Fig. 5.4). Thus the reduced growth associated with -DIF treatment can now be linked to an induced carbohydrate starvation status of the plant.

DOES STARCH LINK TO LEAF MOVEMENT?

The link between carbohydrate starvation and reduced growth could indicate that the altered leaf movement rhythms under -DIF are in part caused by suboptimal starch degradation. In non-green cells of plants, starch granules accumulate in specialised plastids, the amyloplasts. The amyloplast in the endodermis of root and shoot have been linked to gravitropic responses [291]. Amyloplasts will sediment in the direction of gravity when the cytosol is sufficiently fluid, and it was proposed that the sedimentation of these plastids provides a trigger for sensing gravity in plants, the so-called 'starch statolith hypothesis' [290]. In roots the sedimentation of statoliths triggers a downward growth, while for the shoot the sedimentation triggers an upward growth direction [292]. Diurnal leaf movement patterns can be considered to consist of an anti-gravitropic movement (upward), which alternate with a gravitropic (downward) movement. In +DIF the upward movement takes place during the light period and matches the gravitropic response of the shoot. Starch build-up takes place during the photoperiod and starch content of leaves peaks at the end of the photoperiod [241]. At the onset of starch degradation (beginning of the night) the leaves begin to move down. Thus the change in growth direction related to gravity correlates with a change from starch storage to starch degradation. Thus the anti-gravitropic movement coincides with starch accumulation and the gravitropic movement coincides with starch degradation.

The *pgm* mutant has impaired starch synthesis, which presumably also affects statolith formation in the endodermis cells and indeed gravitropic responses are altered in the *pgm* mutant, although not absent [293]. Interestingly, the phase of diurnal leaf movement of *pgm* mutants under +DIF differed from wild-type and phase of the *pgm* mutant under +DIF (which is five hours later then wild-type +DIF) mimics the phase of wild-type leaf movement under -DIF. In *pgm* mutants, upward movement is completely restricted to the dark period and downward movement takes mainly place during the photoperiod but is already initiated during the late night. Thus the reversal of leaf movement patterns of *pgm* mutants and starch starved plants (-DIF) compared to wild-type plants with optimal starch metabolism strongly indicates that the diurnal leaf movement rhythm is driven by starch accumulation and degradation.

In contrast to *pgm*, the s*tarch excess* (*sex1)* mutant accumulates extra starch due to a defect resulting in the inability to mobilise starch. This mutant shows an increased gravitropic response for the shoot, linked to starch granules being bigger in the *sex1* mutant compared to wild type [294]. In our assay *sex-1* mutants showed the same leaf movement phenotype as *pgm* plants. This indicates that it may not be the physical presence of starch granules in the amyloplast, which is important for the anti-gravitropic response, but rather the starvation status of the plant (which both *sex-1* and *pgm* share). Perhaps, the sucrose released by nocturnal starch degradation is an important regulator of the downward movement. Indeed there is feedback between sucrose levels and the circadian clock, and the clock is particularly sensitive to sucrose in the dark, a response that is mediated by GI [295].



TOWARDS A GENERAL MECHANISMS

Our results have shown that there are differential responses for different circadian output processes under -DIF. These alterations in output processes are schematically depicted in Fig. 6.4, which aims to illustrate the mechanism by which -DIF affects growth.

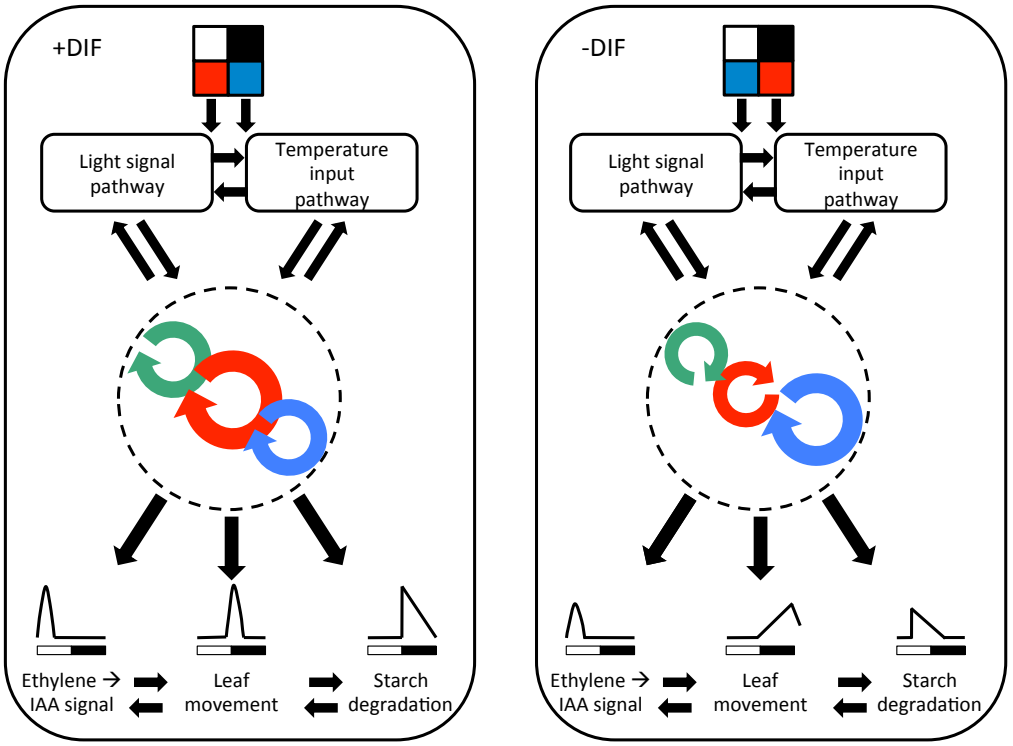
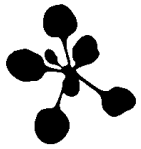


Figure 6.4. Schematic depiction of the synchronisation of clock regulated output processes under +DIF and -DIF. (**A**) Under +DIF light and temperature signalling generate robust circadian feedback and the output processes of the clock maximally correspond with the light and temperature environment. (**B**) Under -DIF, light and temperature input are no longer synchronous. This results in an altered circadian clock and altered timing of output processes. Some processes, such as ethylene emission, remain in the photoperiod, while others, such as upward leaf movement, shift towards the warm period. Starch degradation, which normally occurs in the night (A), is already initiated during the photoperiod under -DIF (B). These observations form the basis for the conclusion that the altered internal coordination between various output processes, reduces rhythmic growth under -DIF. Top square pictograms illustrate the experimental conditions: a full square represents a 24 h diurnal day and is divided into four 12 h sub-squares. The top sub-square indicates the light (white) and dark (black) period. The bottom sub-squares indicate the synchronous temperature conditions, 22°C (red) or 12°C (blue). The circadian clock is depicted in a schematised and simplified form, consisting of a morning (green), a central (red) and an evening (blue) feedback loop. The rhythmic output processes are depicted as black curves relative to the light (white bar) and dark (black bar) period. (*Figure adapted with permission* [61]).

One should remain cautious for over interpretation of the temporal relationships between different output processes. Various circadian output processes can have sequential phases but this does not necessarily imply a causal relationship between these processes. In addition many more output processes may be differentially affected by -DIF, the sum of which probably reduces growth. However, our results confirm earlier observations. Michael and co-workers (2003) also demonstrated differential responses of two circadian output processes after -DIF entrainment. CHLOROPHYL AB BINDING PROTEIN 2 CAB2*::LUC* activity, a circadian output marker related to photosynthesis [296], was more sensitive to light entrainment. In contrast, the circadian output marker CATALAYSE3 [295] *CAT3::LUC* activity was more sensitive to temperature entrainment [203]. The interpretation of this observation by Michael and co-workers differs from our final mechanistic interpretation of the effect of -DIF. They concluded that two Arabidopsis circadian clocks can be distinguished, one sensitive to light and one sensitive to temperature [203]. Our view is that there is one circadian clock, of which the robustness and the control of its rhythmic output depend on, and interact with, the combination of light and temperature cycles to which it is exposed.

LIGHT AND TEMPERATURE SIGNALLING, FROM MODEL TO CROP

Although Went (1944) posed a hypothesis about increasing day temperature difference on plant growth (+DIF) he also addressed the possibility of testing the effects a negative temperature difference between day and night (-DIF) [76]. In fact, as in the case of many inventions, practice preceded theory and -DIF regimes were already used in horticulture even though the molecular basis was not well understood [80]. Crop architecture directly relates to crop quality. Insights into the fundamental mechanisms affected by -DIF in the crop can help develop protocols for more efficient growth control and therefore increase crop quality. This TTI-GG sponsored project, aimed at unravelling the mechanism by which -DIF affects plant growth, and posed not only fundamental questions but also aimed at facilitating the development of improved -DIF protocols, a point best illustrated by the financial and intellectual contribution of 37 different horticultural companies (Supplemental Table S1.1). We took a fundamental approach, and instead of selecting a crop for our research we exploited the resources available in Arabidopsis to unravel causal fundamental processes. This strategy paid off through the screening of the numerous mutants and reporter plants available that were indispensable for this research. However, we did not forget about the crops, and once certain principles of the -DIF effect were clarified, experiments with petunia, tomato and cyclamen demonstrated that the principle interaction observed in Arabidopsis also holds for these crop species. In this thesis we not only provide a fundamental mechanism by which -DIF growth reduction can be better understood. The results described in this thesis also provide leads for further commercial innovation, and this resulted in a follow-up research project (STW) made possible by the continuing contribution of 22 horticultural companies. In the following final paragraph we peak into the future and aim to provide a roadmap for



application of the results described here and how they could potentially be of use in crop production systems.

PERSPECTIVE: PREDICTING AND CONTROLLING PLANT GROWTH

Chapter 5 provides a better understanding of how combined light and temperature cycles influence growth by affecting the clock. All growth related gene activities studied in this thesis display circadian fluctuations. The expression patterns of *PHYB* [280], *PIF* genes [75, 220], ethylene biosynthesis genes [195], nocturnal starch degradation rates [241], and auxin synthesis and signalling [224] are all (in part) regulated by the circadian clock.

From an applied point of view, It is important to note that responses to other environmental conditions that could occur in crop production, such as to salt stress [298], drought stress [299], and pathogens [299], are gated by the clock. During each of these responses ABA levels and sucrose status may change, which feeds back into the circadian oscillator [262, 272]. The complexity of the interaction between clock, entrainment signals, and circadian gaiting makes static growth models of limited value for predicting growth in a crop production, or natural environment. Markers and sensors, which are able to provide the most essential readouts for rhythmic growth and thus allow evaluation of the efficiency of a growth regime, are required for unravelling this puzzle. OSCILLATOR (chapter 2) is a suitable system for measuring clock output under various environmental conditions. Such measurements could be implemented in commercial greenhouses and when integrated in dynamic clock models [300], OSCILLATOR output may be used as a tool to predict various aspects of plant growth under a given set of environmental conditions.

Chapter 4 provides strong indications that growth stimulating PIF3 protein levels during the day limit growth under -DIF. PIF3 levels may be regulated by other means beside -DIF. There is for instance a negative interaction of PHYB protein levels with PIF protein levels [230]. Additional red light could thus be dosed specifically to increase active PHYB (Pfr) levels during the photoperiod and early night. Perhaps, during summer, pulses of red light at the end of the night, when PIF4 and PIF5 proteins are expected to be most abundant [75] could inhibit elongation. Combined with our own results on -DIF, it is tempting to speculate that the PHYB equilibrium is temperature sensitive. This theory is supported by other observations of temperature responses depending on PHYB [301]. Perhaps, strategically applying pulses of low temperature in combination with additional red light could have an additive effect on the PHYB equilibrium, more effectively inhibiting elongation. From a fundamental point of view, proving this would require a marker that allows dynamic *in vivo* measurement of PHYB photo-equilibrium (Pfr vs. Pr). Constructing an *in vivo* sensor in which a nuclear tagged fast maturing reporter protein is fused in-frame to a conserved PIF-PHYB (or PHYB-COP1) interaction domain [302], driven by a constitutive promoter *in planta* could serve that goal (inspired by the DII auxin sensor [225]). If indeed, as expected, this reporter complex is only degraded after being bound by activated PHYB (Pfr) in the nucleus, this would be a technological breakthrough. With such a sensor, the levels of PHYB Pfr versus PHYB Pr under various

conditions and treatments could be quantified in planta. Such a tool, which is probably transferrable to many crop species, could then assist in optimising the use of temperature or red light pulses in horticulture. Apart from tailor made climate control, in the near future we may also witness marker selected breeding of vegetable, flower, and bedding crops for a more favourable growth response to light and temperature pulses.

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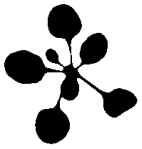

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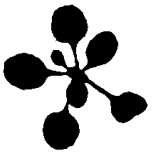

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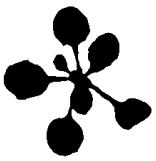

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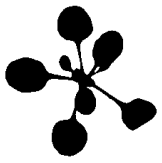

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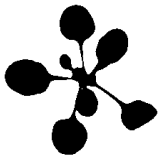

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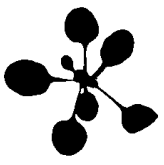


SUMMARY

Light and temperature are important determinants of plant growth and development. Plant elongation is stimulated by positively increasing differences between day and night temperature (+DIF, phased cycles). In contrast, a negative temperature difference (-DIF, antiphased cycles) reduces elongation growth. In **chapter 1** the different responses of plants to light and temperature are described. We focus on how light and temperature are perceived and integrated with physiological and molecular pathways to control plant development and architecture. As both light and temperature converge at the circadian oscillator attention is given to temperature entrainment and temperature compensation of the Arabidopsis circadian clock. Finally we discuss the importance of temperature effects on plant growth for horticulture. -DIF is frequently applied in commercial greenhouses to inhibit unwanted elongation of crops. Despite the economic importance, the response of plants to -DIF was poorly understood. Using *Arabidopsis thaliana,* our research aimed to understand the mechanisms underlying the -DIF response. The main questions that needed to be answered at the start of this project were:

(1) At what time during the diurnal day is growth affected by -DIF?
(2) What are key genes in the diurnal signalling pathways that result in reduced growth under -DIF?
(3) Is the temporal effect of -DIF on growth linked to the circadian clock, and if so how?

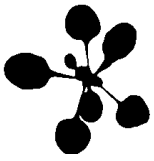
To answer question 1 (when is growth affected by -DIF?) it was important to develop a monitoring system through which growth could be analysed over the full day, including the dark period. This would allow us to determine how growth proceeds over the day and whether there is a specific period of the day at which growth is most affected by the -DIF regime. Elongation in plants is not constant throughout the day, but exhibits a diurnal rhythm. However, the effect of treatments on growth is usually scored as a cumulative effect after many days. Thus the precise relationship between environmental changes and the daily cycles in the growth of the plant remain mostly unnoticed. More detailed analysis can reveal whether the window of growth or the growth rate itself is affected by the environmental conditions. For this purpose, OSCILLATOR, a growth monitoring system, which allows the analysis and parameterisation of diurnal growth of rosette plants was constructed. The demonstration and validation of OSCILLATOR as growth monitoring system is described in **chapter 2**. The system consists of IR sensitive cameras and allows time-lapse imaging and subsequent analysis of leaf growth and leaf movement of Arabidopsis, tomato and petunia. We use this system to examine how fluctuating diurnal temperature cycles affect leaf movement in different Arabidopsis ecotypes, demonstrating that this approach allows

comparison of various genotypes through parameterisation of rhythmic growth. The analysis by OSCILLATOR showed that diurnal growth is accompanied by a cyclic movement of the growing leaves, and parameters (phase and amplitude) of this diurnal leaf movement can be used as a proxy for growth rate. This facilitated the characterisation of the effect of -DIF on growth.

To answer question 2 (what are key genes affected by -DIF) we tested many different mutants impaired in either light signalling, hormone perception, or hormone biosynthesis and studied their response to -DIF in comparison with wild-type plants. **Chapter 3** describes how, using this approach, we unravel the light and hormonal signalling processes that mediate the effect of -DIF on leaf movement. Pharmacological treatments combined with the genetic screens identify ethylene signalling as limiting for leaf growth and movement under -DIF. We demonstrate that specifically the activity of the ethylene biosynthesis gene *ACC synthase 2* activity in the petiole relates to the -DIF leaf phenotype. In addition, the effect of -DIF on ethylene sensitivity and biosynthesis is shown to depend on active PHYB.

To further characterise how light and hormone signalling affect growth under -DIF, we set out to identify factors limiting cell elongation. In **chapter 4,** local cell elongation in the hypocotyl is linked to local auxin signalling capacity. We demonstrate that ethylene, similar to its role in rosette leaves, becomes limiting in this tissue under -DIF as a result of reduced auxin production. While previously overall auxin was shown to be reduced in Arabidopsis inflorescence tissue developed under -DIF, we now demonstrate that it is mainly the effect of tissue specific auxin signalling that limits growth under -DIF. Moreover, we show that auxin can complement growth inhibition under -DIF in wild-type plants but not in ethylene signalling or biosynthesis mutants, placing the effect of auxin on growth upstream of ethylene. Downstream, ethylene signalling activates the growth promoting transcription factor PIF3, which is known to activate genes controlling cell elongation. In contrast, PIF5 acts upstream, possibly regulating the input of the signalling cascade. Remarkably, PIF4, which is a main regulator of heat induced hypocotyl elongation, is not required for the response to -DIF.

To answer question 3 (does -DIF affect the clock?) we used luciferase reporter plants and developed a unique luminometer set-up with which we could monitor gene promoter activity in mature rosette plants under different diurnal light regimes. This system was used in penultimate **chapter 5** where we demonstrate that an altered function of the circadian clock under -DIF is responsible for altered output processes identified in the other chapters. Analysis of expression patterns of core clock genes under diurnal conditions reveals that -DIF reduces the amplitude of most clock genes and differentially shifts the phase of core clock components. The magnitude and direction of these shifts differ for each clock gene, suggesting that -DIF alters the coordination within the circadian clock itself. We subsequently showed that the phase shifts occurring under -DIF relate to a temperature compensation mechanism controlled by *GI*. *GI* was previously identified to be required for temperature compensation in the amplitude of clock controlled genes at low and high temperature. Moreover, *GI* was identified to be responsible for the effect of -DIF on the phase of clock



genes. Indeed, *gi* loss-of-function mutants are insensitive to the effects of -DIF on growth. We demonstrate that under –DIF starch biosynthesis during the day, and starch degradation rates at night are altered. Carbohydrate availability during the night is essential for growth and therefore part of the sugars generated during the photoperiod are stored as starch. Throughout the night this starch is degraded in a controlled rate, which is adjusted to the predicted length of the dark period. The starch degradation rate under different photoperiod lengths is therefore tightly controlled by the circadian clock in anticipation of the expected dawn, to prevent running out of carbohydrates at the end of the night. Indeed, under -DIF starch metabolism is disturbed, resulting in an apparent starch shortage at the end of the night. This was monitored by activation of a reporter gene for carbohydrate starvation under -DIF. Furthermore, the phase of leaf movement of starch mutants under control (+DIF) conditions resembles the phase of wild-type plants developing under -DIF, indicating that the carbohydrate status of a plants determines rhythmic leaf movement.

In **chapter 6** the results obtained in this thesis are discussed and a conceptual model that aims to integrate all findings with recently published literature is proposed. In this model, -DIF affects growth by directly affecting the phase and amplitude of clock genes, which in turn control downstream processes such as starch metabolism and hormone signalling pathways. The auxin and ethylene signalling pathways affected by -DIF show significant crosstalk and interconnect with the circadian clock at several positions, by direct interaction with the PIFs, which are regulated by PHYB, of which transcription is under circadian control. Therefore, special focus is given to the unique position of the photoreceptor PHYB in this model. PHYB is essential for PIF protein stability and in addition is an important component for light entrainment of the clock. Finally we discuss the potential applications of the results described for horticulture and speculate on possible ways to improve the efficiency of DIF like treatments.

SAMENVATTING

Licht en temperatuur cycli zijn belangrijke factoren voor plantengroei en -ontwikkeling. Strekkingsgroei in planten wordt gestimuleerd door een positief verschil tussen de dag (licht) en nacht (donker) temperatuur (+DIF, gefaseerde cycli). In tegenstelling tot een positief verschil, leidt een negatief verschil (-DIF, anti-fase cycli) tussen de dag- en nachttemperatuur (koude dag, warme nacht) tot gereduceerde strekking. In **hoofdstuk 1** worden de verschillende responsen van planten op licht en temperatuur, die bekend zijn uit de literatuur beschreven. De focus ligt op hoe licht en temperatuur waargenomen en geïntegreerd worden met de fysiologische en moleculaire mechanismen die plantontwikkeling en -architectuur bepalen. Zowel licht als temperatuur hebben een wisselwerking met de circadiaanse klok en de sturing en compensatie van de circadiaanse klok door beide factoren staat centraal in dit hoofdstuk. Aan het einde van hoofdstuk 1 wordt ingegaan op het belang van de effecten van licht en temperatuur voor de tuinbouw. -DIF wordt frequent gebruikt in de glastuinbouw om ongewenste strekking van gewassen te remmen. Ondanks het economische belang van deze toepassing was het onderliggende mechanisme van het effect van -DIF op plantengroei grotendeels onbekend. Gebruik makend van de modelplant *Arabidopsis thaliana* (gewone zandraket) richtte ons onderzoek zich op het ontrafelen van het mechanisme dat in de plant verantwoordelijk is voor het effect van -DIF. De belangrijkste vragen aan het begin van het onderzoek waren:

(1) Gedurende welk moment van een etmaal beïnvloedt -DIF de groei?
(2) Welke sleutelgenen zijn betrokken in de signalering die resulteert in gereduceerde groei bij -DIF?
(3) Is het eventueel tijdsgebonden effect van -DIF afhankelijk van de werking van de circadiaanse klok, en zo ja, hoe?

Om vraag 1 (wanneer beïnvloedt -DIF groei?) te beantwoorden was het van belang om een systeem te ontwikkelen waarmee groei gedurende zowel de dag als nacht geanalyseerd kon worden. Dit zou ons in staat stellen te observeren hoe groei over een etmaal (24 uur) verloopt en of er een specifiek tijdstip is waarop -DIF ingrijpt op de groei. Strekking in planten is niet constant maar vertoont een dagelijks ritme. Het effect van behandelingen wordt echter veelal gemeten als een cumulatief effect na meerdere dagen. Daardoor kon voorheen de precieze interactie tussen veranderingen in de omgeving en hun directe effect op groei gemakkelijk gemist worden. Een meer gedetailleerde analyse van groei kan aanduiden of het tijdstip van groei, of de groeisnelheid zelf, wordt beïnvloed door omgevingsfactoren. Voor dit doeleinde ontwikkelden we OSCILLATOR, een camera systeem waarmee plantengroei gedurende het hele etmaal geanalyseerd en geparameteriseerd kan worden. In **hoofdstuk 2**

beschrijven en valideren we dit OSCILLATOR systeem. Het bestaat uit infrarood gevoelige camera's die time-lapse opnames van groei- en bladbeweging in Arabidopsis, tomaat en petunia gedurende dag en nacht mogelijk maken. Met dit systeem kunnen bijvoorbeeld verschillende genotypes met elkaar vergeleken worden. We demonstreren dit door ritmische bladbeweging van verschillende Arabidopsis accessies te vergelijken. De analyses met OSCILLATOR tonen aan dat groei gepaard gaat met continue cyclische bladbeweging. De beweging van de bladgroei wordt vertaald naar amplitude en fase en kan als een proxy voor groeisnelheid worden gebruikt. Dit faciliteerde de verdere karakterisering van het effect van -DIF op groei. Voor het beantwoorden van vraag 2 (Wat zijn de sleutel genen betrokken bij het -DIF effect?) werden verschillende mutanten getest die genetisch beperkt waren in lichtperceptie, hormoonperceptie, of hormoonproductie. De respons van deze mutanten op -DIF werd bestudeerd en vergeleken met de response van wild type planten. In **hoofdstuk 3** wordt beschreven hoe deze aanpak leidde tot de ontrafeling van licht en hormoon gestuurde signaleringsprocessen die het effect van -DIF op bladbeweging reguleren. Door farmacologische behandelingen in combinatie met de genetische screens, werd ethyleen geïdentificeerd als limiterend voor bladgroei en -beweging bij -DIF. Specifiek de expressie van het ethyleen biosynthese gen *ACC SYNTHASE 2* (*ACS2*) in de bladsteel is gerelateerd aan het -DIF blad fenotype. Daarnaast wordt aangetoond dat het effect van -DIF op ethyleengevoeligheid en -biosynthese afhankelijk is van actief PHYTOCHROME B (*PHYB*). Om deze interactie tussen licht en hormoonsignalering verder te onderzoeken werd gezocht naar factoren die celstrekking limiteren bij -DIF. In **hoofdstuk 4** wordt de lokale auxine signalleringscapaciteit aan stengelstrekking gelinkt. We demonstreren dat ethyleen, gelijk aan de rol in het blad, bij -DIF limiterend wordt voor strekking in de stengel. Dit is het gevolg van verminderde auxine productie. Voorheen werd reeds aangetoond dat de gemiddelde auxine niveaus bij -DIF verlaagd waren in Arabidopsis bloemstengels in vergelijking met +DIF. Nu laten we zien dat het voornamelijk een weefsel-specifiek auxine tekort is dat verantwoordelijk is voor de groeireductie bij -DIF. Exogeen toegediend auxine kan in wild type het effect van -DIF op cel strekking opheffen maar dit is niet het geval in ethyleen perceptie of biosynthese mutanten. Dit duidt aan dat auxine upstream van ethyleen de groei reguleert. Downstream wordt ethyleensignalering limiterend voor de transcriptionele activatie van de groei stimulerende transcriptie factor PIF3, waarvan bekend is dat deze genen activeert die cel strekking faciliteren. In tegenstelling tot PIF3 acteert PIF5 upstream, waar deze transcriptie factor mogelijk de input van de hormoonsignaleringscascade reguleert. Opvallend is dat PIF4, een bekende regulator van hitte-geïnduceerde stengelstrekking, niet noodzakelijk is voor de -DIF response.

Om vraag 3 (Beïnvloedt -DIF de circadiaanse klok?) te beantwoorden gebruikten we luciferase reporter planten in combinatie met een zelf geconstrueerd uniek luminometer systeem waarmee we gen promoter activiteit konden kwantificeren in volwassen rozetplanten bij de verschillende licht- en temperatuurcondities. Dit systeem wordt gebruikt om in **hoofdstuk 5** aan te tonen dat een veranderde klokfunctie verantwoordelijk is voor de

veranderde outputprocessen die beschreven werden in de voorgaande hoofdstukken. Analyse van expressiepatronen van klokgenen toonde aan dat -DIF de amplitude van de expressie van de meeste klokgenen reduceert en differentieel de fase verschuift. De grootte en richting van deze wijzigingen zijn verschillend voor elk van de geteste klokgenen, hetgeen er op duidt dat -DIF de coördinatie van de circadiaanse klok verstoort. Vervolgens demonsteren we dat de faseveranderingen in klokoutput gelinkt zijn aan het temperatuurcompensatie mechanisme waarin GI een belangrijke rol speelt. GI werd voorheen geïdentificeerd als essentieel voor de compensatie van de amplitude van klokgenen bij hoge en lage temperatuur. Hier identificeren we GI als verantwoordelijke factor voor de veranderingen in klok output bij -DIF. GI loss-of-function mutanten zijn ongevoelig voor het effect van -DIF op groei. Verder demonstreren we dat de snelheid en timing van zetmeelafbraak verandert bij -DIF in vergelijking met +DIF. De beschikbaarheid van koolhydraten gedurende de nacht is essentieel voor groei en daarom wordt een deel van de suikers die in de fotosynthese aangemaakt worden opgeslagen als zetmeel. Gedurende de nacht wordt dit zetmeel met een gecontroleerde snelheid afgebroken, welke is afgestemd op de geanticipeerde ochtend. De afbraaksnelheid van zetmeel wordt strak gereguleerd door de circadiaanse klok bij verschillende daglengtes. De klok anticipeert de ochtend en voorkomt dat de plant te snel al het zetmeel verbruikt. Bij -DIF wordt de zetmeelvoorraad te snel afgebroken, hetgeen resulteert in een koolhydraat tekort. Dit werd bevestigd met een koolhydraat 'starvation reporter', een gen dat sterk opgereguleerd wordt als de plant over te weinig suikers beschikt. Bovendien vertonen planten met bekende mutaties in zetmeelopbouw of -afbraak een -DIF bladbewegingsfenotype bij +DIF controle condities. Dit duidt erop dat de koolhydraatstatus van de plant samenhangt met de ritmische bladbeweging.

In **hoofdstuk 6** worden de resultaten die in dit onderzoek zijn verkregen bediscussieerd en presenteren we een conceptueel model dat tracht alle bevindingen met recente literatuur te integreren. In dit model beïnvloedt -DIF de groei door direct in te grijpen op de ritmische expressie van klokgenen die op hun beurt downstream groeiprocessen, zoals zetmeelmetabolisme en hormoonsignalering, reguleren. De auxine en ethyleensignalering die door -DIF beïnvloed worden vertonen onderlinge interacties en zijn verder op meerdere posities met de circadiaanse klok verbonden. Bijvoorbeeld door directe interactie met de PIFs, die door PHYB gereguleerd worden, waarbij *PHYB* transcriptie weer door de klok gereguleerd wordt. Daarom wordt speciaal aandacht besteed aan de fotoreceptor PHYB. Het PHYB eiwit is verantwoordelijk voor stabiliteit van het PIF eiwit en is daarnaast een belangrijke component voor licht 'entrainment'. Tot slot bediscussiëren we de potentiële applicaties van de beschreven resultaten voor de glastuinbouw en speculeren we over mogelijke manieren om de efficiëntie van DIF-achtige behandelingen te verhogen.

“There are no passengers on spaceship earth. We are all crew”

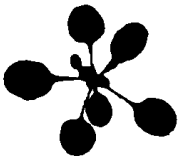
- Marshall McLuhan

ACKNOWLEDGEMENTS

Right now I feel inspired to write what is to be the last part of this thesis, the acknowledgements. I have been looking forward to writing this part ever since I started to practice 'academic writing'. Not just because it is supposed to be the last thing you write or because it is apparently the only thing you will read (probably to see if your name pops up somewhere). No, the reason I am going to enjoy writing this is, too be very blunt, that I find academic writing (a tad bit) boring. Scientific writing is stripped of all decoration; the bare minimum characters needed to convey the message are carefully sculptured out from bulk text, making the result sometimes feel soulless to me. I felt there was little room for style and creativity in putting the other chapters on paper (except for the figures, in which I honestly invested too much time). In the text I made some subtle jokes here and there, but they are for insiders only and that was about as far as I dared to go. The urge to create elaborate, magical-realistic, novel sentences is something I actively need to resist with every academic report, proposal, or paper I write. But in this part I am apparently allowed to uncork all of it (within the political and religious censorship boundaries set by Wageningen UR).

Academic or not, pieces of text need to be connected; no different is it for people. One of the first people who kindly pointed out to me that I needed to let go my novelistic writing style and instead try to write more concise (minder wollig) is Prof. Titti Mariani. It is therefore fitting that Titti is also the very first person to which I express my gratitude regarding this thesis. I remember the wonderful time in Nijmegen very well. I was extremely lucky to be a young (naïve) MSc. student who had a Professor (Titti) as direct supervisor. Because of that, I sometime jokingly refer to that period as my first PhD. Already then, Titti recognised potential pitfalls and talents in me. Titti, it is amazing how well you intuitively grasp what makes people tick and then provide them with what they specifically need. Working with you really made me enthusiastic about science, hence my academic path so far.

The other place where I am allowed to freely let my inner mad scientist out is the lab and greenhouse. That feels like " home" and Titti gave me all the space I wanted. As I was "science-ing" around in Nijmegen, first in the UL (a soulful, cockroach infested Soviet style



chaotic lab) which was (sadly?) demolished and replaced with a completely new building (which in contrast to other new buildings actually had offices) I met the second person I would like to thank. Wouter, if someone had told me back then that this guy who pissed of Richard so often for no apparent reason, would play such a prominent role in my scientific career as a colleague and a friend, I would not have believed it. I still remember the first AFLP acrylamide gel we poured, or rather injected, together. Bubbles, loads of bubbles, mostly in my gel, yet Richard was maniacally occupied with tapping only on your glass plate to kill the occasional bubble. Later, when I rolled into my Master thesis in Wageningen, you became my supervisor, and again, I remember bubbles. We literally flushed buckets, perhaps swimming pool worthy quantities, of Arabidopsis, tomato and later Petunia exudates through tiny stacked C18 columns. A system that slowly turned into a makeshift, multi-tube, hosing machine, based on the law of communicating vessels, which could have been a prop in a David Fincher movie. If I look back at photographs of that time, I notice that next to all the tubes and puddles of water and/or acetone (?) there is a young version of me with a big smile on his face. In the end when all is said and done, those smiles are more valuable than anything else we strive for. There was a period in which I nearly lost that smile, but it fortunately proved to be a resilient feature.

Often we were compared to Statler and Waldorf, the two disagreeable old men from the Muppet Show. That makes me jump straight to Carolien, you know why. I enjoyed working with you a lot. You have a sixth sense for correct hypotheses. I am still amazed that you were, unaware and independent of the French and the Japanese efforts, complementing *max* mutants with GR24. It's a shame they got it quicker, but it shows your talent none the less. I am still proud to have been part of one of the labs where the strigolactone saga started.

Back to the Muppets, I find the comparison with Statler and Waldorf quite hilarious; they pretend to be absolutely no part of the ridiculous show "they observe". And as all the other Muppets get hopelessly entangled, they watch and comment from the balcony: 'It was terrible!' '*Horrendous*!' 'Well it wasn't that bad.' '*Oh, yeah?*' 'Well, there were parts of it I liked!' '*Well, I liked a lot of it.*' 'Yeah, it was GOOD actually.' '*It was great!*' 'It was wonderful!' '*Yeah, Bravo!*' 'More!' '*More!*'

As most things in my life my Master thesis slid naturally into a PhD position and the paperwork lagged behind. I was halfway my first year and actually had not received any diplomas yet. I remember I more or less acquired my Bachelor diploma a few months before my Masters. Sander, I remember the fear on your face when you found out (through Titti) that I actually had not graduated (yet).

Sander, you and I are quite alike in many ways and I suspect that because of this, sometimes things did not go as smoothly as we both would have wished. I diverge, doubt, double check, run around in circles, jump ahead, drift off, and I prefer to avoid finishing over trying and failing. Often we enhanced each other, looking back, mostly for the better. I am still grateful that in the ambitious project we started you postulated a hypothesis that was as enticing as logical. It was, most importantly, spot on. Your ideas are as brilliant as plentiful

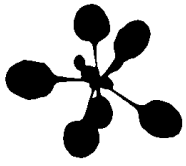
and in the massive, sometimes incomprehensible flow of new ideas there are real raw diamonds. I always felt my job was to (intuitively) know how to sieve and polish them. Perhaps the only regret I have is that I divided the gems over the chapters, maybe patiently making a small collection and selling them together would not have been a bad idea. You were right on the clock. The clock is behind it all and we were the first to demonstrate that even 13 million years of evolution was not enough to prepare Arabidopsis for cold days in combination with warm nights! By manipulating the universal rule: light=heat, into: light≠heat, the clock became desynchronised, which allowed us to observe exactly what was going wrong with the clock itself and with all the stuff that obeys the clock. One can learn more from observing something going wrong, than from situations that are organised to perfection. Whenever I got entangled in emotion based rationalised disputes, Harro always knew exactly what to say and to put it in perspective. Harro, thanks, you are an excellent manager and I admire your ability to prioritise, observe calmly, and make decisions under pressurewithout regretting them later. I think Harro, but all of us, are also quite lucky to have Rina around. Rina, thanks for all your help with forms, refunding and bookings!

Back to ticking clocks, believe it or not, as I write this, some parts of my thesis are not even finished. I remember confusion, empathy and worry in the faces of some of my former colleagues as they observed me run around fanatically in the lab during my last months of 'near unemployment'. Marieke, Wilco, Natalia, Desalegn, Xing, Phong, Julio, Kerstin, Thierry, Tanya and all the others who frequently asked me if I still had time to do all that new stuff in the terminal stage of my PhD. The answer was simple, no, but I guess I needed it. And most of that stuff was very useful and went straight into this thesis, of which I am quite proud.

Actually there are many excellent students to thank for useful stuff that went straight into this thesis: Cheon MengFoong you were a great help for the GUS staining work. Van Hoang Thi Nguyen, you firefly, you burned your way though many Luciferase plates and plants while I was attempting to collect Arabidopsis in Australia. Your work was performed precisely and independently and most of it you find back in chapter 5. Finally Mastaneh Ahrar, I enjoyed working with you. The hypothesis was a bit crazy but I feel there was great potential in the approach and you explored that very well.

When all students left and my contract ended, I lost my spot in the lab. I did not fully surrender; I threw all my stuff into a big yellow crate and took refuge in the luminometer room. I came to love that place. It allowed me to remain 'the mad scientist' without distressing too many of the excellent technicians: Francel, Diaan, Mariëlle, Leo, Marieke, Carin, Miriam. I like to think that, apart from the mess and chaos, you maybe also enjoyed me buzzing around in your domain. In my opinion, an empty lab is worse than a messy one. I definitely enjoyed working with you all. Whenever I felt a bit lonely (a rare feeling in Radix), I popped into the 'real' lab and had a chat with Mr. Julio, Mr. Anderson, Mr. Song, or Mr. Bas.

Julio, I had a lot of fun with our NASA expedition. I was as excited as a child launching a



homemade model rocket when we filled those paint sprayed wells with that mysterious black powder. Keep that stuff close, there is great potential… Um dia, meu amigo!

To me, a big part of the lab was Bas. In my PhD mind you where the textbook example of a Postdoc. I respect your knowledge, attitude, and work ethic. I am very fortunate to have worked with many such talented people throughout my career. Tanya and Francel, you are also great examples; it's all about getting the chemistry right! I believe that without Tanya and Francel not a single paper I have on my list would have been possible. Speaking of chemistry, Chris, I am honoured to have a soon to be Organic Chemistry Doctor as one of my paranymphs! Of course brilliant people are not limited to Wageningen. Early on, when we discovered that -DIF disrupted leaf movement patterns I went to the department of Ecophysiology in Utrecht, the specialists in this area. A little bit nervous but with high expectations I pitched my research. Martijn, I was so happy to see you genuinely excited about the effects of this bizarre treatment we were working on. You must have seen potential in the approach, potential that dawned on me much later. The collaboration we established is dear to me and you were like a shadow supervisor. Especially your writing skills are phenomenal. Martijn, you are the best writer I worked with and your (correcting and teaching) contributions to this thesis are more then significant. Thank you very much for all those rounds of tedious corrections of a work that slowly but steadily morphed into chapter 3. That was my crash course paper writing. Ronald, many thanks, you intuitively gave me the freedom to use all the equipment I needed for my quest to prove the involvement of ethylene. You also very efficiently and very valuably helped us to focus and sharpen our work in chapter 3. And even regarding the follow up work you managed to convey the essence of a problem with just one simple remark or question at a conference or meeting. Thanks!

Thinking of Radix, I automatically think of Unifarm. My precious Weiss growth cabinets were first located very close to the lab and the office, but had to move to Radix Serre. All the time I tortured those beautiful pieces of German engineering with ridiculous climate settings, while Taede and Gerrit carefully maintained them. In the end I had four of those babies running in antiphase. The only thing cooler (and more cost efficient) would be 24h access to the Radix and Klima building. The few times when the cabinets did break down, Taede and Gerrit did everything in their power to rescue ongoing experiments. A big thanks to all Unifarm people for their commitment and involvement. This project was in collaboration with many companies whom I all would like to thank, but in particular I am grateful to Arjan Stolte for being an excellent chairman. You saved me a lot of time and this is greatly appreciated.

Throughout my promotion there was Manickam. From the moment we met, I was enticed by your calmness and serenity. Looking back, I guess that next to that serenity, a darker part of you must have boiled at times. You became a true friend and no matter what crazy metaphor we would throw at each other we would somehow get it. Many lost their way in those metaphors and I guess your metaphors drove some folks quite desperate. Often to my amusement, I shamefully admit. I think the only explanation for our innate ability to decipher each other's cryptology must be that our mutual understanding is beyond words.

Finally, I arrive at the true pillar of my faith in myself, Johanna, I love you so much, without your support and care I would not have finished this endeavour. When you went to Australia I missed you very much and realised what great team we make. We are completely different but complement each other so well! And the time and effort you invested into transforming my 'mess' into this beautiful layout can only be interpreted as true love, for which I am deeply grateful! Talking about people breaking a sweat for my layout, Theresa and Sam, I deeply owe you and despite the fact you personally find the cover to dark I really like it and feel it fits perfect with my work and philosophy. Black or white, when things become mixed, grey, it gets messy. Therefore the world must be grey and me myself an idealist.

In MPIPZ I made so many more friends, although in academics friends and colleagues are hard to distinguish, especially when the time people stick around is so short. Corinna, Wilma, Dorothee, Agata, Ding Jia, Sigi, Mariane, Birgit and all the others! What a great team you all make. You made me feel at home, although for a hardliner plant physiologist quantitative genetics felt at first a bit alien to me. I had never seen more then 100 Arabidopsis plants together and started right away with 2200 individual plants. Genetics is all about planning. Thanks Maarten, for your patience and understanding with my lingering perfectionism in finishing and publishing the work presented here. You are the finest example of a group leader I have met so far and I am honoured to be you Postdoc.

At home, even when working late, two peculiar creatures always kept me company. Dickie and Ko, you stole my heart. Despite the killing instinct, cats instinctively reside in the now and therefore have zero bad karma. When I feel inner turmoil I pretend to be a cat watching a mouse hole. I focus on my breath, attentive to the now, eye on the mouse hole. Thanks cats, for being great gurus.

Finally I would like to express great gratitude to my parents and family: Pap, Mam, Carsten, Klaas, Geartsje, Marcel, Roely, Frank, Marlies, Anouk, Janou and all dear family. The many trips to your houses or gatherings always provided me with a refuge and haven when work had once again invaded my private home and mind.

Mijn dank is bijzonder groot voor Mam en Pap, bedankt voor al jullie steun, ik zou niet zijn wie ik ben zonder alle zorg, liefde en offers die jullie mij als vanzelfsprekend geschonken hebben. Jullie vertrouwen in mijn kunnen heeft me altijd gestimuleerd en gemotiveerd op mijn reis langs het bizarre pad van het leven.

Tschüss und auf Wiedersehen liebe Leute,

Ralph

About the author

List of publications

Curriculum Vitae

Graduate school EPS education statement

PEER REVIEWED PUBLICATIONS

Bours, R., van Zanten, M., Pierik, R., Bouwmeester, H., and 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 physiology *163*, 882-895.

Bours, R., Muthuraman, M., Bouwmeester, H., and van der Krol, A. (2012). OSCILLATOR: a system for analysis of diurnal leaf growth using infrared photography combined with wavelet transformation. Plant methods *8*, 29.

Kohlen, W., Charnikhova, T., **Bours, R.**, Lopez-Raez, J.A., and Bouwmeester, H. (2012). Tomato strigolactones: A more detailed look. Plant signaling & behavior *8*.

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J.B., Reinhardt, D., **Bours, R.**, Bouwmeester, H.J., and Martinoia, E. (2012). A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature *483*, 341-344.

Kohlen, W., Charnikhova, T., Liu, Q., **Bours, R.**, Domagalska, M.A., Beguerie, S., Verstappen, F., Leyser, O., Bouwmeester, H., and Ruyter-Spira, C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host Arabidopsis. Plant physiology *155*, 974-987.

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., Cardoso, C., Lopez-Raez, J.A., Matusova, R., **Bours, R.**, et al. (2011). Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in Arabidopsis: another belowground role for strigolactones? Plant physiology *155*, 721-734.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., **Bours, R.**, van der Krol, S., Shibuya, N., Joosten, M.H., and Thomma, B.P. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science *329*, 953-955.

BOOK CHAPTERS

van Zanten, M., **Bours, R.**, Pons, T.L., and Proveniers, M.C.G. (2013). Acclimation and adaptation to warm environments. In Temperature and plant development, K.A. Franklin and P.A. Wigge, eds. (Wiley-Blackwell publishers).

Ruyter-Spira, C., López-Ráez, J., Cardoso, C., Charnikhova, T., Matusova, R., Kohlen, W., Jamil, M., **Bours, R.**, Verstappen, F., and Bouwmeester, H. (2013). Strigolactones: a cry for help results in fatal attraction. Is escape possible? In Isoprenoid synthesis in plants and microorganisms, T.J. Bach and M. Rohmer, eds. (New York: Springer), pp. 199-211.

CURRICULUM VITAE

Ralph Bours was born on 9 July, 1983 in Maasbracht, the Netherlands. After obtaining his HAVO (Hoger Algemeen Voortgezet Onderwijs) diploma in 2000 from Stedelijk Lyceum in Roermond, he started a Bsc in Organic Chemistry at Hogeschool Zuyd in Heerlen, where he obtained his propedeuse. He switched fields and in 2003 started a BSc in Biology at the Radboud University in Nijmegen. This study included a two-month internship in the department of Microbiology, Radboud University (RU), of Prof. dr. ir. M. S.M. Jetten.

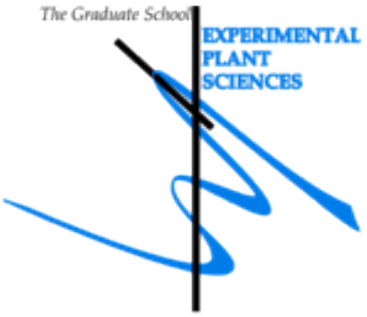
After his BSc he completed a major in Biology which included a six month MSc internship in the department of Plant Cell Biology, RU, of Prof. dr. C Mariani. Here he explored the possibilities of using *Solanum dulcamara* as model specie for studying adventitious root initiation. In addition, he completed a seven month MSc internship in the Laboratory of Plant Physiology, Wageningen University, of Prof. dr. H.J. Bouwmeester, where he was involved in the early characterisation of strigolactones as plant hormones. He completed his MSc in Biology in 2009. Before graduation he was appointed a PhD position at the Laboratory of Plant Physiology, Wageningen University. The aim and results of this PhD project are presented in this thesis.

The approach and results presented in this thesis contributed to a follow-up project, granted by STW to Dr. ir. A van der Krol and Dr. ir. W van Ieperen, in which 22 companies continue to financially and intellectually support research into compact plants.

Throughout his PhD, Ralph was involved in teaching and supervising several BSc and MSc students. He attended multiple (international) courses and congresses (poster/oral presentation). In addition he reviewed papers for the international journals Plant Physiology and The Plant Journal.

Currently, Ralph holds a Postdoc position at the Max Planck Institut für Pflanzenzüchtungsforschung (Plant Breeding) in Köln. In the group of Prof. M. Koornneef he investigates the genetics of shoot branching in *Arabidopsis thaliana* by exploiting natural variation.

EDUCATION STATEMENT OF THE GRADUATE SCHOOL



Experimental Plant Sciences

Issued to: Ralph Bours
Date: 2 June 2014
Group: Laboratory of Plant Physiology, Wageningen University & Research centre

1) Start-up phase	Date
► **First presentation of your project**	
Mechanism(s) behind hysiological treatments resulting in plant growth reduction for producing compact plants	Apr 04, 2009
► **Writing or rewriting a project proposal**	
Rewriting Compact cultivation TTI Project	Feb 2013
► **Writing a review or book chapter**	
Van Zanten M, Bours R, Pons TL, Proveniers MCG. (2013) Acclimation and adaptation to warm environments. In: Eds. Franklin KA & Wigge PA: Temperature and Plant Development (Wiley-Blackwell publishers)	Aug 2012-2013
Ruyter-Spira C, López-Ráez J, Cardoso C, Charnikhova T, Matusova R, Kohlen W, Jamil M, Bours R, Verstappen F, and Bouwmeester H. (2013) Strigolactones: A Cry for Help Results in Fatal Attraction. Is Escape Possible? In: Eds. Bach TJ, Rohmer M. Isoprenoid Synthesis in Plants and Microorganisms (Springer New York), 199-211.	Jul 2010-2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	Date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
► **EPS theme symposia**	
EPS Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Jan 30, 2009
EPS Theme 3 symposium 'Metabolism and Adaptation, Wageningen University	Feb 10, 2011
EPS Theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Jan 19, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012

ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
▶ **Seminars (series), workshops and symposia**	
Invited seminars (Jan de Ruijter, Wim Soppe, Otoline Leyser)	Feb-Nov 2009
Invited seminars (Koichi Yoneyama, Takahito Nomura, Kaori Yoneyama, Adam Prize, Kirsten Bomblies, Ian Henderson, Christian Hermans)	Jan-Dec 2010
Service XS Seminar, Wageningen	Sep 2011
Mini Symposium parasitic plants	Oct 07, 2011
Invited seminars (Ian Baldwin, Bertus Beaumont)	Jan 2012
▶ **Seminar plus**	
▶ **International symposia and congresses**	
Fleuroselect conference, Ratingen, Germany	Nov 04-06, 2009
IPGSA 2010 Tarragona Spain	Jun 28-Jul 02, 2010
Auxin 2012 Hawaii	Dec 09-14, 2012
▶ **Presentations**	
Invited speaker: Fleuroselect conference	Nov 05, 2009
Oral: TTi-GG Project Meeting, Bleiswijk	Apr 13, 2010
Poster: NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
Oral: Bessensap, Museon, Den Haag	Jun 07, 2010
Poster: IPGSA	Jun 28-Jul 02, 2010
Poster: TTI-GG Congress	2010
Oral: TTI-GG congress	2011
Poster: TTI-GG Congress	Sep 19, 2012
Oral: NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
Poster: Auxin congress, Hawaii, USA	Dec 09-14, 2012
Invited speaker: EPS Theme 3 Symposium 'Metabolism and Adaptation', Wageningen University, Wageningen, The Netherlands	Mar 11, 2014
▶ **IAB interview**	
Meeting with a member of the International Advisory Board	Feb 17, 2011
▶ **Excursions**	
Subtotal Scientific Exposure	*20.7 credits**

3) In-Depth Studies	**Date**
▶ **EPS courses or other PhD courses**	
EPS PhD Summerschool on Environmental Signaling, Utrecht University	Aug 24-26, 2009
Hacking the Biological Clock: Circadian Rhythm and Photosynthesis, Lorentz Centre, Leiden	Apr 10-13, 2012
▶ **Journal club**	
Literature discussions at PPH	2008-2012
▶ **Individual research training**	
Confocal training MPIZ, Cologne, Germany (3 days) Dr. Kohlen en Dr. Smelzer	Oct 2012
Training Photoacoustic laser measurements of plant volatiles, Utrecht University, Ronald Pierik (2 days)	May 2011

Subtotal In-Depth Studies	*6.3 credits**
4) Personal development	**Date**
► **Skill training courses**	
Communication Training Personal Coach `De tweede Verdieping'	Jan, 17, 2009
ExPectationS (EPS Career day)	Nov 18, 2011
MPIPZ course: Funding possibilities for young european scientists	Oct 10, 2013
► **Organisation of PhD students day, course or conference**	
Organisation of Conference Compact Cultivation Van der Knaap	Jun 24, 2009
Organisation of Conference Compact Cultivation Bleiswijk	Apr 13, 2010
Organisation of Conference Compact Cultivation Wageningen	May 2011
Organisation of Conference Compact STW	Sep 21, 2012
► **Membership of Board, Committee or PhD council**	
Organising host and chairman of the TTI"Stuurgroep meeting" 1.1	Apr 01, 2009
Organising host and chairman of the TTI"Stuurgroep meeting" 1.2	Nov 24, 2009
Organising host and chairman of the TTI "Stuurgroep meeting" 2	Nov 09, 2010
Organising host and chairman of the TTI "Stuurgroep meeting" 3	Jun 07, 2011
Organising host and chairman of the TTI "Stuurgroep meeting" 4	Nov 19, 2012
Subtotal Personal Development	*6.6 credits**

TOTAL NUMBER OF CREDIT POINTS* **44.1 credits**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was financially supported by Top Technological Institute Green Genetics (TTI-GG) (2CFL009RP) through Wageningen University (2009-2013).

Financial support from Kieft Seed/PanAmerican Seed, via Willem Koopman, the Netherlands and the Laboratory of Plant Physiology, Wageningen University for designing and printing this thesis is gratefully acknowledged.

Colophon

Cover design: Theresa Bisdorf and Samuel Wulf (http://angelghost.deviantart.com/)
Cover photography: Marcel Ohlenforst (www.marcelohlenforst.nl)
Layout design: Johanna Rienks

Printed by: BOXPress, Den Bosch, the Netherlands

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